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Applying Different Methods To Evaluate the Freshness of Large Yellow Croacker (*Pseudosciaena crocea*) Fillets during Chilled Storage

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ABSTRACT: The freshness of large yellow croaker (*Pseudosciaena crocea*) fillets was evaluated over 20 days of chilled storage under vacuum packaging. The physicochemical properties [pH, *K* value, thiobarbituric acid (TBA), texture profile analyses (TPA), color], microbiological properties [total viable count (TVC)], sensory attributes, transcriptomics (levels of transcripts coding for cathepsin L), and functional properties of proteins [emulsion activity (EA) and emulsion stability (ES)] were assessed at 0, 5, 10, 15, and 20 days of storage. This study also investigated the effect of the storage time on fish muscle proteome in large yellow croaker using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS). The parameters that were the most sensitive to storage duration were color (C^*_{ab}), TPA (springiness, chewiness, and resilience), pH, *K* value, TVC, levels of transcripts coding for cathepsin L, EA, ES, and sensory attributes. The three altered proteins were successfully identified. Therefore, these parameters might be considered suitable indicators for evaluating the freshness of large yellow croaker fillets during chilled storage under vacuum packaging.

KEYWORDS: *Pseudosciaena crocea, freshness, real-time PCR, two-dimensional gel electrophoresis, food quality*

INTRODUCTION

The large yellow croaker, *Pseudosciaena crocea*, is a commercially important marine fish species in southeastern China and also represents the largest yield for a single species in Chinese marine net-cage farming because of its good taste and high nutritional value. Studies on large yellow croaker have investigated its genome,[1] immunity and physiology,[2] and breeding.[3] However, little information is available on the post-mortem freshness changes of large yellow croaker fillets during chilled storage.

Due to new lifestyle habits and the younger generations' increasing consumption of prepared products, the consumption of fillets is expected to increase in the future. In aquaculture, fillet quality parameters are important determinants of profitability. Freshness is the most important attribute when fillet quality is evaluated and depends primarily on the handling, processing, and storage. Their high levels of moisture, nutrient content, and pH render fillets easily perishable, often going bad within a short period of time post-mortem under refrigerated conditions. Several post-mortem changes are caused by the breakdown of the cellular structure and biochemistry, protein degradation, lipid oxidation, the production of undesirable compounds, and microorganism growth. The changes that occur in fish muscle post-mortem have a significant impact on fillet freshness and consumer acceptance. Therefore, the evaluation of large yellow croaker fillet freshness post-mortem is important and of economic and scientific interest. Some studies have evaluated the freshness and quality of fish by focusing on chemical, physical, histological, and microbiological indicators. Given that the results of these analyses do not always correlate well, it is important to use all of these methods to define the freshness of a particular species of fish.[4−8]

For these reasons, the objective of our study was to evaluate post-mortem freshness changes in large yellow croaker fillets using sensory, microbiological, physicochemical, transcriptomics, and proteomics analyses over a period of 20 days. All of the evaluated parameters were used to identify those factors that are most sensitive to post-mortem changes to explore the changes occurring in the fillet that may be related to freshness.

MATERIALS AND METHODS

Materials. Forty live commercial-sized large yellow croaker with an average weight of 700 ± 50 g were purchased from an aquatic farm in Zhoushan, Zhejiang province, China.

Fish and Tissue Collection. The fish were removed from the water and sacrificed by hypothermia (30 min of immersion in ice-cold water). The samplings was performed at the time of death by taking a fragment of white muscle tissue (1.5 × 1.5 × 1.5 cm) from the lateral−dorsal muscle quadrant (epiaxial) of each fish, and a total of 40 muscle samples were vacuum-packed in a polyethylene bag and stored for up to 20 days post-mortem in a refrigerator at 0 °C. The fillets were used on each sampling day to perform the different analyses. Fish samples were taken randomly on days 0, 5, 10, 15, and 20 of storage to assess the microbiological, physicochemical, and sensory parameters; the levels of transcripts coding for cathepsin L; and proteomics.

The experiment was carried out at the College of Food Science and Biotechnology of Zhejiang Gongshang University (Hangzhou, China). All procedures were approved by the Animal Care Committee of the University of Zhejiang Gongshang and conducted according to the guidelines of the Zhejiang Province Committee on Animal Care.

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Microbiological Analysis. The total viable counts (TVC) were determined in plate count agar following the Chinese National Standard (2008) (GBT 4789.2-2008). A sample (10 g) taken from the fillets was transferred aseptically into a polyethylene tube containing 90 mL of 8.5 g/L sterile NaCl water and then homogenized for 1 min. Further decimal dilutions were made, and then 0.1 mL of each dilution was pipetted onto the surface of the plate count agar (Base Bio-Tech, Hangzhou, China) plates, which were incubated for 72 h at 30 °C.

Physicochemical Analyses. *Colorimetric Measurement.* Flesh color was measured at three points in the fillet. A Minolta Chroma meter CR400 (Minolta, Osaka, Japan) was used for the color measurements. In this system, the L^* scale represents lightness (L^* = 0 for black, L^* = 100 for white), the a^* scale represents red/green ($+a^*$ intensity in red, $-a^*$ intensity in green), and the b^* scale represents yellow/blue ($+b^*$ intensity in yellow, $-b^*$ intensity in blue). The color intensity is expressed by a chroma value (C^*_{ab}); the hue (H^0_{ab}) corresponds to the name of the color as found in its pure state on the spectrum. These values were calculated according to the formulas

$$C^*_{ab} = (a^{*2} + b^{*2})^{1/2},\ H^0_{ab} = \arctan(b^*/a^*)$$

The instrument was calibrated against a white standard under the same light conditions and temperature. The analysis was performed four times on each fillet surface.

Determination of Texture. Texture profile analyses (TPA) were performed as described by Li et al.[9] using a TA-XT2i Texture Analyzer (Stable Micro System, Surrey, UK). Texture variables such as hardness, gumminess, adhesiveness, cohesiveness, chewiness, and springiness were calculated as described by Bourne.[10] The samples, measuring 1.5 × 1.5 × 1.0 cm, were compressed perpendicularly using a 5 mm cylindrical probe (P/5). The testing conditions were two consecutive cycles at 25% compression, cross-head movement at a constant speed of 0.5 mm/s, and a trigger point of 0.05 N. Four measurements were run on each fillet just above the midline.

Determination of pH. A 10 g sample of the fish flesh was homogenized in 100 mL of distilled water, and the resultant mixture was filtered. The pH of the filtrate was measured using a digital pH-meter (FE20/EL20; Mettler Toledo, Shanghai, China).

Thiobarbituric Acid Reactive Substances (TBARS) Determination. Thiobarbituric acid (TBA, mg MDA/kg muscle) value was determined according to the method described by Botsoglou et al.[11]

K Value Determination. ATP and its breakdown products were analyzed according to the method of Li et al.[12] As for the K value, it was defined according to the formula[13]

$$K\ \text{value}\ (\%) = [(\text{HxR} + \text{Hx})/(\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx})] \times 100$$

Sensory Evaluation. Raw fillets were used for the sensory analysis. A panel of seven judges was trained according to standard ISO 8586-1[14] to evaluate the appearance, odor, and texture of the flesh. The sensory evaluation was based on a five-point scale (5, extremely desirable; 0, extremely unacceptable) to determine the samples. The shelf-life criteria assumed that rejection would occur when at least half of the panel members rated the sensory attributes below 4.

Real-Time PCR Analyses of Large Yellow Croaker Cathepsin L mRNA. *Large Yellow Croaker Cathepsin L cDNA Sequences.* An RNA stability time course study on the large yellow croaker would provide quantitative measurements of RNA degradation in post-mortem samples and might be used as an indicator of the post-mortem interval.[5,30] Therefore, we first investigated the integrity of the total RNA extracted from large yellow croaker muscle stored for 20 days post-mortem at 0 °C. Subsequently, the presence of specific transcripts encoded by the large yellow croaker cathepsin L genes was determined by real-time PCR analyses after isolation of the cDNA sequences by molecular cloning and sequencing techniques.

A BlastN search was performed on the Genbank nucleotide database for cathepsin L in other fish species. Primer design was based on the alignment of two cathepsin L coding sequences available in the NCBI Genbank database: *Lates calcarifer* (accession no. AY795481) and *Ictalurus punctatutus* (accession no. EU915299). A strategy based on the regions of strong nucleotide conservation was used to design the primers. The sequences of the primers used for the molecular cloning and sequencing of the cathepsin L were the following:

1 F1 (5′-gdvtgrtbtgggagawraac-3′) and R1 (5′-trgcratvccaca-syggttbt-3′)
2 F2 (5′-atgaaycrbttyggdgayatg-3′) and R2 (5′-ccagctgtthttba-cratcca-3′)

(r = a or g, y = c or t, s = c or g, w = a or t, h = a or c or t, b = c or g or t, v = a or c or g, d = a or g or t).

cDNA Synthesis. RNA samples were extracted and purified from large yellow croaker muscle using an RNA cleanup kit according to the manufacturer's protocol (Bio-Rad, USA). Spectrophotometric analysis at A260/280 nm provided the concentration of RNA for cathepsin L. After extraction, RNA was reverse transcribed into cDNA with MMLV reverse transcriptase kit (Invitrogen) following the manufacturer's instructions. To perform PCR, a 4 μL aliquot of the resulting cDNA was amplified with platinum PCR supermix high fidelity (Invitrogen) to a final volume of 25 μL containing 10 pmol of each designed cathepsin L primer set.

The PCR conditions were as follows: 2 min at 94 °C followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 68 °C. The PCR products from each target gene amplification were then cloned using the *pUCm-T* cloning vector system (Promega) and sequenced in both directions.

Quantitative Real-Time PCR Analysis. The transcript copies of the target gene were quantified by comparison with a standard curve constructed using the known mRNA copy number of this gene. To this end, forward and reverse primers were designed on the basis of the mRNA sequences of large yellow croaker cathepsin L that were identified in this study (Genbank accession no. JN835256). The real-time PCR primers were F1 (5′-cagggtcagtgtggctcttgct-3′) and R1 (5′-cgttgccctctggtctggaaca-3′), which were designed on the basis of the cathepsin L sequences using Primer Premier 6.0, and the gene-specific fluorogenic probes were designed by Beacon Designer (Applied Biosystems, USA).

The β-actin gene (Genbank accession no. EU443733) was used as an endogenous control for normalization. Standard curves for the cathepsin L gene and the endogenous control were constructed using 10-fold serial dilutions of the corresponding plasmid. Quantitative PCR was performed for each cDNA sample on a Bio-Rad iQ 5 real-time PCR detection system (Bio-Rad, USA). Predefined amounts of mRNA at 10-fold dilutions were subjected in triplicate to real-time PCR, including the following: ddH_2O, 10.5 μL; SYBR Premix ex Taq (2×), 12.5 μL; PCR-F (10 μM), 0.5 μL; PCR-R (10 μM), 0.5 μL; and template cDNA, 1.0 μL in a 25 μL reaction volume. The RT-PCR conditions were as follows: 1 min at 95 °C followed by 45 cycles consisting of 10 s at 95 °C, 25 s at 60−63 °C (collect fluorescence), and a melting point curve analysis at 55−95 °C.

The standard curves were run on the same plate of the samples. For each sample, the amounts of the cathepsin L gene and β-actin reference were determined from the appropriate standard curve. The cycle threshold (C_T) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The C_T values were used to create standard curves to serve as a basis for calculating the absolute amounts of cathepsin L mRNAs in the total RNA.

Emulsion Activity (EA) And Emulsion Stability (ES). EA and ES were measured according to the method of Sathivel et al.[15] with some modifications. Minced fish muscle (3 g) was mixed with 40 mL of chilled 0.8 M NaCl (pH 7.4) and homogenized in a polytron homogenizer (Kinematica, Switzerland) at 12000 rpm for 1.5 min. The resultant extract was centrifuged using a refrigerated centrifuge (REMI R24, India) at 20000*g* for 20 min at 4 °C, and the supernatant containing the protein solution was collected. The protein concentrations in the extracted solutions were estimated using the Bradford Protein Assay Kit from Beyotime Institute of Biotechnology (Shanghai, China). These kits extracted 12 mL of 0.5% protein solutions in a 50 mL centrifuge tube, to which 1.2, 2.4, 3.6, 4.8, 6.0, 7.2,

8.4, 9.6, 10.8, and 12 mL of 100% pure soybean oil (Dongling Co., Ltd., Guangzhou, China) were added. The mixture was homogenized using an emulsion probe at 10000 rpm for 2 min in a 50 mL plastic centrifuge tube. Next, 25 mL of 0.1% SDS solution was added to 250 μL of these emulsions, the resultant solution was oscillated for 20 s, and then the sample absorbances were measured in duplicate using a spectrophotometer (Biomate 3, Thermo Spectronic, USA) at a wavelength of 500 nm. A series of absorbance values was observed when the oil capacity of the protein of each fillet emulsion reached a maximum and the emulsion collapsed to form a water-in-oil emulsion. At that point, the optimum oil phase volume (3.6 mL) was measured by calculating the quantity in milliliters. The EA was expressed as the maximum absorbance value for the optimum oil phase volume (3.6 mL) for 3 g of fillet muscle. The EA was determined from the absorbance measured immediately after emulsion formation, and the ES was estimated by following the time-dependent change in the absorbance readings at 500 nm.

Two-Dimensional Gel Electrophoresis. Fish white muscle samples are suitable for comparative proteomics, and the influence of individual differences can be avoided by using the method reported by Weinkauf et al.[16] with some modifications. Approximately 100 mg of frozen samples was crushed in a mortar containing liquid nitrogen and mixed with 5.0 mL of a solution containing 8 M urea, 2 M thiourea, 4% CHAPS (w/v), 65 mM DTT, 0.8% v/v Carrier Ampholyte (pH 3−10), and a protease inhibitor cocktail. The protein concentration of the sample groups was determined according to Bradford's method using a BSA standard curve.

Samples containing 1.5 mg of total protein were loaded in the rehydration step and separated in horizontal 2-DE using ReadyStrip IPG strips (Bio-Rad, Richmond, CA, USA). Isoelectric focusing (IEF) was performed by running a nonlinear immobilized pH gradient (IPG) strips (0.5 × 180 mm, pH 5.0−8.0) at 50 V for 10 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 11 h using a Bio-Rad Protean IEF Cell (Bio-Rad). The IPG strips were transferred onto 12% second-dimensional gradient slab gels after equilibration, reduction, and alkylation. The proteins were further separated by an SDS discontinuous system at 60 mA for 5 h using a Bio-Rad PROTEAN II xi 2-D Cell. The anode and cathode buffers were Tris-CAPS buffer with the addition of 15% methanol or 0.1% SDS, respectively. After this second-dimensional separation by SDS-PAGE, the gels were stained with Coomassie Brilliant Blue G-250.

Protein Identification. Each sample was run in triplicate, and the resultant 2-DE pictures were analyzed by PDQuest 2-D analysis software (Bio-Rad). Matched spots of interest were manually excised from the gels, destained, and subjected to O/N tryptic digestion. Peptide mixtures were then collected by squeezing with acetonitrile and centrifugation, then acidified, dried, resuspended in formic acid, and stored at −20 °C. Protein identification and mass spectrometry techniques were performed as described by Lu et al.[17]

Statistical Analysis. Values are expressed as the mean ± standard deviation. Descriptive statistics, one-way ANOVA, multiple comparison with the Tukey test, and factorial analysis were performed using SPSS software (version 18.0) (SPSS, Chicago, IL, USA). The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Microbiological Analysis. The changes in the microflora of large yellow croaker fillets during storage under vacuum conditions at 0 °C are given in Figure 1. The total viable counts (TVC) exhibited significant linear increases as a function of the storage duration, yielding very high coefficients ($r = 0.963$, $P < 0.05$). The ICMSF[18] has established a microbiological acceptability limit of 7 log CFU/g for human consumption. The initial (day 0) TVC (3.14 log CFU/g) indicates that the simple fillets were of good quality. The fillet shelf life was determined using regression analysis (log CFU/g = 0.201×day + 3.14, $r = 0.963$, $P < 0.05$). On the basis of the regression equation, the TVC limit would have been reached on day 19 of storage. Generally,

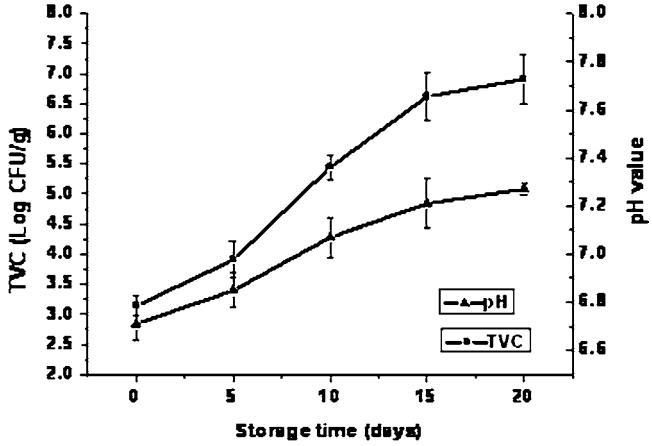

Figure 1. Post-mortem changes in pH and TVC of large yellow croaker fillets during storage under vacuum conditions at 0 °C for 20 days (bars represent the standard deviation from triplicate determinations).

the total viable count is a sensitive parameter; therefore, in the present study, the total viable count was used for the microbiological analysis of large yellow croaker fillets. Similar results were obtained by Taliadourou et al.[19] and Li et al.[9]

Physicochemical Analyses. *Color Measurements.* The surface color parameters of large yellow croaker fillets during cold storage are shown in Table 1. With the exception of L^* and a^*, the color parameters showed a significant correlation with storage time. The value of b^* was significantly correlated with the storage time ($r = 0.525$); the b^* value increased with storage time until reaching a maximum (−2.22 ± 0.30) on day 15 and then decreasing until reaching a minimum on day 20 (−0.45 ± 0.08), reflecting an evolution toward gray-blue tones as the muscle aged. The chroma value (C^*_{ab}) showed significant changes, varying between 2.42 ± 0.02 (day 0) and 1.10 ± 0.08 (day 20) with a correlation coefficient of $r = -0.504$, indicating a reduction in color intensity. Chaijan et al.[20] have attributed color loss to the oxidation of proteins with hemo groups in fish muscle during storage. Furthermore, TBA values were significantly correlated with H^0_{ab} ($r = -0.522$). Due to its autoxidative capacity, hemoglobin is a possible catalyst of lipid oxidation according to Richards et al.[21]

Texture Measurements. The results of the texture parameter analyses for large yellow croaker fillets during storage are also shown in Table 1. The average values for springiness, chewiness, and resilience displayed significant day-to-day changes ($P < 0.05$) and correlated significantly with the storage time ($r = -0.543$, -0.523, and -0.487, respectively). Fish muscle texture involves both fat and collagen content, which are related to muscle fiber density and depend on a number of intrinsic biological factors. The death of the fish triggers autolytic processes that make the muscle softer and less elastic.[22] Our results indicated that denaturation could occur in the myofibrillar protein of large yellow croaker muscle, affecting its texture as shown by the springiness, chewiness, and resilience data during the storage period.

Furthermore, adhesiveness was significantly correlated with b^* ($r = -0.574$) and C^*_{ab} ($r = 0.573$); chewiness was significantly correlated with C^*_{ab} ($r = 0.517$); and springiness was significantly correlated with b^* ($r = -0.58$), C^*_{ab} ($r = 0.748$) and L^* ($r = -0.604$). The color and texture parameters may be used to support the data obtained for the micro-

Table 1. Color Measurements, Instrumental Texture, and Spots/Proteins Identified by MALDI-TOF-MS/MS Analyses during Cold Storage of Large Yellow Croaker Fillets under Vacuum Conditions at 0 °C[a]

	time of storage					
	0 days	5 days	10 days	15 days	20 days	r^b
L^*	46.47 ± 2.88	51.22 ± 2.06	48.43 ± 1.90	46.74 ± 2.09	49.46 ± 3.86	0.075
a^*	1.01 ± 0.31	0.52 ± 0.18	0.62 ± 0.08	0.55 ± 0.21	1.00 ± 0.13	0.004
b^*	−2.20 ± 0.08a	−1.73 ± 0.14a	−2.14 ± 0.30a	−2.22 ± 0.30a	−0.45 ± 0.08b	0.525*
C^*_{ab}	2.42 ± 0.02a	1.81 ± 0.06ab	2.23 ± 0.09ac	2.29 ± 0.04ac	1.10 ± 0.08b	−0.504*
H^0_{ab}	−1.14 ± 0.05	−1.27 ± 0.07	−1.29 ± 0.04	−1.33 ± 0.07	−0.42 ± 0.11	−0.045
hardness	270.86 ± 18.82	276.91 ± 18.49	297.22 ± 8.61	245.77 ± 4.62	276.66 ± 6.46	−0.065
adhesiveness	−10.84 ± 0.55	−24.61 ± 0.54	−17.23 ± 0.57	−11.37 ± 0.41	−23.13 ± 0.35	−0.144
springiness	1.05 ± 0.08a	0.86 ± 0.07b	0.91 ± 0.04b	0.92 ± 0.05b	0.85 ± 0.07b	−0.543*
chewiness	130.93 ± 7.43a	94.52 ± 5.12b	113.04 ± 3.78ab	98.21 ± 1.33b	91.922 ± 3.41b	−0.523*
gumminess	124.47 ± 9.35	106.83 ± 3.73	123.89 ± 2.95	107.12 ± 4.06	106.99 ± 2.05	−0.326
cohesiveness	0.47 ± 0.06	0.40 ± 0.05	0.42 ± 0.04	0.44 ± 0.04	0.40 ± 0.07	−0.257
resilience	0.21 ± 0.03a	0.14 ± 0.02b	0.14 ± 0.01b	0.15 ± 0.03b	0.14 ± 0.04b	−0.487*

spot	protein name	accession no.	theor pI/ M_w, Da	protein score	total ion CI%
5109	similar to actin, γ2 propeptide	gil224070339	5.52/201976.9	186	100
3010	muscle creatine kinase	gil45361591	6.32/43148.6	89	99.99
5108	actin, α skeletal muscle isoform 4	gil296230233	5.27/37575.6	118	100

[a]Mean ± standard deviation. Data within the same row with different lower case letters are significantly ($P < 0.05$) different. [b]r, correlation value. * indicates a significant correlation ($P < 0.05$).

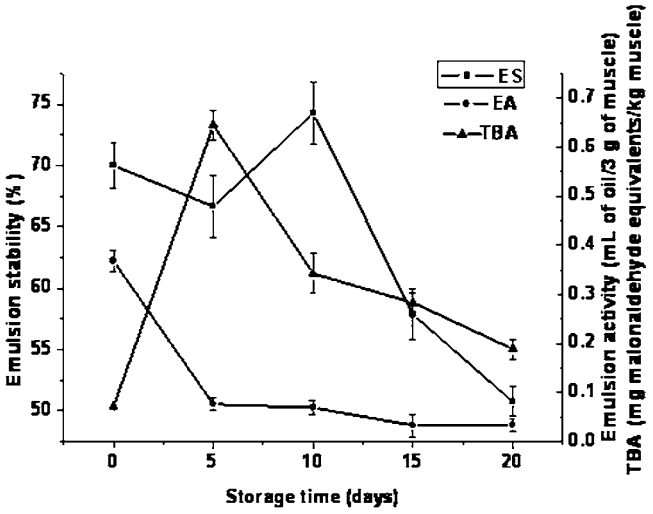

Figure 2. Post-mortem changes in TBA, EA, and ES of large yellow croaker fillets during storage under vacuum conditions at 0 °C for 20 days (bars represent the standard deviation from triplicate determinations).

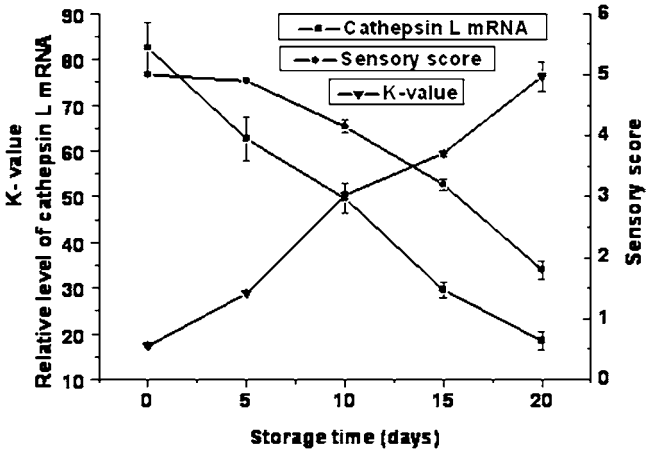

Figure 3. Post-mortem changes in relative cathepsin L mRNA levels, K value, and sensory score of large yellow croaker fillets during storage under vacuum conditions at 0 °C for 20 days (bars represent the standard deviation from triplicate determinations).

biological, sensory, transcriptomics, and protein functional properties in this study.

pH Measurement. Changes in the pH of the muscle of vacuum-packaged large yellow croaker fillets over 20 days of storage are shown in Figure 1. The initial pH of the fish samples was 6.71. The post-mortem pH values are influenced by the species, catching season, diet, activity level, and other factors. In the present work, the pH exhibited statistically significant ($P < 0.05$) changes for large yellow croaker fillets over the entire period of storage in vacuum packaging. The pH values exhibited a significant correlation with storage time (r = 0.957), gradually increasing with each sampling day. The increase in pH might be attributed to an increase in the volatile bases produced by either endogenous or microbial enzymes and/or the buffering capacity of muscle. This result was similar to that of Etemadian et al.[23] Their study revealed that pH measurements could be useful for monitoring changes in large yellow croaker fillets.

Determination of TBARS. Lipid oxidation in fish flesh depends on numerous factors such as the species, preservation temperature, and fat composition. TBA value has been broadly used to describe the degree of lipid oxidation, and the presence of TBARS is due to second-stage autoxidation, during which peroxides are oxidized to aldehydes and ketones. In this study, the TBA values of large yellow croaker fillets during refrigerated storage are shown in Figure 2. The TBA value ranged from 0.07 (day 0) to 0.19 mg MDA/kg (day 20), with all of the daily differences being statistically significant ($P < 0.05$). However, the TBA values did not correlate significantly with time. Interestingly, the TBA values are below the thresholds for spoiling in lipid spoilage indices.[24] Therefore, the lipid oxidation observed in this study was minimal.

In our study, the TBA variations showed a very significant correlation with H^0_{ab} (r = 0.522) and EA (r = 0.522), as the

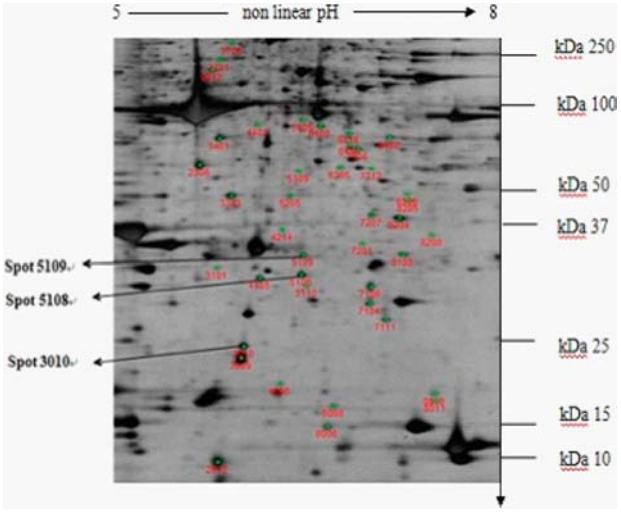

Figure 4. Coomassie Brilliant Blue G250-stained 2-DE gels of fish muscle. A total of 1.5 mg of protein was loaded, and 2-DE was performed using a pH range of 5−8 in the first dimension and SDS-PAGE (12%) in the second dimension. Using PDQuest 2-D analysis software, a cutoff value of 2.5 was used to select out protein spots that were significantly different (nested ANOVA, $P < 0.05$) in expression level between the 0 and 20 day samples. The selected protein spots were numbered for further analysis. From left to right, 5−8 nonlinear p*I* gradient; from top to bottom, 250−10 kDa range.

oxidative reactions can be catalyzed by the endogenous protease of the fish itself, which may contribute to the changes in the color and protein functional properties by forming covalent bonds with muscular proteins.

Determination of K Value. The *K* value, which is the index of the degradation of ATP, is used as a freshness index in many species.[25] Variation of *K* value in the muscle of large yellow croaker during superchilling storage under vacuum conditions is shown in Figure 3. A gradual increase in *K* value was observed with storage time. The initial *K* value in fish samples was 5.07 ± 0.11%, which was in agreement with that of Li et al.[2] The *K* value of fillets rose continuously and reached 45.58 ± 1.22% on day 20 of cold storage. Saito et al.[13] depicted fish products with *K* values <20% as very fresh ones, <50% as moderately fresh, and >70% as not fresh. On the basis of these *K* value categories, the fish samples could be considered very fresh until at least the fifth day and moderately fresh at the end of storage.

Sensory Analysis. The loss of freshness changes the sensory parameters, which have a direct effect on the customer's acceptance of the product. The first sensory changes to occur in stored fillets are related to appearance and texture.[26] In agreement with Huss' findings,[26] the first change we observed in the fillets was a deterioration in muscle appearance, springiness, chewiness, and resilience on day 5 of storage.

Changes in the sensory score of large yellow croaker fillet during storage are shown in Figure 3. On day 0 of storage, the

Table 2. Rotated Factor Loading Matrix (Varimax) for the Communality Attributes and Values and Factor Statistics

		factor[a]				
		1	2	3	4	5
time of storage		−0.913*	0.367	0.042	0.03	0.16
color	L^*	−0.3	−0.629	0.239	−0.465	0.267
	a^*	0.062	0.252	0.248	0.715*	−0.348
	b^*	−0.69	−0.208	0.547	−0.207	−0.216
	C^*_{ab}	0.699*	0.342	−0.362	0.453	0.017
	H^0_{ab}	0.079	0.107	0.727*	−0.425	−0.282
texture	hardness	−0.059	−0.549	0.566	0.549	0.237
	adhesiveness	0.459	0.721*	−0.252	−0.213	0.184
	springiness	0.778*	0.48	0.072	0.254	−0.076
	chewiness	0.734*	0.169	0.415	0.266	0.399
	gumminess	0.482	−0.051	0.565	0.172	0.642
	cohesiveness	0.546	0.589	−0.086	−0.492	0.294
	resilience	0.693	0.395	0.225	−0.453	0.057
physicochemical analysis	pH	−0.898*	0.308	−0.062	0.232	0.128
	K	−0.893*	0.381	0.071	0.058	0.197
	TBA	−0.173	−0.688	−0.571	−0.039	0.178
microbiological analysis	TVC	−0.906*	0.358	0.014	0.05	0.206
sensory analysis	sensory	0.847*	−0.467	−0.239	0.004	−0.013
transcriptomics analyses	cathepsin L mRNA level	0.908*	−0.313	−0.002	−0.048	−0.133
protein functional properties analyses	EA	0.853*	0.092	0.372	−0.046	−0.319
	ES	0.732*	−0.366	−0.16	0.046	0.283
eigenvalue		9.526	3.651	2.573	2.184	1.431
total variance, %		45.362	17.384	12.251	10.399	6.813
cumulative eigenvalue		45.362	62.747	74.998	85.397	92.21

[a]* indicates a loading >0.7.

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large yellow croaker fillets had a pleasant appearance, odor, and texture that were characteristic of fresh fish. On day 20, the total score of appearance, odor, and texture reached 1.8, which is lower than the acceptable limit (score = 4). Generally, odor was a more sensitive attribute than appearance and texture; therefore, odor was used for the sensory evaluation of large yellow croaker fillets and the determination of freshness. The results of the sensory evaluation (appearance, odor, and texture) correlated relatively well with storage time (r = −0.959) and b^* (r = −0.611), C^*_{ab} (r = 0.521), TVC (r = −0.941), pH (r = −0.894), K value (r = −0.954), levels of transcripts coding for cathepsin L (r = 0.906), EA (r = 0.593), and ES (r = 0.833).

The results of the sensory analysis demonstrate that the selected attributes are good indicators of fillet deterioration during cold storage. Similar results have been obtained for other marine species.[4,27] Nevertheless, the results of the sensory analysis must be confirmed by microbiological analysis, which is based on microbiological standards specifically developed for fish and aquaculture products.

Cathepsin L mRNA Analysis. *Cathepsin L cDNA Sequences.* Cathepsin L is believed to contribute to the autolytic degradation of fish muscle because it is far more active at acidic pH and has been shown to digest both myofibrillar proteins (actomyosin) and connective tissue.[28]

Two cDNA fragments were obtained using the primers designed for cathepsin L. Then, by connecting the sequences of the partially overlapping clones, a partial coding sequence (773 bp) for large yellow croaker cathepsin L was determined and deposited in Genbank under accession no. JN835256.

Levels of Transcripts Coding for Cathepsin L. The action of proteolytic enzymes contributes to the progressive deterioration of fillet quality during post-mortem storage. An increasing number of studies address the post-mortem modifications and degradation of these proteases at the protein level;[6] however, few studies have been performed on the dynamics of their specific mRNAs in fish muscle tissues during post-mortem storage.

In this study, we identified for the first time the cDNA sequences coding for cathepsin L in large yellow croaker and investigated the stability of total RNA and the presence of cathepsin L mRNAs in muscle fragments of large yellow croaker in relation to post-mortem storage time. The levels of transcripts coding for cathepsin L in the muscle of large yellow croaker stored for 20 days post-mortem at 0 °C are shown in Figure 3. Cathepsin L mRNA levels exhibited a significant correlation with storage time (r = 0.961), gradually decreasing with each sampling day. Similar to what was observed for cathepsin L transcript code levels, the mRNA levels differ significantly with the storage time. Statistical analysis of these results shows a significant decrease (P < 0.05) in the level of transcripts from day 0 to day 20 post-mortem when stored at 0 °C, whereas no significant differences were detected between day 5 and day 10 or between day 15 and day 20 post-mortem.

Because it is usually assumed that RNA is highly unstable and degrades rapidly after death, only a few studies have dealt with the post-mortem RNA integrity of skeletal muscle in fish.[5,29,30] In samples stored at 0 °C, the level of transcripts of cathepsin L exhibited a significant correlation with storage time, showing significant differences from day 0 to day 20 post-mortem (P < 0.05). The intense muscular activity of fish slaughtered by hypothermia leads to a rapid decline in ATP reserves and promotes the formation of free radicals pre-mortem, which contributes to rapid RNA degradation. Some mRNA molecules exhibit slower turnover rates due to various activities of intracellular ribonucleases, such as the cellular microenvironment interfering with enzyme activity because of an increasing pH. In addition, the exogenous ribonucleases originating from bacteria may contribute to overall RNA degradation.

Our study is the first to examine the post-mortem level of cathepsin L mRNA in the muscle of large yellow croaker over a long interval post-mortem during cold storage. The data presented here are consistent with previous publications on the post-mortem stability of mRNA in cow,[31] pig,[32] and beef cattle.[33] In conclusion, the results of this study revealed that real-time PCR is suitable to quantify the degree of total RNA fragmentation and the number of copies of specific mRNAs. Overall, RNA degradation is a relatively slow process under the conditions investigated here, and the transcript levels of cathepsin L are present for up to 20 days post-mortem in the muscle of large yellow croaker stored at 0 °C.

EA and ES of Protein Functional Properties. A classic emulsion can be defined as a heterogeneous system consisting of at least two immiscible liquid phases. EA denotes the ability of the protein to rapidly adsorb at the water−oil interface during emulsion formation, preventing flocculation and coalescence. The EA and ES changes of large yellow croaker proteins during storage under vacuum conditions at 0 °C are also given in Figure 2. The EA value exhibited a significant correlation with storage time (r = 0.784), gradually decreasing on each sampling day with some significant differences between different storage lengths. Protein denaturation during storage could have contributed to the decreased solubility by unfolding the protein and exposing the hydrophobic groups located in the interior. The lower solubility of hydrolyzed protein is caused by smaller peptides having fewer available polar residues that can form hydrogen bonds with water. Statistical analysis of these results shows significant differences (P < 0.05) in the EA value of samples from day 0 to 20 post-mortem but no significant differences between days 5 and 10.

An ES value of 52−61% was reported for enzymatically hydrolyzed Atlantic salmon (*Salmo salar*) muscle protein.[34] Protein solubility and hydrophobicity play an important role in emulsion properties. Proteins that retain their tertiary structure at the interface will maintain an extensive intermolecular network through the physical entrapment of fat globules within the protein matrix, formed largely via protein−protein interactions, that forms a more stable emulsion.

The ES value also exhibited a significant correlation with storage time (r = 0.759). As shown, the ES value decreased on day 0 of storage at 0 °C from a value of 0.406, increased on day 5 up to day 10 of storage, and decreased during further storage. Statistical analysis of these results also shows some significant differences (P < 0.05) in the ES value from the value after 15 days of storage at 0 °C up to 20 days post-mortem but no significant differences on the days 0, 5, and 10.

2-DE Gel and Protein Identification. Altered protein expression was quantitatively detected in 41 spots in fish muscle (Figure 4). The altered protein spots were excised and analyzed by MALDI-TOF-MS/MS. Spots 3010, 5108, and 5109 were successfully identified by Mascot search with reliable protein scores (N > 88) and total ion CI% (N > 99%) (Table 1). We found that protein spots 3010, 5108, and 5109 were up-regulated with significant changes in expression and might play an important role in evaluating the post-mortem changes in freshness during chilled storage, as these proteins were mainly

associated with muscle integrity, the degree of the injuries and necrosis of muscle, and muscle-specific creatine kinase.

Factorial Analysis. The factorial analysis using the principal components method to extract factors produced five factors that explain 92.21% of the variance (Table 2). Factor 1 explains 45.36% of the variance, whereas factors 2, 3, 4, and 5 represent 17.38, 12.25, 10.40, and 6.81%, respectively. The variables most closely associated with factor 1 are C^*_{ab} (color); springiness and chewiness (texture); pH and *K* value (physicochemistry); microbiological counts; sensory attributes; level of cathepsin L mRNA transcripts; and EA and ES (protein functional properties) with factor loadings of −0.70, 0.79, 0.73, −0.90, −0.90, −0.91, 0.85, 0.91, 0.85, and 0.73, respectively.

When fish quality is evaluated, freshness is one of the most important attributes. The freshness and quality of the end-product depend on different biological and processing factors that affect the post-mortem fish changes.[35] Post-mortem changes in fish muscle have an important impact on the aquaculture industry. Storage time is a key determinant of the ultimate product quality. In our study, the storage time variable exhibited a very high factor loading (0.913) for factor 1, which reveals the important effect that this variable has on spoilage.

Therefore, the physicochemical, microbiological, and sensory parameters; levels of transcripts coding for cathepsin L; and protein functional properties used in this study proved useful in assessing the freshness changes of large yellow croaker fillets at 0, 5, 10, 15, and 20 days of storage. The parameters that were most sensitive to variations over storage time were color (C^*_{ab}), TPA (springiness, chewiness, and resilience), physicochemistry (pH, *K* value), microbiological counts (TVC), sensory attributes, the level of cathepsin L mRNA, and protein functional properties (EA and ES). Protein spots such as 3010, 5108, and 5109 might play an important role in evaluating the changes in fillet freshness during chilled storage. These spots may therefore be considered good indicators for evaluating the spoilage of large yellow croaker fillets stored under vacuum packaging during chilled storage. The results of these analyses indicated the optimal freshness for large yellow croaker fillets stored under these conditions.

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Notes

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