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Influences of Bleeding Conditions on the Quality and Lipid Degradation of Cobia (*Rachycentron canadum*) Fillets During Frozen Storage

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Abstract

The effects of bleeding methods (iced water bleeding and air bleeding) on the quality and lipid degradation of cobia fillets during frozen storage for 24 weeks at -20 ± 2 °C were investigated. Bleeding significantly retarded lipid hydrolysis and oxidation progress, resulting in lower free fatty acid (FFA), lipid hydroperoxides (PV), thiobarbituric acid-reactive substances (TBARS) and higher phospholipids content (PL) obtained in bled samples. A higher polyunsaturated fatty acid (PUFA) content was observed in the bled samples compared to that of the unbled samples. Ice water bleeding showed the best bleeding method in terms of blood removal, leading to lower heme and non-heme iron contents in the fish muscle. Heme pigments in the fish muscle oxidised during frozen storage, resulting in decreased heme iron content and increased non-heme iron content. The development of lipid hydrolysis and oxidation was in high correlations with the heme and non-heme iron content remained in the fish muscle. Oxidation of lipid and heme pigments was the main cause of the flesh discolouration during frozen storage, resulting in decreased lightness (L* value) and increased yellowish (b* value). Cobia need to be bled before processing to maintain the quality of the product during frozen storage.

Keywords: Cobia, bleeding, lipid oxidation, colour, frozen storage

Introduction

Marine aquaculture is a sector with a high growth globally to improve fish supply and to compensate the decline in wild catch. Cobia (*Rachycentron canadum*) is a relatively new species and has gained as one of the promising candidates for marine culture, mainly due to its fast growth rate, white muscle, high nutritional value and high commercial price. Cobia aquaculture has been farmed in over 23 countries and territories, half of them in the Asian-Pacific region (Nhu et al., 2011). According to the FAO FishStat (2015), the global production of cobia was about 40,329 tons in 2014. Vietnam is the third largest producer of farmed cobia with the estimated production of 1,500 tons in 2008, following the major production of China and Taiwan (Nhu et al., 2011). Cobia fillets contain a high content of polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic (EPA; 20:5*n*-3) and docosahexaenoic acids (DHA; 22:6*n*-3) (Taheri, Motallebi, Fazlara, Aghababyan, & Aftabsavar, 2012). The PUFAs have beneficial health effects to consumers (Murray & Burt, 2001), but they are highly susceptible to oxidation (Masniyom, Benjakul, & Visessanguan, 2005). Lipid degradation consists of lipid hydrolysis caused by

enzyme activity (e.g. lipase and phospholipase) and lipid oxidation. Lipid oxidation is one of the major deteriorative reactions taking place in fish muscle during processing and storage, limiting the shelf-life of fishery products (Duun & Rustad, 2008). Lipid oxidation results in deterioration of flavour, odour, colour, texture and the production of toxic compounds (Richards & Hultin, 2002). Lipid oxidation depends on different factors, such as the amount of lipid present, the degree of unsaturated fatty acids in the muscle, packaging method and conditions during processing and storage (Nguyen, Thorarinsdottir, Thorkelsson, Gudmundsdottir, & Arason, 2012; Taheri et al., 2012). Blood and blood components in the fish muscle are proved to have contributions to lipid oxidation of fish products during processing and storage (Richards & Hultin, 2002). It is well known that heme proteins, including myoglobin (Mb) and hemoglobin (Hb), play an important role in lipid oxidation as prooxidants (Richards, Kelleher, & Hultin, 1998). Mb and Hb are firstly oxygenated to form oxyMb and oxyHb and are then oxidised to form metMb and metHb. The latter metMb and metHb are oxidised to yield perferryl-Mb and ferryl-Mb, perferryl-Hb and ferryl-Hb radicals, which catalyse lipid oxidation (Harel & Kanner, 1985; Kanner, German, Kinsella, & Hultin, 1987; Rao, Wilks, Hamberg, & Ortiz de Montellano, 1994). The iron ions released from heme proteins are thought to promote lipid oxidation (Faustman, Sun, Mancini, & Suman, 2010; Richards & Hultin, 2002). The blood lipids, mainly found as lipoproteins, of bony fish account for around 1.2% to 3% (Lizenko, Regerand, Bakhirev, & Lizenko, 2008; Richards & Hultin, 2002). The main components of blood lipids of fish are phospholipids accounting for about 48% of the total lipids, while triglycerides and cholesterols account for 31% and 21%, respectively (Lizenko et al., 2008). It can be suggested that blood lipids contribute to lipid oxidation of fish muscle since phospholipids are known to be highly susceptible towards oxidation (Pamplona, 2008).

The total blood volume of fish ranges from 1.5% to 7% of the total body weight, depending on fish size and species. The majority of the blood is localized in internal organs, while only 20% is localized in muscular tissues (Huss, 1995). Bleeding of fish is generally carried out to remove most of the blood from the muscles. It is well accepted that bleeding of live fish has positive effects on the quality of fish products by retarding the lipid oxidation (Maqsood & Benjakul, 2011; Nguyen, Karlsdottir, Olafsdottir, Bergsson, & Arason, 2013; Richards & Hultin, 2002; Sakai & Terayama, 2008; Sohn et al., 2007). However, the effects of bleeding on lipid oxidation might be different from species to species (Sakai & Terayama, 2008) and depends on the bleeding conditions, including bleeding time, bleeding medium and temperature (Nguyen et al., 2013).

In Vietnam, cobia are filleted and frozen without bleeding. Therefore, it is associated with rancidity development and discolouration during storage, likely due to lipid oxidation mediated by blood retained in the fish muscle. Thus, the objective of the present study was to investigate the effects of bleeding conditions on the quality and lipid degradation of cobia fillets during frozen storage. Another objective was to evaluate the effects of bleeding conditions on residual blood in the fish muscle.

Materials and Methods

Materials

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Farmed cobia were caught from the cages in Nha Trang bay, Vietnam in January, 2015. The average weight of the fish used in this study was 4.5 ± 0.5 kg. The fish were divided into three groups (each group contained 14 fish). Fish of the first group were unbled (Unbleeding). Fish of the second group were cut the blood vessel between the heart and the gill (throat cut) and bled in the air for 15 min (Air bleeding). Fish of the third group were cut the blood vessel between the heart and the gill (throat cut) and bled in iced water at the temperature of 4 ± 1 °C for 15 min (Iced water bleeding). After bleeding, the fish of three groups were immediately iced in Styrofoam boxes and transported to the JK Fish Processing Company (Nha Trang, Vietnam) where the fish were gutted and filleted. The fillets of each group were individually air packaged in polyethylene bags and arranged in trays prior to freezing. The packaged fillets were frozen in an air blast freezer at -35 °C for 3 h.

All of the chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland).

Storage and sampling

The frozen fillets of three groups were packaged in carton boxes and stored at -20 ± 2 °C for 24 weeks. Samples were evaluated on the arrival and after 2, 4, 6, 8, 12, 16, 20 and 24 weeks of storage. At each sampling point, three fillets of each group were collected randomly and thawed at 2 ± 1 °C for overnight. The thawed fillets were used for determination of colour, water content, lipid hydrolysis and lipid oxidation as well as heme and non-heme content.

Colour measurement

The intensity of the flesh colour was measured by using the Minolta CR-300 chromameter (Minolta Camera Co., Ltd; Osaka, Japan) in Lab* system (CIE, 1976) with CIE IlluminantC as described in Nguyen, Jonsson, Thorarinsdottir, Arason and Thorkelsson (2011). Three positions on the flesh side of the fillets (n = 3) were measured. The average *L**, *a** and *b** value of three measurements for each fillet was used to calculate the mean and standard deviation for each group.

Chemical determinations

Water content was determined as the weight loss during drying at 103 ± 1 °C for 4 h according to ISO 6496 (1999). Results were expressed as percentage of wet weight (n = 3).

Total lipids (TL) of the fish samples were extracted according to the Bligh and Dyer (1959) method. The lipid content was determined gravimetrically and the results were expressed as percentage of wet muscle basis. The rest of extracts was used for determination of free fatty acid content, phospholipids and fatty acid composition analysis.

Lipid oxidation

Lipid hydroperoxides (PV) was determined by the ferric thiocyanate method (Santha & Decker, 1994). The results were expressed as μmol lipid hydroperoxides per g of sample.

Thiobarbituric acid-reactive substances were measured by method of Lemon (1975) with modifications. A 5.0 g sample was homogenized with 10 mL of 7.5% trichloroacetic acid (TCA) using an Ultra-Turrax homogenizer (Kika Labortechnik, T25 basic, Staufen, Germany) at 2400 rpm for 10 s. The homogenate was centrifuged at 1500 × *g* for 15 min at 4 °C (Heraeus Biofuge Stratos Reconditioned 75005289R, Rotor 3335, DJB Labcare Limited, England). A mixture of 0.5 mL of supernatant and 0.5 mL of 0.02 M thiobarbituric acid solution was heated in a water bath at 95 °C for 40 min. The heated samples were cooled down on ice and absorbance was read at 530 nm (Sunrise Microplate Reader, Tecan Austria GmbH, A-5082 Grödig, Austria). The results were expressed as µmol malondialdehyde per kg of sample (µmol MDA/kg) and calculated using a standard curve prepared from 1,1,3,3-tetraethoxypropane (TEP).

Lipid hydrolysis

Free fatty acid content was determined on the TL extract by the method of Bernardez, Pastoriza, Sampedro, Herrera and Cabo (2005), based on complex formation with cupric acetate-pyridine followed by absorbance reading at 710 nm (Sunrise Microplate Reader, Tecan Austria GmbH, A-5082 Grödig, Austria). The FFA content was calculated as micromolar oleic acid (OL) based on standard curve spanning a 2-22 µmol range. Results were expressed as g oleic acid equivalents per 100 g of total lipids extract (g oleic acid Eq/100 g TL).

Phospholipid content (PL) of the fish muscle was determined on the TL extract by using a spectrophotometric method (Stewart, 1980), based on complex formation of phospholipid with ammonium ferrothiocyanate, followed by absorbance reading at 488 nm (Sunrise Microplate Reader, Tecan Austria GmbH, A-5082 Grödig, Austria). The PL content was calculated using a standard curve prepared from phosphatidylcholine (PC). The results were expressed as g phosphatidylcholine equivalents per 100 g of total lipids extract (g PC Eq/100 g TL).

Fatty acid composition determination

The fatty acid composition of the samples was determined by gas chromatography (Varian 3900 GC, Varian, Inc., Walnut Creek, CA, USA) following derivatization of extracted lipids to fatty acid methyl esters (FAME), according to the AOCS (1998). The FAMEs were separated on a Varian 3900 GC equipped with a fused silica capillary column (HP-88, 100 m x 0.25 mm x 0.20 µm film, Agilent Technologies), split injector and flame ionisation detector fitted with a Galaxie Chromatography Data System (Version 1.9.3.2 software). The setting of the oven was as follows: 100 °C for 4 min, then increased to 240 °C at a speed of 3 °C /min and held at this temperature for 15 min. The injector and detector temperature were 225 °C and 285 °C, respectively. Helium was used as the carrier gas and the column flow rate was set to 0.8 ml/min; with a split ratio of 200:1. The programme was based on AOAC (2000). The result of each fatty acid was expressed as g per 100 g lipid.

Determination of total heme and non-heme content

Total heme content of fish muscle was determined according to the method of Gomez-Basauri and Regenstein (1992) with slight modification. Ground sample (2 g) was transferred into a 50 mL polypropylene centrifuge tube

and 20 mL of cold 20 mM phosphate buffer (pH 6.8) was added. The mixture was homogenised (Ultra-Turrax T-25 basic, IKA, Germany) at a speed of 13,500 rpm for 10 sec. The homogenate was then centrifuged (TJ-25 Centrifuge, Beckmann Coulter, USA) at 3000 × *g* for 30 min at 4 °C. The supernatant was filtered with a Whatman No. 1 filter paper. Total heme content was determined by direct spectrophotometric measurements at 525 nm (Sunrise Microplate Reader, Tecan Austria GmbH, A-5082 Grödig, Austria). The total heme content was expressed as mg/100 g sample.

Non-heme iron content was determined according the method of Schricker, Miller, and Stouffer (1982) with a slight modification as described in Maqsood and Benjakul (2011). Ground sample (1 g) was placed in a 10 mL screw cap test tube, and 50 μL of 0.39% (w/v) sodium nitrite was added. A mixture (4 mL) of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65 °C (Memmert, D-91126, Schwabach, Germany) for 22 h, and then cooled down at room temperature (25-30 °C) for 2 h. The supernatant (400 μL) was mixed with 2 mL of the non-heme iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm (Sunrise Microplate Reader, Tecan Austria GmbH, A-5082 Grödig, Austria). The colour reagent was prepared by mixing a 1:20:20 ratio (v/v/v) of: (1) bathophenanthroline disulfonic acid (0.162 g, dissolved in 100 ml of double-deionised water with 2 mL thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution.

The non-heme iron content was calculated from an iron standard curve. The iron standard solution, ranging from 0 to 2 ppm, (400 μL) was mixed with 2 ml of the non-heme iron colour reagent. The concentration of the non-heme iron was expressed as mg/100 g sample.

Statistical analysis

The data sets obtained were analysed by General Linear Modelling (GLM) to investigate the main effects of bleeding conditions on the indicators of quality and lipid oxidation. Means were compared by using ANOVA and Duncan's Multiple-Comparison Test using NCSS 2000 software (NCSS, Kaysville, Utah, USA). Significance of differences was defined at the 5% level ($p < 0.05$).

Results

Changes in water and lipid contents

Changes in lipid and water content of the cobia fillets during frozen storage for 24 weeks are shown in Table 1. The lipid content of all samples decreased slightly with increasing storage time for 24 weeks. The lipid content of unbled samples was lower than that of the air bled samples throughout the storage period. The iced water bled samples had a significantly ($P < 0.05$) higher lipid content compared to that of the unbled samples from week 8 to week 24 of storage time. Similarly, the water content of all samples decreased with extended storage time. A slightly lower water content was observed in the samples without bleeding compared to that of the air bled samples

throughout the storage period. The iced water bled samples remained the highest water content during storage for 24 weeks.

Changes in total heme and non-heme iron contents

Generally, the bleeding significantly affected the heme (Figure 1a) and non-heme iron (Figure 1b) content in the cobia muscle. After freezing (week 0 of storage), the heme and non-heme iron values of the bled samples were significantly ($P < 0.05$) lower than those of the unbled cobia fillets. Furthermore, lower heme and non-heme iron contents were observed in the ice water bled samples compared to those of the air bled samples. The heme iron content of all samples decreased with increasing storage time throughout the storage period for 24 weeks (Figure 1a). The decrease in heme iron content was in accordance with the increase in non-heme iron content as seen in Figure 1b. The non-heme iron content of the bled samples increased slightly during the first 6 weeks of storage and increased rapidly during the subsequent storage time. Whereas, the non-heme iron content of the unbled samples increased significantly after storage for 2 weeks. Significantly lower non-heme iron values were found in the bled samples compared to those of the unbled samples.

Changes in free fatty acids (FFA) and phospholipids (PL) contents

The changes in free fatty acids (FFA) and phospholipids (PL) content of the cobia fillets during frozen storage as affected by bleeding conditions are depicted in Figure 2a and 2b, respectively. After freezing, the FFA content of the unbled samples was higher than that of the bled samples, while a lower PL content was observed in the unbled samples compared to that of the bled samples. The bleeding had a positive effect on retardation of lipid hydrolysis development during frozen storage. The FFA content of all samples increased with increasing storage time (Figure 2a). The unbled samples had a significantly ($P < 0.05$) higher FFA content and lower PL content compared to those of the bled samples throughout the storage period. The FFA and PL content of the ice water bled samples remained lower than those of the air bled samples with extended storage time for 24 weeks.

Lipid oxidation in the cobia fillets during frozen storage

Lipid oxidation in the cobia fillets during frozen storage as influenced by bleeding conditions were measured by monitoring PV and TBARS (Figure 3a and 3b, respectively). Bleeding conditions significantly ($P < 0.05$) affected the lipid oxidation progress during frozen storage. The pattern of changes in PV of all sample groups was similar as seen in Figure 3a. The PV content increased slightly during the first four weeks of storage and then decreased from week 4 to week 8 of storage time. A significant ($P < 0.05$) increase in PV was observed in all samples from week 8 to week 16 of storage period. The PV decreased again with extended storage time from week 16 to week 20 and then increased during the subsequent storage time for 24 weeks. The changes in PV were in harmony with the changes in TBARS as shown in Figure 3b. Generally, the TBARS content of all samples increased slightly during the first 6 weeks of storage. A noticeable ($P < 0.05$) increase in TBARS content was observed in all samples from week 6 to week 12. Subsequently, the TBARS content of the bled samples (air bled and ice water bled samples) was rather stable, whereas the TBARS content of the unbled samples kept on increasing.

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Effects of bleeding conditions on fatty acid profile of cobia muscle

Changes in fatty acid profile of cobia muscle as affected by bleeding conditions after storage for 24 weeks at -20 ± 2 °C are shown in Table 2. The SFA, MUFA and PUFA content of of raw cobia muscle were of 39.12%, 31.51% and 26.86% of total lipid, respectively. After storage for 24 weeks, the lipids of unbled cobia muscle contained 44.12% SFA, 36.56% MUFA and 18.14% PUFA. Whereas, the SFA, MUFA and PUFA content of air bled and ice water bled cobia muscle were of 38.37%, 36.01%, 23.44% and 36.59%, 35.75%, 25.84%, respectively. As expected, the unbled samples had significantly ($P < 0.05$) lower PUFA and higher SFA content compared to that of raw cobia muscle and bled samples. Correspondingly, significantly ($P < 0.05$) higher DHA (C22:6n–3) and EPA (C20:5n–3) values were observed in the bled samples compared to those of unbled samples after storage for 24 weeks.

Changes in colour of cobia fillets

The changes in lightness (L^* value), redness (a^* value) and yellowness (b^* value) of cobia fillets during frozen storage are presented in Figure 4a, 4b and 4c, respectively. After processing, the L^* values of the bled samples were higher than those of the unbled samples. Inversely, higher a^* and b^* values were observed in the samples without bleeding. During frozen storage for 24 weeks, the lightness (L^* values) of all samples decreased rapidly during the first 8 weeks of storage and then were rather stable during the subsequent storage time (Figure 4a). Inversely, a^* and b^* values of all samples increased with increasing storage time (Figure 4b and 4c, respectively). The samples with bleeding had higher L^* values and lower a^* and b^* values compared to those of the samples without bleeding throughout the storage period. The ice water bled samples showed a better flesh colour with higher L^* values and lower a^* and b^* values ($P < 0.05$) in comparison with those of the air bled samples.

Discussion

The lower lipid content in cobia fillets without bleeding was in accordance with the higher lipid oxidation products (i.e. PV and TBARS) obtained in the unbled samples. The decrease in lipid content during storage may be mainly due to lipid oxidation occurred. Similarly, lower water content noted in the unbled samples may be due to higher oxidation rate of lipid and heme proteins in the fish muscle, leading to changes in protein structure. This leads to decrease water holding capacity of the fish muscle, resulting in increased drip loss after thawing. Offer and Knight (1988) have concluded that decreased water holding capacity of the muscle has often been described as an effect of structural alternations such as protein denaturation. The decrease in water content may also be resulted from the drip loss after thawing and water evaporation during frozen storage.

Significantly lower heme and non-heme iron values found in the bled samples reveal that the blood in the fish muscle was effectively removed by bleeding process. The results were in agreement with those published by Maqsood and Benjakul (2011) for Asian seabass and Thiansilakul, Benjakul, and Richards (2010) for red tilapia. However, the heme and non-heme iron contents of cobia fillets were higher than those of Asian seabass and red tilapia. This is thought to be due to the different fish species used, leading to the difference in blood volume and

blood composition. Lower heme and non-heme iron contents were observed in the ice water bled samples compared to those of the air bled samples. The results demonstrate that ice water bleeding method gave more satisfactory blood draining than air bleeding method. This may be ascribed to the higher concentration gradient between the blood vessels and bleeding medium (i.e. ice water) was obtained in the ice water bleeding in comparison with that of air bleeding, leading to higher blood removal rate. Another factor that may have a negative effect on blood removal in air bleeding is the rapid blood coagulation (Richards & Hultin, 2002), leading to block the blood vessels. It has been reported that the clotting time of fish blood in the air is between 5 s and 30 min (Jagadeeswaran & Liu, 1997).

The decrease in heme iron content during storage was probably due to heme breakdown, leading to release non-heme iron (Benjakul & Bauer, 2001). It is well-known that hemoglobin and myoglobin are the most abundant heme proteins in fish blood. Hemoglobin is lost rather easily during handling and storage, while myoglobin is retained by the muscle intracellular structure (Livingston & Brown, 1981). Moreover, Chaijan, Benjakul, Visessanguan, and Faustman (2005) have noted that heme proteins became less soluble due to the deterioration of fish muscle, leading to the decrease in heme iron content. As expected, the heme iron content of the bled samples was significantly ($P < 0.05$) lower than that of the unbled cobia fillets throughout the storage period. Maqsood and Benjakul (2011) have reported the same findings for Asian seabass as the heme iron content of unbled samples was higher than that of the bled samples.

Significantly lower non-heme iron values were found in the bled samples compared to those of the unbled samples. The results suggest that the chemical changes in the unbled fish muscle occurred earlier compared to the bled samples, mainly due to heme protein autoxidation. The increase in non-heme iron content with an increased storage time was in coincidental with the decrease in heme iron content during storage. The increased non-heme iron content is probably due to the disruption of the porphyrin ring during extended storage, resulting in the release of free iron (Gomez-Basauri & Regenstein, 1992; Maqsood & Benjakul, 2011). The denaturation of heme pigments and/or other iron-containing proteins also have contributions to the increase in non-heme iron content (Decker & Hultin, 1990).

The increased FFA values in the cobia fillets during frozen storage were in a negative correlation ($R = -0.76$) with the decrease in PL content, indicating that FFA development was mainly due to PL degradation. It has been well demonstrated during storage of fish products, lipid in the fish muscle is hydrolysed mainly caused by microbial enzyme activity such as lipases and phospholipase A and phospholipase B (Hwang & Regenstein, 1993) and non-microbial enzyme activity (i.e. natural lipase present in the fish muscle) as well as spontaneous lipid hydrolysis (Haas, 2001). Lipases breaks down triglycerides into diglycerides, monoglycerides, free fatty acids and glycerol; while phospholipases cleaves fatty acids from phospholipids. The higher FFA and lower PL values obtained in the samples without bleeding compared to those of the bled samples are believed to be due to the contribution of blood lipids in the fish muscle. It has been reported that the main component of blood lipids of fish are phospholipids accounting for about 48% of the total lipids (Lizenko et al., 2008) and phospholipids are known to be highly susceptible towards hydrolysis and oxidation (Masniyom et al., 2005; Pamplona, 2008). Furthermore, lipoprotein

lipase located on the interior walls of the capillary blood vessels hydrolyses the triacylglycerols, releasing free fatty acids (FAO, 2010).

The increase in PV is believed to drive from the preferential oxidation of phospholipids. The results were in harmony with the decreased PL in all samples. It is well noted that hydroperoxides are primary lipid oxidation products, and their content is much dependent on the ratio between the formation and decomposition of hydroperoxides (Jin et al., 2010). This can explain the PV of all samples increased and decreased during the storage period. The decrease in PV may also be due to interactions of hydroperoxides with protein components (i.e. peptides, amino acids, etc), that has also been reported to form fluorescence compounds (Nguyen et al., 2012). The increase in TBARS content during the first 6 weeks of storage may mainly be attributed to the decomposition of hydroperoxides accumulated in the fish muscle during processing (Nguyen et al., 2012). Further decomposition of formed hydroperoxides led to a significant increased TBARS content from week 6 to week 12 of storage. A highly positive correlation (R = 0.78) between FFA and TBARS was observed in the present study. This suggests that the formation of FFA in the fish muscle may have a contribution to lipid oxidation development. Accumulation of FFA does not affect quality attributes of fish products but has been shown to interrelate with lipid oxidation and have been proposed to have a prooxidant effect on lipids (Waraho, Mcclements, & Decker, 2011). Thiansilakul et al. (2010) have reported fish muscle containing high FFA is more prone to lipid oxidation. FFAs have been shown to undergo as faster oxidation rate than bigger lipid classes of triglycerides and phospholipids (Nazemroaya, Sahari, & Rezaei, 2011).

It is interesting to see that the bled samples had lower PV and TBARS values compared to those of the samples without bleeding. This is believed to be due to the presence of heme and non-heme iron in the fish muscle. Good correlations between heme and non-heme content with lipid oxidation products (i.e. PV and TBARS content) were obtained in this study. According to Richards et al. (1998), heme proteins, including myoglobin (Mb) and hemoglobin (Hb), play an important role in lipid oxidation as prooxidants. Various pathways by which Hb and Mb can promote lipid oxidation have been described, including pseudolipoxygenase activity (Everse & Hsia, 1997), perferryl-Mb and ferryl-Mb, perferryl-Hb and ferryl-Hb radicals catalysed lipid oxidation (Harel & Kanner, 1985; Kanner et al., 1987; Rao et al., 1994) and iron ions released from heme proteins to promote lipid oxidation (Faustman et al., 2010; Richards & Hultin, 2002) as well as lipoxygenase products that are known to enhance lipid oxidation (Pettitt, Rowley, & Barrow, 1989). Moreover, the fish blood also contains large amount of white blood cells, which is believed to generate superoxide, hydrogen peroxide and hydroxyl radical (Gabig & Babior, 1981). The results from the present study were in agreement with those published by Richards and Hultin (2002) for whole trout and light muscle of mackerel, Maqsood and Benjakul (2011) for Asian seabass. On the contrary, Porter, Kennish, and Kramer (1992) have reported that bleeding did not reduce lipid oxidation of salmon muscle during frozen storage. Ando, Nishiyabu, Tsukamasa, and Makinodan (1999) found that bleeding treatment did not sufficiently reduce hemoglobin to a low concentration enough to reduce lipid oxidation of yellowtail during chilled storage. Simply removing a portion of the blood from live yellowtail by bleeding is not sufficient to prevent lipid oxidation in the early stage of ice storage (Sohn et al., 2007). The differences in effect of bleeding on retardation lipid oxidation may be ascribed to the different fish species used, leading to the differences in blood composition.

The different bleeding techniques applied and different storage temperatures may also have effects on lipid oxidation.

The results of lipid oxidation were in accordance with the fatty acid profile of cobia muscle. A lower PUFA content observed in the unbled samples compared to that of the bled samples (air bled and ice water bled samples) is believed to result from faster oxidation of PUFA in the unbled samples, especially EPA (C20:5n-3) and DHA (C22:6n-3) that are highly susceptible to oxidation (Masniyom et al., 2005). The results were in harmony with lower EPA (3.47%) and DHA (8.40%) contents obtained in the unbled samples compared to those of air bled samples (4.70% EPA and 9.92% DHA) and of ice water bled samples (5.05% EPA and 10.33% DHA). The lower PUFA content was in correspondence with the higher SFA and MUFA content (Table 2). The same findings have been reported by Nguyen et al. (2013) for salted cod during storage and Thiansilakul et al. (2010) for seabass and red tilapia during ice storage. Moreover, among the saturated fatty acids (SFAs), palmitic acid (C16:0) was predominant with the value varied in the range of 21.14% - 23.69%. Oleic acid (C18:1n-9) was substantial among the monounsaturated fatty acids (MUFAs) with the value ranging from 20.60% to 22.67%.

The changes in colour parameters after processing confirm that the oxidation of lipid and heme proteins took place during processing even at low temperature. Lower L^* values and higher a^* and b^* values obtained in the unbled samples were in accordance with higher PV, TBARS, heme and non-heme content noted in the unbled samples after processing. It is well documented that b^* value is closely related to lipid oxidation taking place in the fish muscle. This leads to increased yellow/brown hue and decreased blue/green hue (Nguyen et al., 2013). The lower L^* values obtained in the unbled samples may have resulted from yellow/brownish discolouration of the flesh surface. Moreover, the formation of MetHb and MetMb due to oxidation of Hb and Mb is thought to have effects on the flesh colour (Cai, Grunwald, Park, Lei, & Richards, 2013). The changes in colour parameters of the cobia flesh during frozen storage are mainly due to the oxidation of lipid and heme proteins. It was observed in the present study that the increase in TBARS and PV content had positive correlations with b* value (R = 0.72 and R = 0.76, respectively) and negative correlations with L* value (R = -0.73 and R = -0.69, respectively). Moreover, the decrease in L* value was in positive correlation (R = 0.71) with the decreased heme content and in negative correlation (R = -0.78) with the increased non-heme content. The formation of fluorescence compounds between lipid oxidation products and protein components is believed to cause the formation of yellow/brownish discolouration (Lauritzsen, Martinsen, & Olsen, 1999; Nguyen et al., 2013). The results confirm that Hb, Mb and white blood cells were removed from the fish muscle by bleeding. Consequently, lipid and heme proteins oxidation may be retarded, leading to better colour quality.

Conclusion

The results from the present study indicate that bleeding conditions significantly affected the quality, lipid degradation and heme residues in the cobia muscle. Ice water bleeding showed the best bleeding method in terms of blood removal, leading to lower heme and non-heme iron contents in the fish muscle. The development of lipid degradation (i.e. hydrolysis and oxidation) was in high correlations with the heme and non-heme iron content remained in the fish muscle. Oxidation of lipid and heme pigments was the main cause of the flesh discolouration

during frozen storage, resulting in decreased lightness (L^* value) and increased yellowish (b^* value). It can be recommended that cobia need to be bled before processing to maintain the quality of the product during frozen storage.

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Table 1. Changes in lipid and water content of the cobia fillets during frozen storage (n=3, Mean ± SD).

Treatments	Storage time (weeks)						
	0	4	8	12	16	20	24
Lipid content (%)							
Unbled	12.08±0.12^{a}	11.07±0.91^{a}	8.90±1.08^{b}	8.60±0.44^{b}	7.87±0.45^{b}	7.70±1.08^{b}	7.61±0.74^{b}
Air bled	12.71±0.11^{a}	12.22±0.05^{a}	9.87±0.69^{b}	9.36±1.53^{b}	9.04±0.59^{a}	8.37±1.05^{b}	8.03±0.11^{b}
Iced water bled	12.94±0.06^{a}	12.59±0.08^{a}	11.12±1.25^{a}	10.12±0.66^{ba}	10.36±0.84^{a}	9.24±1.12^{a}	9.09±0.59^{ba}
Water content (%)							
Unbled	70.96±0.31^{a}	69.35±0.41^{a}	67.84±0.48^{a}	67.76±0.28^{a}	64.54±1.14^{b}	63.60±0.95^{b}	61.30±0.40^{b}
Air bled	71.43±0.33^{a}	70.35±0.13^{a}	68.12±0.74^{a}	67.87±0.76^{a}	64.46±1.39^{b}	66.48±0.84^{a}	64.82±0.98^{b}
Iced water bled	70.87±0.48^{a}	70.12±0.14^{a}	68.21±0.45^{a}	68.47±0.73^{a}	68.29±0.78^{a}	68.38±0.50^{a}	68.41±1.08^{a}

Different lowercase letters (superscript) in the same column of lipid and water content indicate a significant difference between treatments ($P < 0.05$)

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Table 2. Influence of bleeding conditions on fatty acid composition of cobia lipids of raw material and after frozen storage

Fatty acids (g/100 g lipid)	Raw	Storage for 24 weeks		
		Unbled	Air bled	Ice water bled
C14:0	5.28	6.18	5.02	4.52
C16:0	18.69[c]	23.69[a]	21.18[b]	21.14[b]
C16:1n–9	3.22	2.25	2.29	3.01
C16:1n–7	1.44	1.48	1.56	1.40
C16:2n–4	0.43	0.40	0.45	0.48
C18:0	8.30	9.67	8.01	7.02
C18:1n–11	1.31	1.27	1.49	1.56
C18:1n–9	21.98[a]	22.67[a]	21.48[a]	20.60[a]
C18:1n–7	0.05	4.35	3.92	3.51
C18:1n–5	0.26	0.29	0.30	0.26
C18:2n–6	4.89[a]	1.77[b]	3.00[a]	4.20[a]
C20:0	1.03	1.54	1.23	1.05
C18:3n–3	0.50	0.27	0.30	0.44
C20:1n–11	0.54	0.48	0.54	0.60
C20:1n–9	0.85	1.93	2.92	3.26
C20:1n–7	0.38	0.19	0.27	0.14
C18:4n–3	0.27	0.25	0.31	0.32
C20:2n–6	1.23	0.18	0.20	0.20
C22:1n–9	0.14	1.22	0.80	0.97
C20:3n–3	2.29	0.09	0.26	0.05
C20:4n–6	0.43	1.37	2.19	2.61
C20:4n–3	0.44	0.36	0.39	0.47
C20:5n–3 (EPA)	5.15[a]	3.47[b]	4.70[a]	5.05[a]
C24:0	1.02	1.25	1.13	1.04
C24:1	0.49	0.43	0.43	0.43
C22:5n–3	1.56	1.45	1.62	1.54
C22:6n–3 (DHA)	10.67[a]	8.40[b]	9.92[b]	10.33[a]
SFA	39.12[b]	44.14[a]	38.37[b]	36.59[b]
MUFA	31.51[b]	36.56[a]	36.01[a]	35.75[a]
PUFA	26.86[a]	18.14[b]	23.44[a]	25.84[a]
Others	2,51	1.16	2.18	1.82

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Different lowercase letters (superscript) in the same row indicate a significant difference between treatments and raw material ($P < 0.05$)

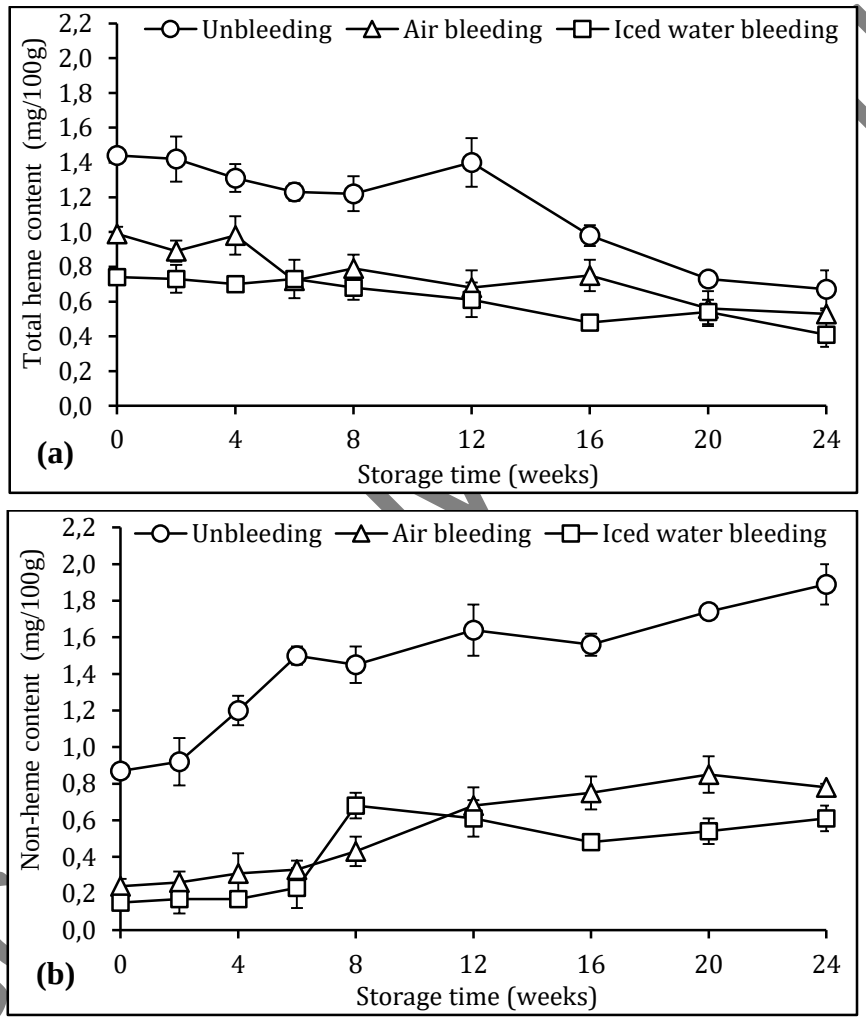

Figure 1. Changes in total heme (a) and non-heme iron (b) of cobia fillets during frozen storage as functions of bleeding conditions [Unbleeding (o), Air bleeding (Δ) and Ice water bleeding (□)].

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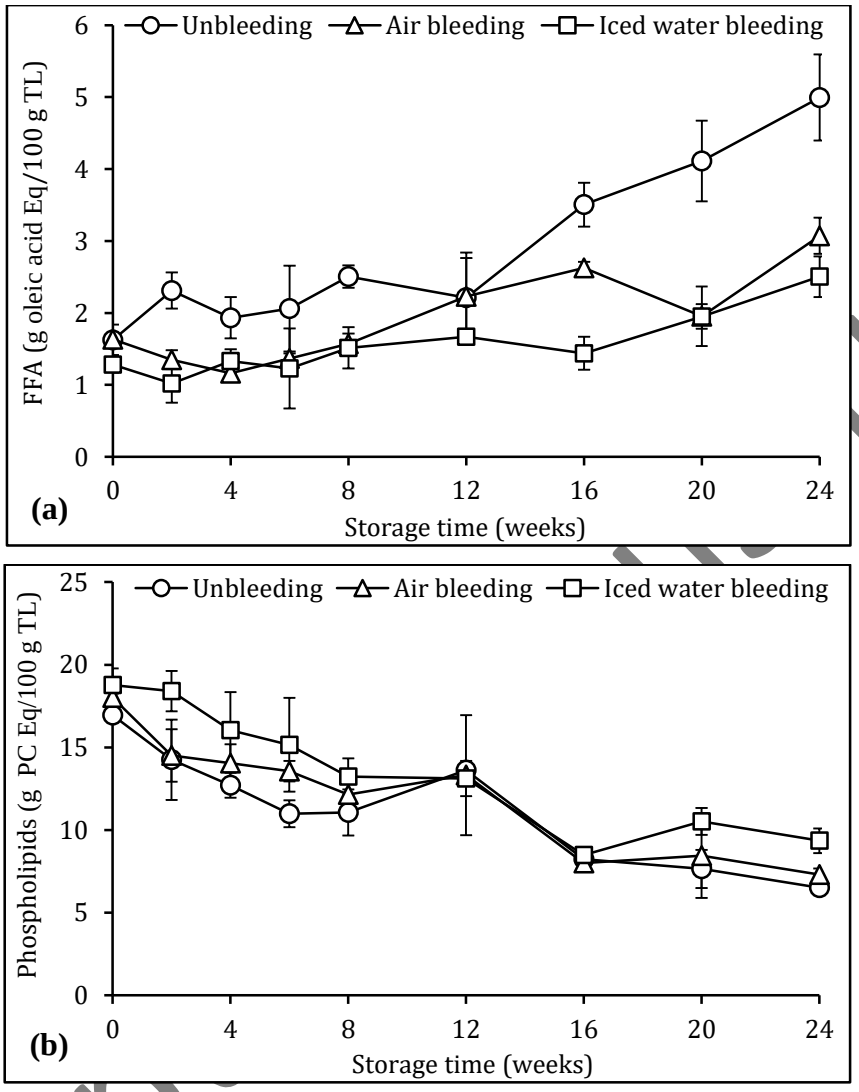

Figure 2. Changes in free fatty acids (FFA) (a) and phospholipids (PL) (b) of cobia fillets during frozen storage as affected by bleeding conditions [Unbleeding (o), Air bleeding (Δ) and Ice water bleeding (□)].

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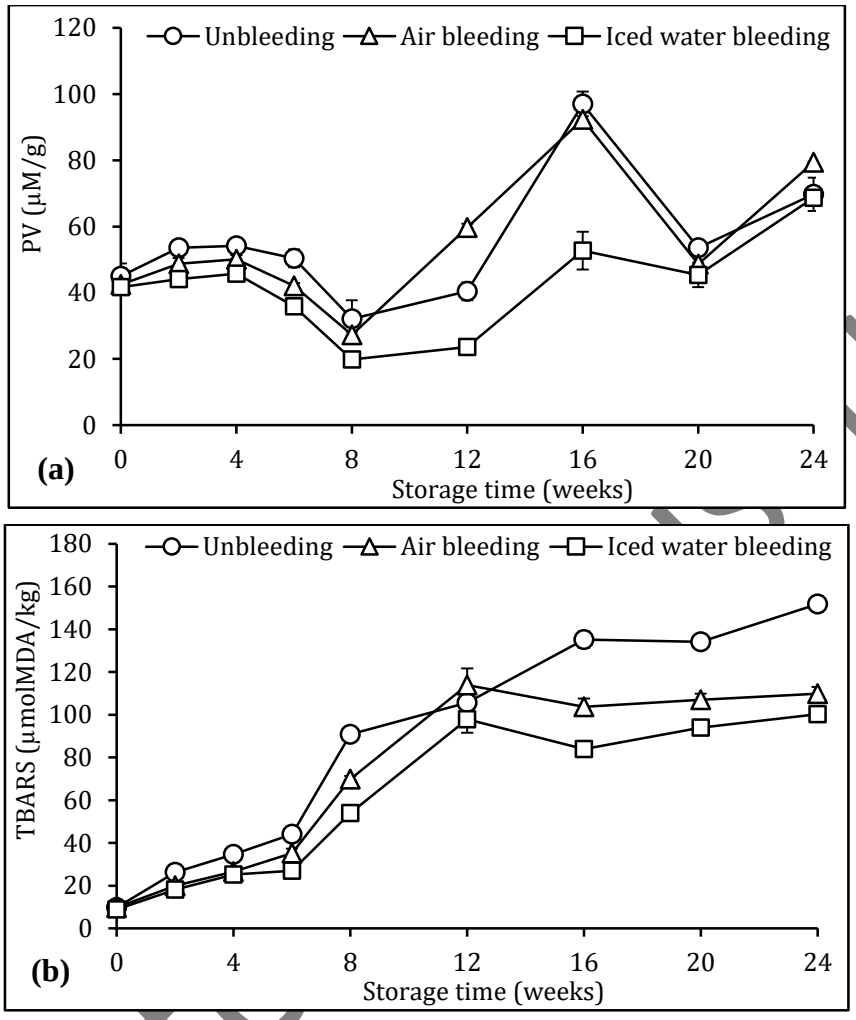

Figure 3. Changes in PV (a) and TBARS (b) content of cobia fillets during frozen storage as affected by bleeding conditions [Unbleeding (o), Air bleeding (Δ) and Ice water bleeding (□)].

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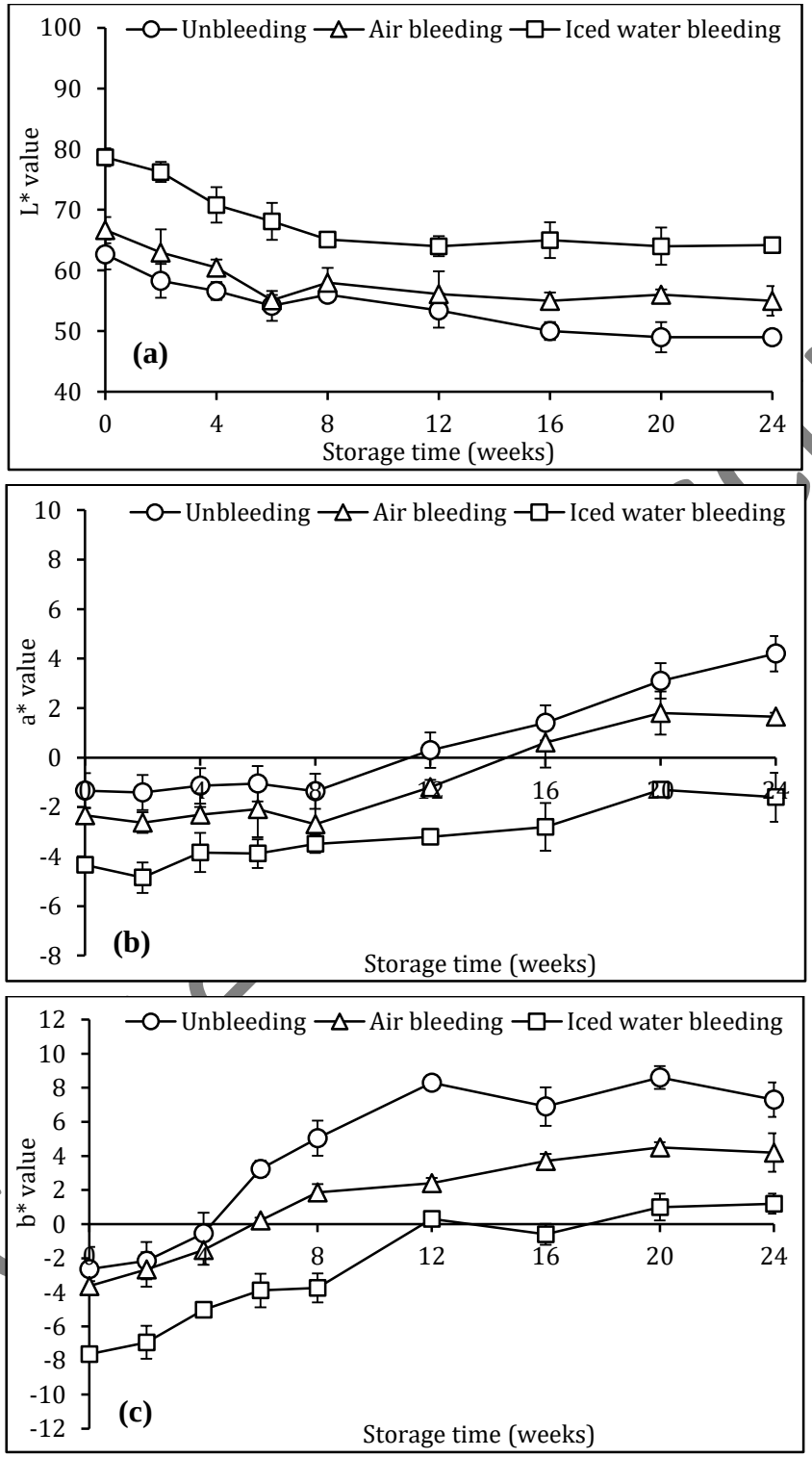

Figure 4. Changes in L* value (a), a* value (b) and b* value (c) of the flesh side of cobia fillets during frozen storage as influenced by bleeding conditions [Unbleeding (o), Air bleeding (Δ) and Ice water bleeding (□)].