

EUTHANASIA OF TILAPIA USING CARBON MONOXIDE FOR COLOR
FIXATION AND COLOR STABILIZATION

By

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This document is dedicated to my loving parents and my sister.

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Tilapia is predominately a white muscle fish that has a small amount of dark lateral muscle. This muscle is an important indicator of fillet freshness, as it changes from a red color to brown on storage. In order to extend the color of the red muscle many tilapia processors are treating their fish with gasses containing carbon monoxide (CO).

The most common treatment method is a post-mortem CO gas treatment of fillets. Currently the application of CO into fish muscle via euthanasia is being performed by a few tilapia processors. There is a lack of information on how the euthanasia of the fish with CO will affect fish quality compared to more conventional applications. The objective of this study was to investigate the color retention and quality of fillets from euthanized tilapia, compared to a 100% CO post-mortem gas treatment.

Live tilapia was placed in a sealed water tank and was euthanized with 100% CO flushed into a circulatory water system. Two studies were performed. In the first study, tilapia was immediately filleted after euthanasia, vacuum packed and frozen (-20°C) for

one month, then thawed and kept exposed to air for 18 days at 4°C. Fillets from non-CO treated fish were subjected to a 30 min treatment with 100% CO. Untreated fillets were used as control. In the second study, CO-euthanized tilapia was gutted, vacuum packed and frozen whole for up to 4 months. For this study, a set of normally slaughtered tilapia was used as control. Before each set of analysis (at 0, 2 and 4 months) the fish was thawed for 24 hours at 4°C. The change in muscle color was analyzed with a digital Color Machine Vision (CMVS) and L*, a* and b* values were recorded. The uptake and stability of CO in the fish muscle and its binding to heme proteins were analyzed with gas chromatography (FID) and spectrophotometry, respectively. The effect of the different treatments on muscle pH and muscle drip loss was also analyzed.

Euthanasia with CO and direct CO treatments on fillets led to a significant ($p < 0.05$) increase in the red color (a*-values) of the muscle, especially the dark muscle. This distinctive cherry red color was maintained for a long period of time for both treatments while a brown color developed for the controls. The color characteristics of the fillets from euthanized fish were more “natural” than those of the 100% CO treated fillets. The UV-Vis spectra and the concentration of CO in the muscle confirmed the uptake of CO by heme proteins and also demonstrated an increase in heme protein stability. CO uptake was significantly higher in dark muscle compared to white muscle. No significant differences were found in pH or drip loss among the treatments.

These results suggest that both CO treatments have a positive effect on color and heme stability, while euthanasia appears to give a more “natural” looking product. In addition, this new method of processing (i.e., euthanasia) has several advantages such as shorter processing time and less product handling.

CHAPTER 1 INTRODUCTION

The variety and the high nutritional content of aquatic foods have created great interest and a high demand for these products. Data provided by the Food and Agriculture Organization (FAO) in 2002[1] shows that the total fish production has reached its highest level ever of 94.8 millions tons in 2000. The high demand for these products is also reflected in an increase of people directly engaged in fisheries and the aquaculture industry. These industries employed an estimated of 35 million people this decade, 7 million more compared to last decade[1]. The high demand for seafood however has depleted many fish stock due to overfishing. World population has been growing faster than the total food fish supply, leading to a decrease in the fish supply per capita[1]. About one billion people rely on fish as their main source of protein [2].The global crisis in capture fisheries and the increasing need for seafood has stimulated the rapid expansion of aquaculture. Aquaculture offers a predictable and consistent supply of high quality seafood [3]. According to the FAO, aquaculture production represented 3.9 % of total fish supply in 1970 and this percentage increased to 27.3% in 2000 [1].

Tilapia and Aquaculture

Tilapia is one of many species that is been aquacultured with great success. Tilapia has a broad tolerance to harsh environmental conditions. They are more tolerant to high salinity, high water temperature, low dissolved oxygen and high ammonia concentrations than most other farmed freshwater fish [4, 5]. Illustrations from Egyptians tombs suggest that tilapia was one of the first fish species cultured, more than 3000 years ago [5].

Tilapia is also known as Saint Peter's fish and it is believed that tilapia was fed to the multitudes by Jesus Christ [5]. From 1950 to mid 1970s tilapia species moved from their native waters in sub-Saharan Africa to Asia and the rest of the world [2, 6]. They were introduced to different parts of the world for various reasons; for instance, they were used as bait for tuna which is how they were introduced to Hawaii [7]. Apparently the introduction of tilapia in the Caribbean, Central and South America was made in order to reduce mosquitoes through aquatic vegetation control [7].

In 2000 farmed tilapia production surpassed 800 thousand metric tons, second only to carp [3, 5]. Tilapia has a high acceptance in the United States and is one of the fastest growing seafood imports into the U.S. along with salmon. According to Knapp [3] in 2002 close to 70 thousand metric tons of fresh and frozen tilapia were imported to the U. S.

Quality of Seafood

Seafood is a highly perishable commodity since it is very susceptible to microbial and chemical spoilage which results in economic losses [8]. Its quality declines soon after harvest and continues once the fish has been processed. The value of the product is highly influenced by its appearance, in particular its color. Tilapia is predominately a white muscle fish that has a small amount of dark lateral muscle. This muscle can however be an important indicator of fillet freshness, as it changes from a red color to brown during storage. Maintaining the color of the dark muscle during processing, transport, storage and display is essential and has an influence on the consumer perception of the product. In order to extend the color of the red muscle and reportedly its shelf life, many tilapia processors are treating their fish with carbon monoxide (CO) and filtered wood smokes (FS) containing CO [9]. Several different treatment methods exist, the most common

being exposure of the fillets briefly (<30 min) to CO gas immediately after harvest and processing while the muscle is still respiring (personal communication, B. Olson, Clearsmoke Technologies). Euthanasia of fish using CO dissolved in water has been proposed by Kowalski [10] and is currently performed by some tilapia processors (personal communication, B. Olson, Clearsmoke Technologies). This new method incorporates carbon monoxide to the edible muscle of the fish through the respiratory and circulatory system of the animal. Fillets from fish euthanized with 100% CO have a distinctive and stable cherry red color characteristic of a CO exposure/treatment.

CHAPTER 2 LITERATURE REVIEW

Tilapia Taxonomy

Tilapia is a generic term used to designate a group of commercially important food fish that belongs to the Cichlidae family [4]. Tilapias have been classified into three genera based on the type of care the parents provided to their young [2, 4]. The genera *Oreochromis* and *Sarotherodon* are mouthbrooders. The eggs are fertilized in the nest but parents immediately pick them up in their mouths and protect them and incubate the young for several days after hatching [2, 4, 5]. A difference between these two genres is that in the *Oreochromis* genera only the female parents practice the mouthbrooding. This genus of female mouthbrooding is the most important in aquaculture and it includes the Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and blue tilapia (*O. aureus*) [2]. The third genus is called *Tilapia*. These species are nest builders. Eggs are fertilized, incubated and protected by a brood parent in a pond bottom built-in nest [4, 5]. In the United States, tilapia is grown for commercial purposes mainly in Arizona, California and Florida. In 2000 U.S. production reached its peak of 20 million pounds, with a value of 30 million dollars. The production decreased to 17.6 million pounds in 2001 but the total value remained the same [11]. However, domestic production of tilapia is minimal compared to U.S. imports. In 2004 U. S. tilapia imports reached 249 million pounds 25% more from 2003 and 68% higher than in 2002 [12].

Quality and Shelf Life of Seafood

Immediately after slaughter, the quality of seafood begins to decline. The initial quality and the type of processing that the fresh product undergoes has a great influence on the shelf life, rate of spoilage and quality of the final product. Some of the factors that influence the spoilage rate of fish are muscle pH, temperature, microbial load, microbial type, amount and type of heme proteins, fat content and fatty acid profile. Refrigeration, frozen storage and modified atmosphere packaging are some of the most common and effective methods to extend the shelf life of seafood. However, these methods normally do not prevent color changes or extend fresh color. In addition, the muscle texture might be affected by some of these methods.

Heme Proteins and Seafood Quality

Myoglobin and hemoglobin are heme proteins whose main function is the retention and transport of oxygen for enzymatic reactions [13]. Myoglobin is a globular protein consisting of a single polypeptide chain. It is found mainly in muscle tissue where it serves as an intracellular storage site for oxygen [13]. Hemoglobin consists of four myoglobins like subunits linked together as a tetramer. It is found in the red blood cells and forms reversible complexes with oxygen in the lung (or gills in the case of fish), where it transports the bound oxygen through the body to be used in aerobic metabolism pathways [13].

Heme proteins consist of a globin part and a heme part. The heme portion of the molecule is responsible for the color of dark muscle. At the center of the heme is an iron (Fe) atom which possesses six coordination sites. Four of them are occupied by nitrogen atoms. The fifth coordination site is bound to nitrogen from a histidine, leaving the sixth site available to complex with electronegative atoms donated by various ligands [13].

Color depends on the oxidation states of the iron atom (Fe^{2+} , Fe^{3+} and Fe^{4+}) in the protein heme group, and the type of ligands (O_2 , CO, NO etc.) bound to the iron atom.

Hemoglobin is the most predominant heme protein found in fish white muscle [14, 15]. The presence of blood, thus hemoglobin, in the muscle leads to changes in color and significant lipid oxidation problems [15, 16]. Soon after death, the heme iron is in the ferrous (Fe^{2+}) valence state [16]. On the surface of fresh muscle oxygen is bound to the ferrous iron yielding oxyhemoglobin/myoglobin which gives the muscle a bright red color. In the interior of the muscle the iron binding site is vacant thus yielding deoxyhemoglobin/myoglobin which gives the muscle a dark purple color. Heme proteins are very sensitive to autoxidation, which is enhanced with temperature increase and pH decrease [17]. Over time, the hemoglobin will oxidize to form methemoglobin (Fe^{3+}). This occurs when oxygen is released from oxyhemoglobin to form ferric (Fe^{3+}) heme iron and the superoxide anion (O^{2-}) [16]. The formation of methemoglobin gives rise to an undesirable brown color. To maintain the red color, the formation of methemoglobin needs to be prevented. This can be achieved by keeping fish at very low temperatures (-50 to -70°C) which is highly impractical and expensive for most species. A more practical and inexpensive means to achieve color stability is by exposing the muscle to CO or filtered smoke which contains CO. The CO molecule will combine with the heme group in hemoglobin and myoglobin to form carboxyhemoglobin/myoglobin and give the muscle a bright cherry red color. The CO molecule binds very strongly to the heme group in hemoglobin and myoglobin, over 200 times stronger than O_2 [18] and will thus displace any oxygen present in the heme. This binding leads to a conformational change

in hemoglobin and myoglobin which makes it very resistant to autoxidation and discoloration [19, 20].

Autoxidation of the heme protein to the met form is also a critical step in lipid oxidation. Met-Hb/Mb reacts with peroxides and stimulates formation of chemical compounds capable of initiating and propagating lipid oxidation [14, 21]. Lipid oxidation is a major cause of quality deterioration of seafoods. It often contributes to the formation of off odors and flavors, and the deterioration of color and texture. Toxic compounds can also arise from lipid oxidation [16]. Fish are particularly sensitive and affected by lipid oxidation due to their highly polyunsaturated fatty acid content [14]. Transition metals such as iron and copper can also catalyze lipid oxidation in fish muscle [14, 22]. Iron is the principal transition metal in seafood and a large portion of iron in fish muscle is found in heme proteins. The amount of iron varies greatly among species. White-muscled fish have lower concentrations of iron than dark muscled fish [14]. In tilapia, most of the iron will come from hemoglobin in the white muscle and myoglobin in the dark muscle. Since CO is expected to retard autoxidation of hemoglobin and myoglobin to the met form it is possible that this treatment may retard lipid oxidation, and thus extend the shelf life of tilapia fillets.

Water Holding Capacity and Muscle pH

Water holding capacity (WHC) of foods can be defined as the ability to hold their own and added water during the application of force, pressing, centrifugation, or heating [23]. Water holding has a great influence on the quality of the final product primarily because of the reduced weight loss during cutting and storage and its ability to retain water during processing [24]. Many factors influence WHC. For example WHC is exponentially related to the protein content of the muscle, as the protein content

increases, WHC increases. The addition of salts also influences the water binding by proteins because of their effects on electrostatic interactions. A change in pH affects as well the conformation of proteins resulting in exposure or burial of the water binding sites [23], as well as increased osmotic pressure within the muscle when is sufficient electrostatic repulsion between proteins [20]. WHC reaches its minimum near the isoelectric points of the major muscle proteins especially myosin (pI~5.4) [25] and rises on either side of this point. After the death of the animal, the anaerobic glycolytic system becomes predominant and ATP is gradually depleted and lactic acid is accumulated leading to a decrease in pH. When the pH is low enough certain critical enzymes are inhibited and glycolysis ceases [13]. The decrease of pH comes from the hydrolysis of ATP [13]. A fast decrease in postmortem pH will cause the denaturation of muscle proteins; the meat produced will be pale soft and exudative (PSE), a condition that is especially troublesome in pork [13, 26]. This phenomenon also occurs in fish; low pH weakens the collagen fibers, they break and “gaping” takes place.

Meat quality and WHC is also influenced by behavioral and physiological status of the animals before slaughter. Stress will exacerbate the drop of pH due to the rising adrenaline levels [26]. An animal is considered in a state of stress if it is required to make abnormal or extreme adjustments in its physiology or behavior in order to cope with adverse aspects of its environment and management [27]. When an animal is under stress, oxygen is not available in sufficient amounts, and the anaerobic pathway becomes predominant and glycogen is depleted. This depletion of glycogen results in an onset of rigor much sooner and in a faster decrease of pH. For example, the time from death to the onset of rigor in unstressed blue tilapia is 6 hours; on the other hand, the time from death

to the onset of rigor is reduced to only 1 hour in stressed tilapias [28]. In one study, CO₂ and live chilling were used on salmon in order to minimize stress and prolong the onset of rigor [29]. The advantage of having a longer onset of rigor is that processing of the raw material can start immediately after slaughter; otherwise the process cannot start until rigor has been resolved. A common practice in Atlantic salmon is to process once rigor mortis has resolved, which takes 3 to 5 days on ice storage [30]. However, it has been shown that there is no major difference between processing pre-rigor salmon and post-rigor salmon [29, 30]. The extension in the onset of rigor is of particular importance to tilapia producers since tilapia is processed pre-rigor.

The tilapia industry uses many ways to slaughter the animal. The most common is to transfer tilapia from the pond to a small tank where they are taken one by one and their branchial artery is severed. They are then transferred back to the small tank until they bleed to death. It has been suggested that bleeding the fish has no effect on the quality of the final product since most of the blood in fish is located on the venous side of the cardiovascular system meaning that by gutting most of the blood will be removed along with intestines [31]. The transferring of the tilapia from the pond to the small receiving tank and then the bleeding may thus be an unnecessary and overly stressing slaughtering method. The euthanasia of tilapia can reduce this stress.

As mentioned above, salmon industry uses CO₂ as anesthesia in order to avoid stress and to comply with the concept of humane slaughter [29]. Carbon monoxide dissolved in the water where the fish is can also act as an anesthesia for fish. In addition of the benefit of having lower stressed fish, CO will enhance the color of the filleted fish and it can help preserve better the final product.

Effects of Carbon Monoxide on Fish Muscle

The bright red color of fish is one of the main attributes that indicates its freshness and quality. The red color arises primarily from the oxygenated and reduced forms of heme proteins which have been discussed in previous sections. The oxidation of these proteins yields a highly undesired brown color. Appearance plays a very important role in consumer buying decisions. The main objective of the use of carbon monoxide is to maintain the attractiveness of the red color characteristic of fresh seafood. As written previously, carbon monoxide binds to heme proteins and forms a very stable complex. Kristinsson and coworkers [20] reported that carboxyhemoglobin was very stable to oxidation even at extreme pH values and temperatures. These results suggest that muscle treated with carbon monoxide may retain its red color even under abusive conditions. The same authors also demonstrated that the Hb-CO complex had decreased pro-oxidative activity in a model system and may thus extend product shelf life with respect to rancidity.

Due to its high price and its high content of red muscle, tuna steaks have been the focus of study with respect to the use of carbon monoxide for color preservation. Different studies using different concentration of carbon monoxide and different exposure times have revealed that there is a significant increase in red color as well as color stability when CO is used. For example, tuna steaks treated with 99.5% CO gas for 4 hr showed a significant increase in a*-value (redness) compared to untreated tuna [32]. There were no significant differences between L*(lightness) and b* (yellowness) values between the gas treated tuna and control [32]. Balaban and coworkers [33] reported that exposure to 4% CO increased a*-value and preserved color stability for up to 12 days in refrigerated storage. Danyali [34] compared CO with filtered smoke (FS) treatments and

found little difference between the two with respect to color, heme protein oxidation, lipid oxidation, water holding, and texture. It was however reported that 100% CO led to a reduced microbial growth, and thus could possibly extend shelf life [34]. Studies also show that a higher level of CO leads to more color increase and better color stability [34, 35].

Few studies have been published on applying CO or FS treatment on tilapia and investigating the effect on quality. Ishiwata and coworkers [36] conducted a survey of the concentration of CO in flesh from a variety of fish sold in a local market and also exposed tilapia to CO for 60 minutes at room temperature and measured CO concentration. They reported that the blood colored parts of tilapia exposed to CO were bright red contrasting with the dark brown color exhibited by the untreated tilapia.

Kristinsson and coworkers [37] reported that tilapia treated with 100% CO did develop less lipid oxidation products compared to untreated tilapia. Kristinsson and coworkers [19] later found that isolated tilapia carboxy-hemoglobin had dramatically increased stability to autoxidation (i.e., browning) under different environmental conditions compared to oxyhemoglobin, and also had less pro-oxidative activity in a model linoleic acid emulsion system. Leydon and coworkers [38] recently reported that commercially obtained previously frozen tilapia fillets treated with filtered wood smoke had increased color stability, less lipid oxidation and microbial growth than fresh commercially obtained tilapia fillets. The filtered smoke treated fillets were however rejected by a trained sensory panel of 3 people [38]. This study did however use commercially obtained samples, one previously frozen and one fresh, from two different sources, which makes it difficult to interpret the data.

Carbon Monoxide and Euthanasia

All studies reported on CO and FS application of seafood employ gas treatments post-mortem on fish steaks or fillets. The practice of euthanizing fish with CO to incorporate CO into muscle is being performed by the industry on tilapia (personal communication, B. Olson, Clearsmoke Technologies). Kowalski [10] issued a patent application in which he described the incorporation of tasteless smoke or carbon monoxide by means of euthanasia; however no supporting data was found. No other research studies have been performed to the best of the author's knowledge.

Carbon monoxide is a colorless, odorless and tasteless gas that has about the same density as air, but sustained inhalation of CO has caused many fatalities due to its competitive binding to hemoglobin [39]. At levels of 5% CO-Hb fetuses can be affected and individuals experience many effects. Levels above 10% are life threatening for heart and lung patients, whereas above 30% CO-Hb healthy individuals are at risk and death can rapidly occur at levels above 50% [40]. Carbon monoxide poisoning will severely alter the oxygen transport characteristics of the circulatory system since about 90% of the oxygen consumed is carried to the tissues by hemoglobin [41]. Carbon monoxide attaches to hemoglobin similarly to oxygen but with a binding constant that is 210- 270 fold stronger [40]. Consequently, CO displaces oxygen from hemoglobin. Due to its great binding affinity, even at low levels of exposure to carbon monoxide, carboxy-hemoglobin will accumulate. Carbon monoxide reduces both the oxygen-carrying capacity of circulating blood by direct displacement and the release of the hemoglobin-bound oxygen to the tissues by shifting the oxygen-hemoglobin dissociation curve [40].

One limitation of killing fish with CO is that it has a relatively low solubility in water. It was reported by Daniels and Lide [42, 43] that CO has a solubility of 1.774×10^{-5}

mole fraction solubility in water at 25 °C and 101.325 kPa. This solubility however is very similar to the solubility of oxygen in water (2.293×10^{-5} mole fraction solubility at 101.325 kPa)[43] . This suggests that O₂ and CO dissolved volumes are approximately equal. The volume of O₂ or CO dissolved in water is dependent on the partial pressure of the gas and the temperature. The solubility increases as the temperature decreases.

Research Objectives

The overall objective of this study was to investigate the effect of euthanizing tilapia by dissolving carbon monoxide directly into the water and comparing to 100% CO post-mortem gas treatment of fillets and no gas treatment. The effect on color, color stability, CO uptake and stability, muscle pH and water holding capacity were investigated for both products stored fresh, as well as frozen and defrosted products.

CHAPTER 3 MATERIALS AND METHODS

Euthanasia of Tilapia with Carbon Monoxide (CO)

Live tilapia were obtained from Evan's Farm in Pierson, FL. The facility produces hybrids from a genetic cross of predominantly aurea tilapia, *Oreochromis niloticus* and *Oreochromis mossambicus*. All the production is destined for local consumption and local restaurants.

The tilapia were transferred live to the laboratory and kept in holding tanks prior to euthanasia. A tank was constructed from transparent Plexiglas® (36"x 16"x 12") where tilapias were euthanized with CO saturated water (Figure 3-1). 100% CO was flushed into a circulatory system which allows the water to saturate with the gas (Figure B-1). CO was introduced to the animal muscle tissue by its respiratory and circulatory systems. All the experiments were performed at ambient temperature (21°C). Tilapias were maintained in the euthanizing tank as much time as needed until they all were confirmed dead by visual inspection. On average, 31 minutes were needed for the completion of the euthanasia process (Table B-2). During every trial, thirteen tilapias were euthanized. To flush the remaining CO out of the tank, air was flushed in and the CO converted to CO₂ by passing it through a Hopcalite catalyst tube (Figure 3-1).

After euthanasia, two studies were performed. In the first study these tilapia was immediately filleted, vacuum packed in high density polyethylene (HDPE) bags and frozen at -20°C. After 1 month fillets were thawed at 4°C for 24 hours and stored aerobically at 4°C for 18 days. In order to compare the effectiveness of euthanasia with

100% CO flushed directly into the water, 100% CO post-mortem gas treated and control fillets were stored under the same conditions for the same amount of time (Figure 3-2).

In the second study, tilapia were only gutted, placed in HDPE bags, vacuum packed and stored at -20°C for up to 4 months. A set of normally slaughtered (no CO) fish was also gutted, vacuum packed and stored at -20°C . The latter was used as control. Three sampling points were chosen; 0, 2 and 4 months. The whole fish was thawed for 24 hours at 4°C , then filleted and analyzed (Figure 3-3)

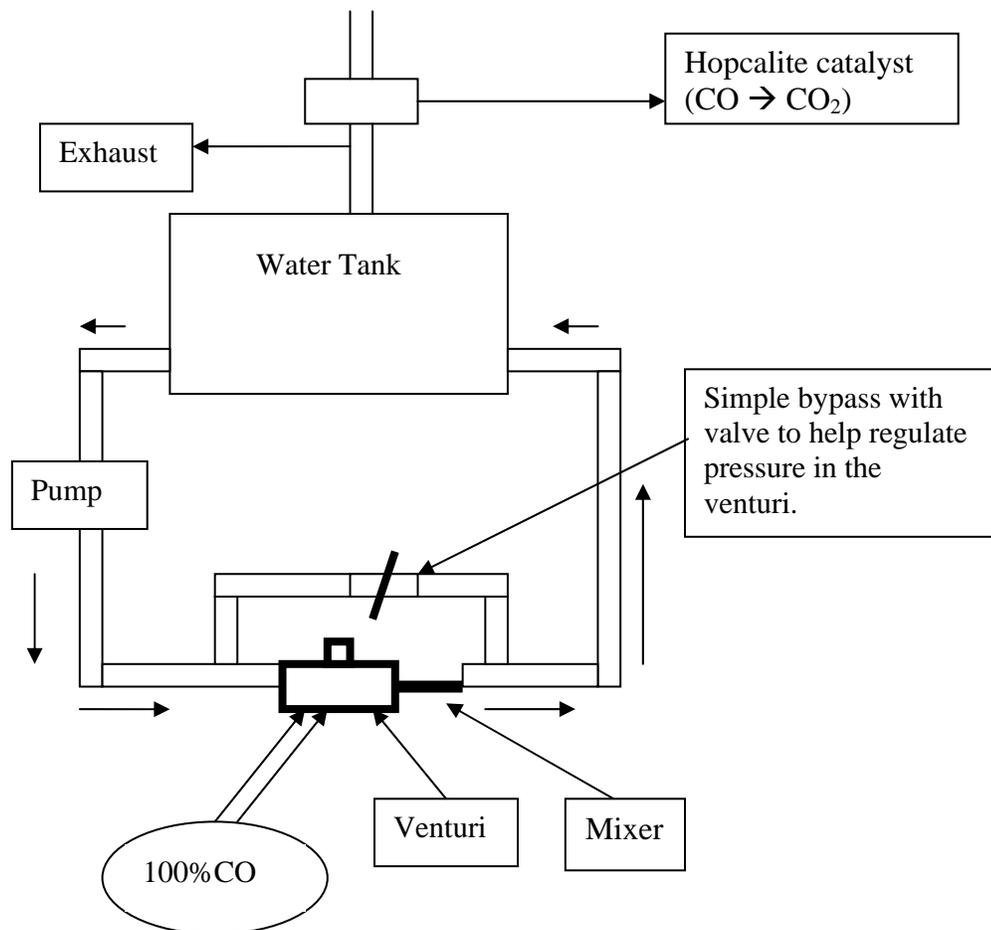


Figure 3-1. Recirculating water-CO system for the euthanasia of tilapia.

100% CO Post-Mortem Gas Fillets Treatment of Tilapia

Live tilapia were killed with ice and by bleeding. The 100% CO post-mortem gas treatment can be applied only to the first study since the later study used whole fish. After death, the fish was filleted immediately before rigor and split in two groups: one subjected to CO gas treatment and the other (control) subjected to no treatment. Fillets were placed in a gas tight stainless steel drum on thinly netted stainless steel shelves and 100% CO applied for 30 min at 4°C. After gas treatment the CO was converted to CO₂ by passing it through a Hopcalite catalyst tube (same one as in Figure 3-1). Untreated, CO-treated and euthanized fillets were then placed in HPDE bags, vacuum packed and frozen for one month. Fillets were then defrosted at 4°C and kept at 4°C for 18 days and analyzed every 3 days.

Color Analysis

A digital Color Machine Vision System (CMVS) was used following the procedures outlined by Balaban and coworkers [33] for detailed color analysis of RGB and L*-(lightness), a*-(redness), and b*-(yellowness) values along with hue values and identifying important color blocks for each treatment. The L*, a* and b*-values were reported. The color analysis was done separately for the white and the dark lateral muscle. The front side of the fillets contains the dark muscle which was used for the analysis of the red muscle. The reverse side which contains practically no red muscle was used for the analysis of the white muscle.

Quantification of CO in Fish Muscle

The method from Miyazaki and coworkers [44] was used to determine the concentration of CO in white and dark muscle separately. Briefly, 6 g of muscle were minced and introduced into a 60 ml head space bottle. 3 drops of 1-octanol (antifoaming

agent) and 12 ml of 10% sulfuric acid were added. The sulfuric acid denatures heme proteins which causes them to release CO. The mixture was shaken for 10 sec, and then incubated for 5 minutes at 40°C. After incubation, the tubes were shaken at room temperature for 15 minutes and 100 µl of the head space gas was injected into an Agilent gas chromatography system equipped with a stainless steel Poropak Q column (3.17 mm i.d. x 1.82 m; 80-100 mesh) a methanizer (to convert CO to CH₄) and a FID detector. Helium was used as the carrier gas with a flow rate of 29.7 mL/min. The injection port, column, methanizer and detector temperatures were maintained at 100°C, 35°C, 320°C and 250°C respectively. The reducing gas was hydrogen with a flow rate of 40.0 L/min. The retention time and area of the CH₄ peak were compared to those obtained with a calibration CO gas. CO levels were then calculated based on a standard curve constructed by injecting different known levels of 100% CO. For the first study, three fillets were retrieved from the cold room (4°C) and analyzed on different days after thawing (day 0, 2, 4, 6, 10, 14, 18). These days were chosen due to the rapid decrease of CO concentration in the muscle during the first days of exposure to normal atmospheric conditions [36].

Heme Protein Extraction and Spectroscopic Analysis

Spectroscopic analysis can reveal how CO is taken up into the muscle and how stable it is bound to heme proteins on storage. The heme extraction method of Huo and Kristinsson [45] was used. A 10 g sample of tilapia white and red muscle (separately) were mixed with 100 ml of 20 mM Na₂HPO₄ buffer (pH 8), followed by homogenization with a Ultra-Turrax T19 homogenizer at lowest speed. The sample was filtered through Whatman #1 filter paper at 4°C followed by centrifugation at 3000x g. All these steps were done in the cold room to avoid protein denaturation and loss of CO from the heme.

The UV-visible absorbance spectra of the collected supernatant were then read from 350-700 nm. The max wavelength of the heme peak of CO-Hb/Mb is 419 nm, 414 nm for oxyHb/Mb and 408 and below for metHb/Mb.

Muscle pH

The stress and its influence on the animal were followed by measuring the pH of the muscle. A sample of 5 g red and white muscle was mixed with 45 mL of deionized water at 4°C and the mixture homogenized with an Ultra-Turrax T19 homogenizer, on ice. The pH was then measured using a Ross Sureflow biological epoxy probe (Thermo Orion, Beverly, MA) attached to a pH meter (Denver Instruments, Fort Collins, CO). The pH meter was calibrated using pH 2, 4, 7, and 10 buffers at 4°C.

Drip Loss

Drip loss analysis was performed on fillets from the first study only. Six fillets from each experimental group (euthanized, gassed and control) were analyzed for drip loss for 18 days. Measurements were acquired every third day for 18 days (day 0, 3, 6, 9, 12, 15 and 18). The fillets were kept in open bags during the 18 days of measurements to allow for air to access the fillets. Each fillet was very delicately blotted for any loose liquid on its surface before its weight was recorded. The bag was meticulously cleaned and dried before the fillet was replaced in the bag. Thaw loss and drip loss were calculated based on the weight difference between the different storage days.

Statistical Analysis

Each analysis was conducted in a minimum of triplicate samples. Analysis of variance (ANOVA) and t-test were used to determine significant differences between treatments and among treatments. The Statistical Analysis Software (SAS) and Microsoft Excel were used for the treatment of the data.

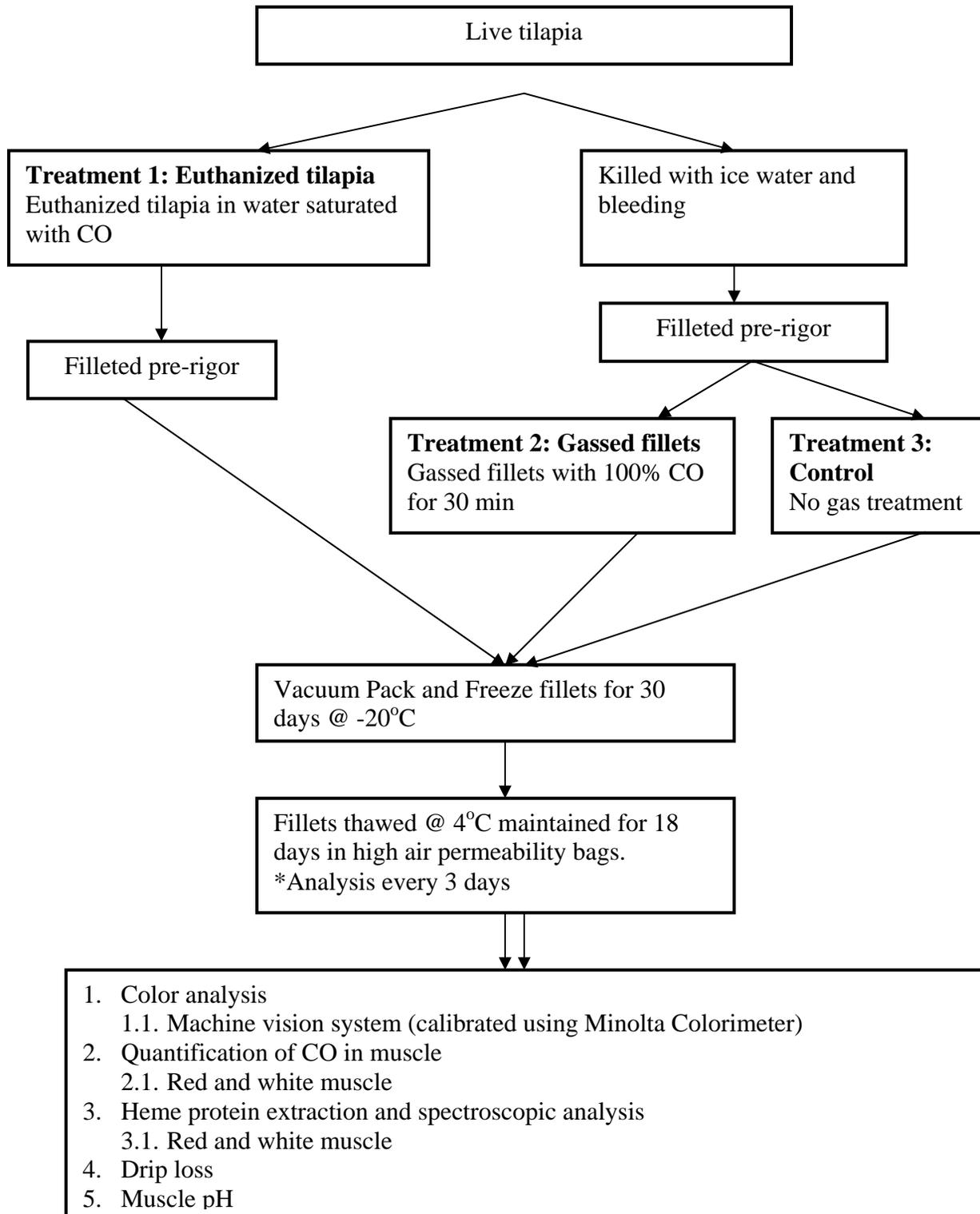


Figure 3-2. Experimental design for the first study where tilapia CO treated or untreated and then filleted, frozen (30 days), defrosted and stored at 4°C for 18 days.

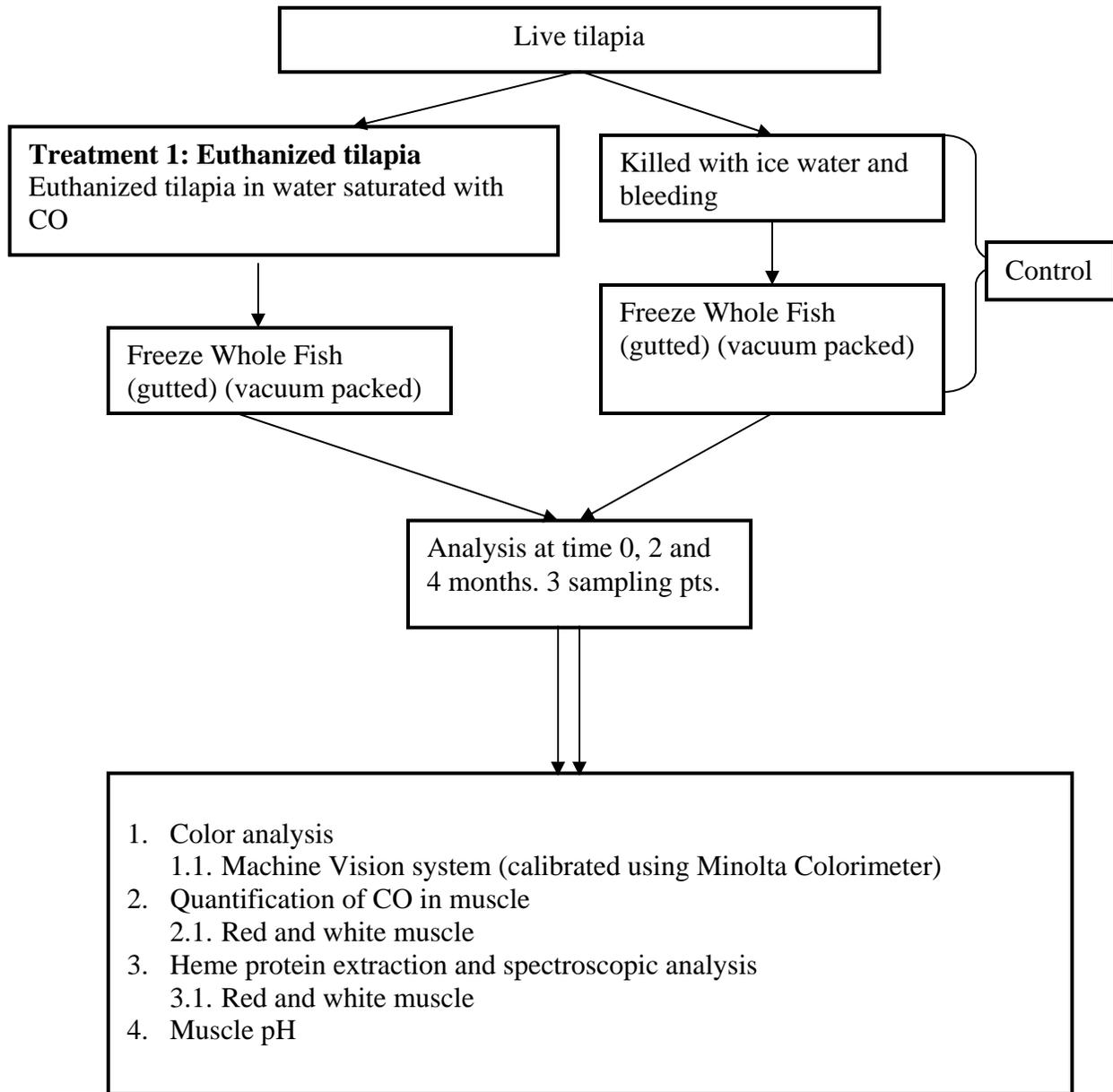


Figure 3-3. Experimental design for the second study where whole tilapia was either euthanized or left untreated, and then frozen for up to 4 months.

CHAPTER 4
RESULTS AND DISCUSSION

Study 1: Frozen Fillets

Effect of Carbon Monoxide on Color

The main reason behind the introduction of carbon monoxide in fish processing is to preserve and enhance the red color of the muscle. A Color Machine Vision System (CMVS) was used to analyze the color and color change during the study. The CMVS was chosen because it analyzes every pixel of the sample compared to other color methods that read only a small part of the sample. Other methods such as the Minolta color meter may be good for fillets that are uniform in color. However, tilapia has a red lateral muscle that gives the fillets an uneven color.

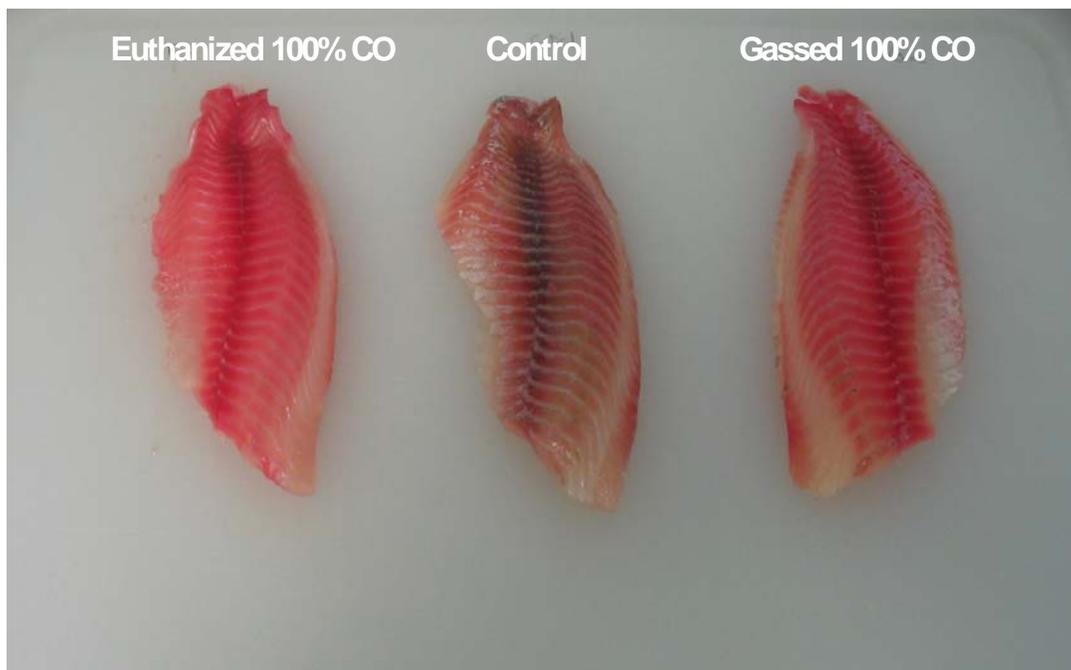


Figure 4-1. “Red” muscle side of 100% CO euthanized, control and 100% CO gassed tilapia fillets.

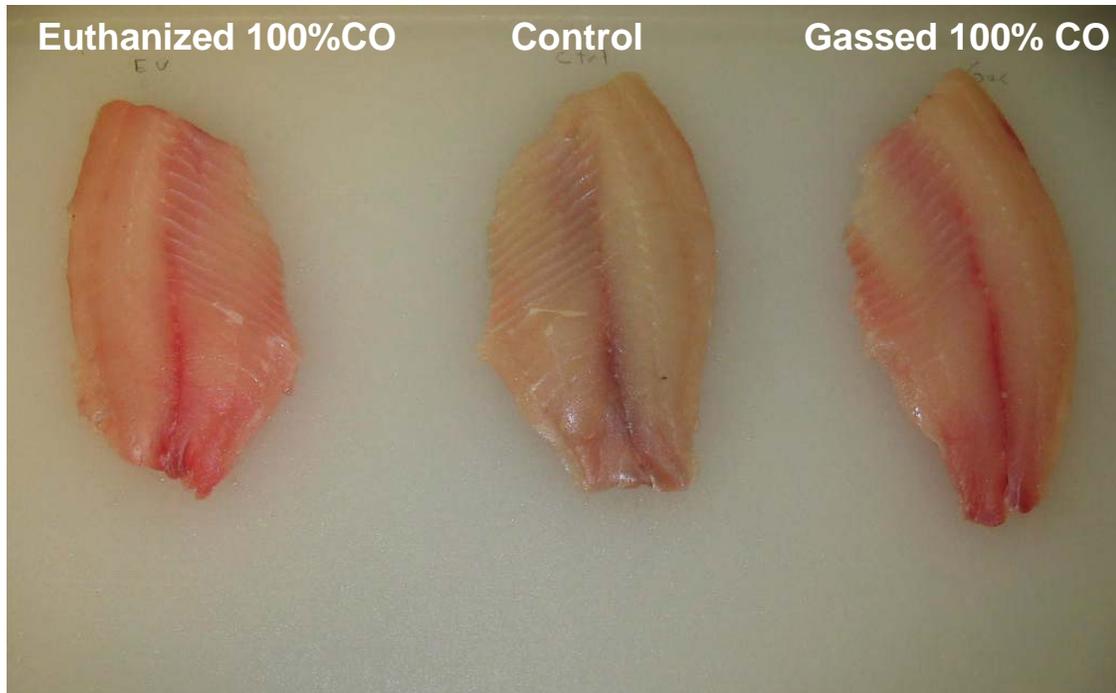


Figure 4-2. “White” muscle side of 100% CO euthanized, control and 100% CO gassed tilapia fillets

Fillets from fish euthanized with 100% CO have a distinctive and stable cherry red color characteristic of a CO exposure/treatment. Figures 4-1 and 4-2 show sample fillets of each treatment for the “red” muscle side and the “white” muscle side of a tilapia fillet. “Red” muscle was defined as the side of the fillets that includes the red muscle (Figure 4-1). The other side was considered as “white” muscle (Figure 4-2).

The degree of redness (a^* -values) is the most important indicator of quality and freshness in species rich in red muscle such as tilapia, Spanish mackerel, mahi-mahi, tuna and swordfish [33, 34, 46]. The effect of the two treatments was very noticeable compared to the control fillets (Figures 4-1 and 4-3). Figure 4-3 shows the increase of a^* -values in the red muscle of the euthanized and the post-mortem gas treated fillets and the control. The 100% CO post-mortem gassing method of fillets significantly increased ($p < 0.05$) a^* -values from 18.36 to 23.63. The a^* -values from the euthanized fillets also increased significantly ($p < 0.05$) from 17.24 to 27.48. The a^* -values of the fillets from the

ethanized fish remained significantly higher ($p < 0.05$) until day 9 compared to both treatments. From day 12 to day 15 euthanized a^* -values were still significantly higher than control a^* -values ($p < 0.05$) but no significant differences were found among euthanized and gassed post-mortem fillets. At day 18 there was no significant difference among any of the three treatments ($p < 0.05$). The post-mortem gassing of the fillets maintained significantly higher a^* -values ($p < 0.05$) compared to the control samples until day 6.

The frozen storage negatively affected the a^* -values of the control and the post-mortem gassed fillets ($p < 0.05$). Freezing did not affect a^* -values of the euthanized samples. Control samples were affected the most by the freezing and thawing as there was a significant decrease ($p < 0.05$) in a^* -values from 17.245 to 9.50. Gassed fillets were also affected by the freezing, but to a lesser extent. Euthanized fillets had significantly better stability ($p < 0.05$) during the freezing period, being 8.46 and 17.89 points over CO treated fillets and untreated fillets respectively. The a^* -values for the 100% CO post-mortem gas treatment were also significantly increased ($p < 0.05$) compared to the control values. After 6 days at 4°C there was a significant drop ($p < 0.05$) in a^* -values for all treatments. The decrease in a^* -values at day 6 corresponded to a decrease in the heme peak wavelength and also in the CO concentration (see Figures 4-6 and 4-8 later) suggesting that the CO is escaping from the hemoglobin and myoglobin (discussed below). These results are consistent with results on CO-treated tuna, mahi mahi and Spanish mackerel, which all show an increase in a^* -value on treatment, but a gradual decline after defrosting [33-35, 46].

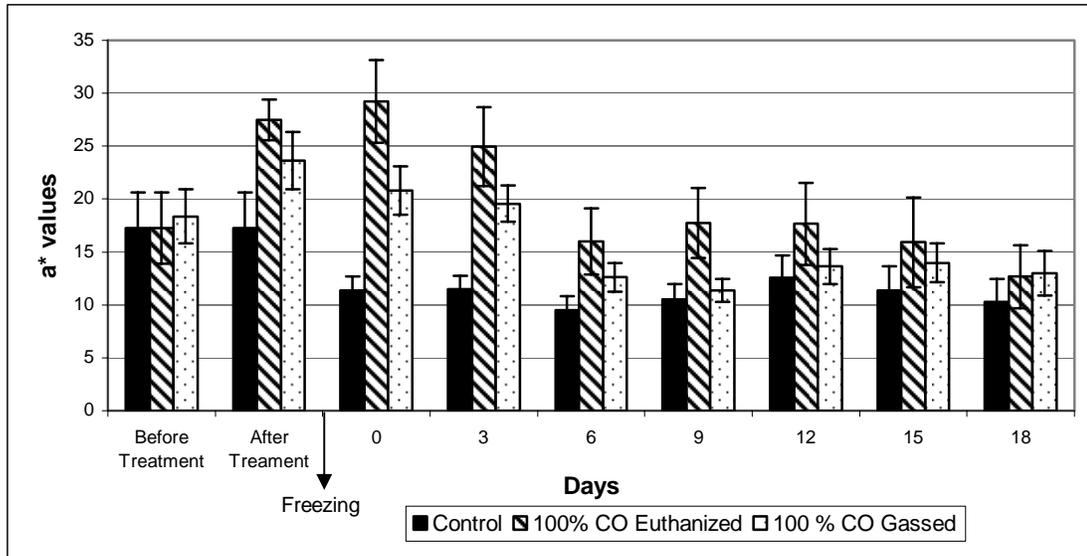


Figure 4-3. Effect of CO treatments and no treatment (control) on the a*-values (redness) of tilapia fillet red muscle before freezing, after freezing and subsequent storage at 4°C for 18 days.

Control and gassed fillets' L* (lightness) values decreased significantly ($p < 0.05$) during the frozen storage. On the other hand, L* values from the euthanized fillets were not significantly ($p < 0.05$) affected until day 6 (Figure 4-4). These results suggest that the euthanasia with 100% CO yielded more “natural” fresh looking fillets than the post-mortem gassing.

Both of the treatments did not have a significant effect on b*(yellowness) values until day 18 where control values increased significantly compare to the euthanized samples (Figure A-1). This slight increase can be attributed to the oxidation of heme proteins and possibly also to lipid oxidation[47]. The reduction in the a*-values for the control corresponds to met-hemoglobin [18], which can produce a brown-yellowish appearance to red muscle, which would explain the increase in b*-value.

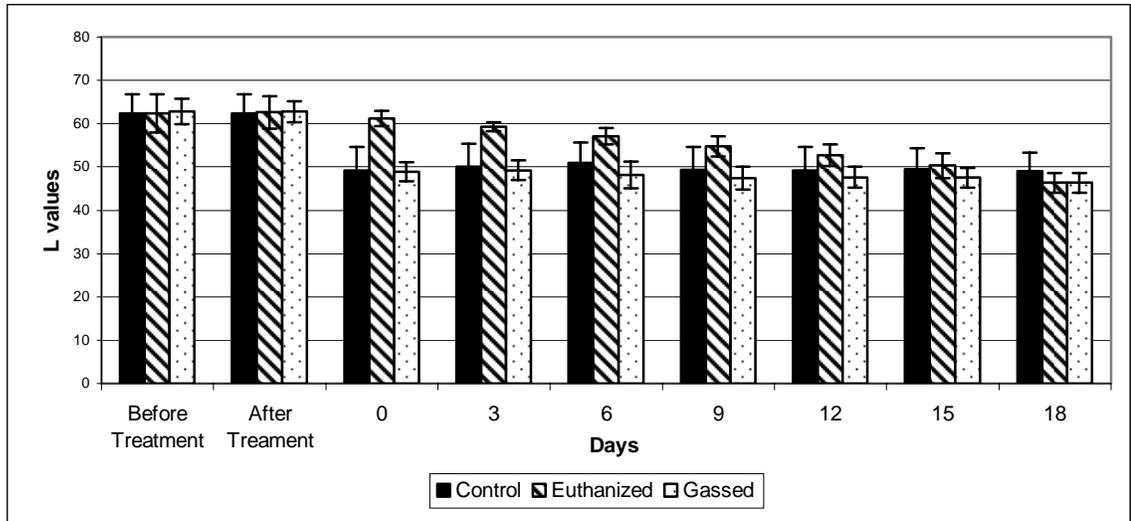


Figure 4-4. Effect of CO treatments and no treatment (control) on the L*-values (lightness) of tilapia fillet red muscle before freezing, after freezing and subsequent storage at 4°C for 18 days.

White muscle is the predominant muscle type in tilapia. White muscle has significantly lower amounts of heme proteins than red muscle [48]. Although levels of heme protein are less in the white muscle, its red color was still significantly ($p < 0.05$) influenced by both of the CO treatments (Figure 4-5). Fillets from tilapia euthanized with 100% CO had a pinkish tone to the white muscle which was reflected in a significant ($p < 0.05$) increase in a^* -values for the entire study (Figure 4-5). 100% CO post-mortem treatment produced significantly ($p < 0.05$) higher a^* -values to the white muscle until day 6. From day 9 to the end of the study no significant differences were found among post-mortem gas treatment and control fillets.

After both CO treatments, euthanized fillets had a^* -values significantly ($p < 0.05$) higher than a^* -values from post-mortem gassed fillets. This difference was still significant ($p < 0.05$) after the frozen storage and thawing process (at day 0). At days 3 and 6 and after day 15 no significant ($p < 0.05$) differences were found. During day 9 and 12 significantly ($p < 0.05$) higher a^* -values were again found. These results suggest higher

stability of a^* -values for the euthanized process compared to the 100% CO post-mortem treatment.

The white muscle side of the tilapia fillet contains a small central line of red muscle (Figure 4-2) that increased the overall a^* -values of the white muscle and gave a higher standard deviations. In addition, the lack of bleeding of the euthanized fillets may also have influenced the results since more blood would leave more hemoglobin in the tissue and thus more CO binding and a larger effect on red color. Overall, the results seen for a^* -values in the white muscle followed similar trends as those seen for the dark muscle.

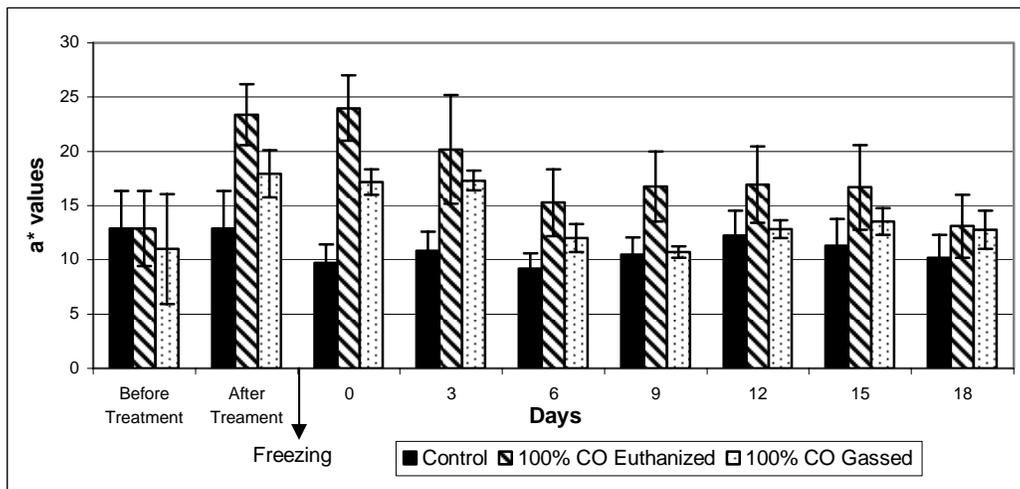


Figure 4-5. Effect of CO treatments and no treatment (control) on the a^* -values (redness) of tilapia fillet white muscle before freezing, after freezing and subsequent storage at 4°C for 18 days.

Heme Spectroscopic Analysis

Both of the treatments influenced the red color of the fillets according to the CMVS. This increase in color and color stability comes from the binding of CO to heme proteins. The complex carboxy-hemoglobin has been proven by Kristinsson and coworkers [19, 20] to be more stable against oxidation compared to oxy-hemoglobin. The oxidation of heme proteins is the main reason why the red color decreases over time and changes to brown [13]. Figure 4-6 shows a representative spectrum of all three oxidation

states at which hemoglobin/myoglobin is found during these experiments (i.e., Met, Oxy and Carboxy).

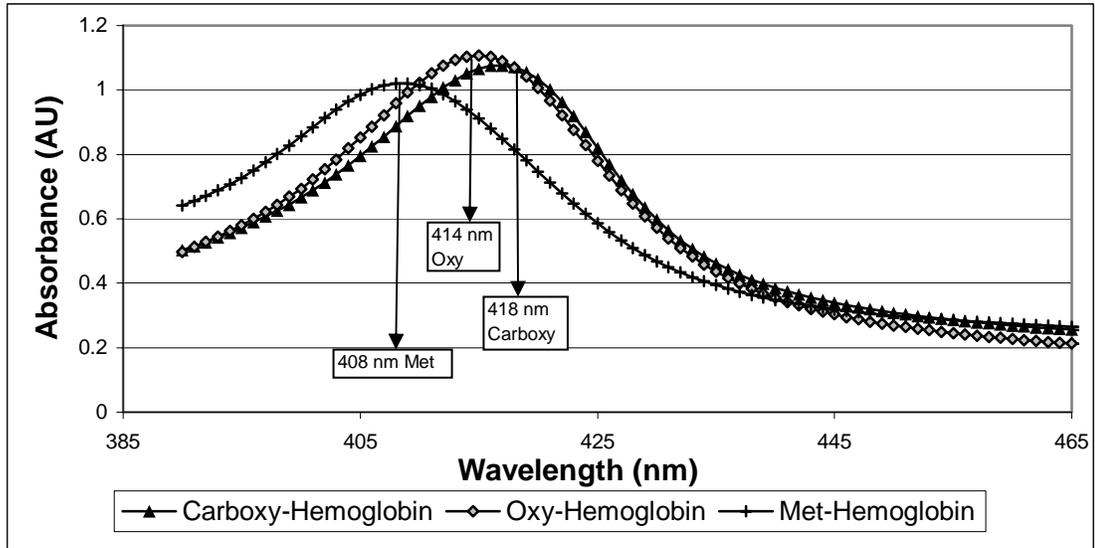


Figure 4-6. Representative spectra for met-hemoglobin (408 nm), oxy-hemoglobin (414 nm) and carboxy-hemoglobin (418 nm)

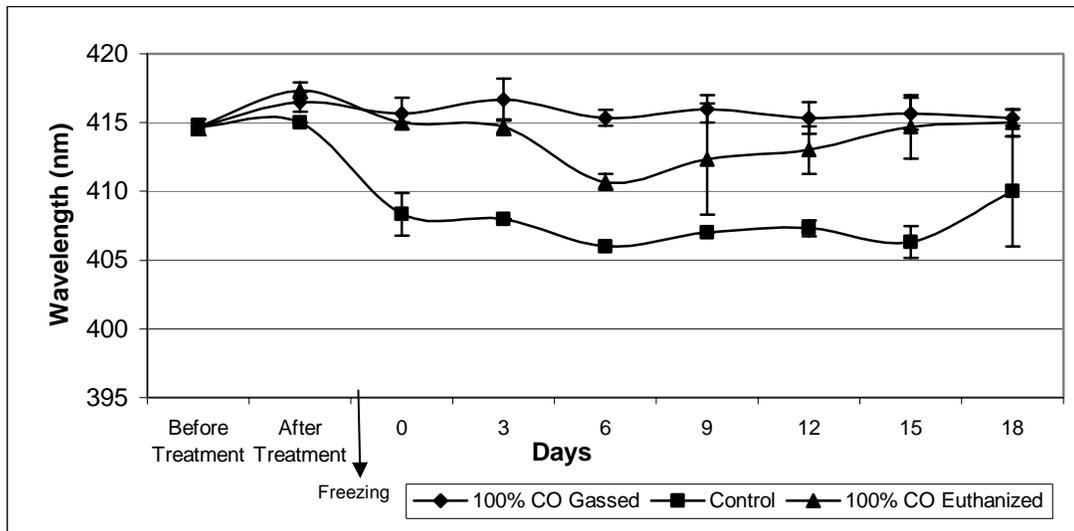


Figure 4-7. Maximum heme peak values for red muscle extracts from euthanized and 100% CO gassed tilapia fillets and untreated tilapia stored at 4°C for 18 days

From Figures 4-7 it can be seen that the gas treatment as well as the euthanasia process led to a substantial increase in the heme peak wavelength, which indicates that CO is being bound to the heme proteins. A wavelength of 418 nm suggests maximum

binding. Before treatment heme peaks wavelength of ~415 nm were found for all the treatments. This reading corresponded to mostly oxy-hemoglobin. After the gas treatment the heme peak wavelengths reached 416.5 nm on average while a reading of 417.3 nm was observed from the heme extracted from the euthanized tilapia. These UV-Vis spectra of the heme proteins extracted from the muscle of the gassed and euthanized tilapia demonstrated that there was a mixture of oxy-hemoglobin and carboxy-hemoglobin present in the extracts. Similar results were found by Danyali [34] and Garner [46] where muscle from tuna and Spanish mackerel, respectively, were exposed to different concentrations of CO. These high heme peak wavelengths were maintained through the frozen storage proving that the CO was still bound to the heme proteins what explains the high a^* -values reported in Figure 4-3. On the other hand, the heme peak for the control decreased after freezing to about 408 nm which shows that it was already oxidized. This explains the decreased in a^* -values obtained after freezing in the control samples (Figure 4-3). The significantly higher wavelength peaks seen for both CO treatments after 1 month of frozen storage compared to the control shows how the heme proteins are significantly stabilized when they are bound to CO. A period of 30 minutes of direct exposure of fillets to CO or euthanasia of fish with CO was therefore enough to significantly stabilize the heme proteins during freezing as well as after thawing. There was however a difference in the CO binding between the two CO treatments. The gassed fillets appeared to have more bound CO since wavelength remained mostly constant during the cold storage, while the heme peak wavelength decreased on storage at 4°C for the euthanized samples. This decrease suggested that the euthanized samples were loosing CO. This fact is reflected in the a^* -values, where a sudden decrease was noticed

at day 6 (Figures 4-3 and 4-5). The heme peak wavelength did however increase after this decrease, suggesting that CO was still present in the muscle and was rebinding to the heme. It is interesting to note that euthanized fish had higher a^* -values than the gassed fillets, but show less binding of CO to the heme according to the UV-vis analysis. This was unexpected as the redness of fish muscle is dictated by the level of CO binding to the heme proteins, and thus an opposite result would have been expected. It is possible that during extraction some of the CO may have been lost (e.g. during homogenization which may have denatured the heme proteins) thus giving lower values. However, all samples were extracted identically. Another possibility is that the euthanized fish had lower overall CO levels in the muscle compared to the gassed fish, and therefore during homogenization, some CO could have been lost and since it did not have the excess CO present in the gassed fillets, the CO lost did not get replenished. This is supported by the data on muscle CO levels presented below (Figures 4-9 and 4-10).

Similar results were found for the white muscle (Figure 4-8). Heme proteins extracted from control fillets had heme peak wavelengths below 408 nm implying that heme proteins were already oxidized and met-hemoglobin was formed. For some samples heme proteins were below detection limits. This is because the level of heme proteins in white muscle is very low. Higher heme peaks were observed for the treated samples compared to the control. Some of the wavelength values for the euthanized fish suggest that the heme proteins were in part oxidized at days 3, 6, and 9. This is interesting, as the a^* -values for the euthanized fish white muscle were higher than those for the gassed fillets, and thus one would have expected higher wavelength for the euthanized fish. The same contradiction was seen for the red muscle. The wavelength then rose again,

suggesting rebinding to CO, similar to what was observed with the red muscle. However, due to the high a^* -values maintained by the treated fillets compared with the control fillets and the lack of browning, it can be concluded that these wavelengths may come from some free blood that was present at the surface of the fillets. The free surface blood is expected to oxidize faster than blood in the fillets.

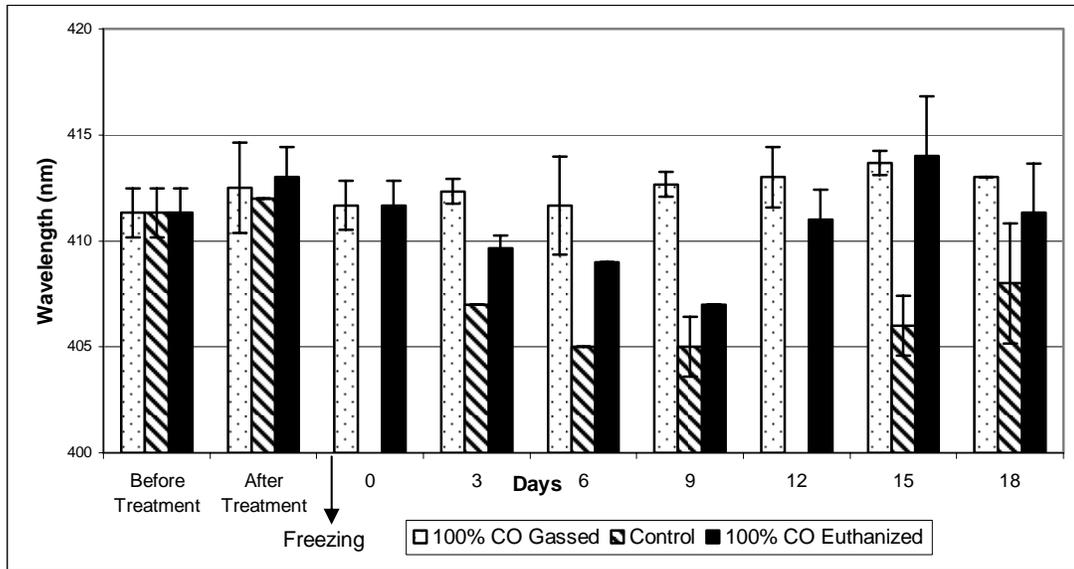


Figure 4-8. Maximum heme peak values for white muscle extracts from euthanized tilapia, 100% CO gassed tilapia fillets and untreated tilapia stored at 4°C for 18 days.

The heme spectroscopic analysis revealed that treated products with CO can be differentiated from the untreated ones just by analyzing its UV-Vis spectra. Untreated product should not have a heme peak wavelength higher than 414 nm, while treated products will have heme peak wavelengths higher than 414 nm revealing the carboxy-hemoglobin/myoglobin complex.

Carbon Monoxide Quantification

The concentration of CO in the muscle was quantified using a GC equipped with a flame ionization detector (GC-FID). Few methods are available to measure CO in fish muscle. The Japanese health authority uses a method called the “A method” [36]. This

method however requires large amounts of muscle for analysis and results have demonstrated that it lacks sensitivity[45]. Due to the small amount of red muscle present in tilapia fillets a different method was chosen [44] which recovered more CO from the muscle than the “A method”.

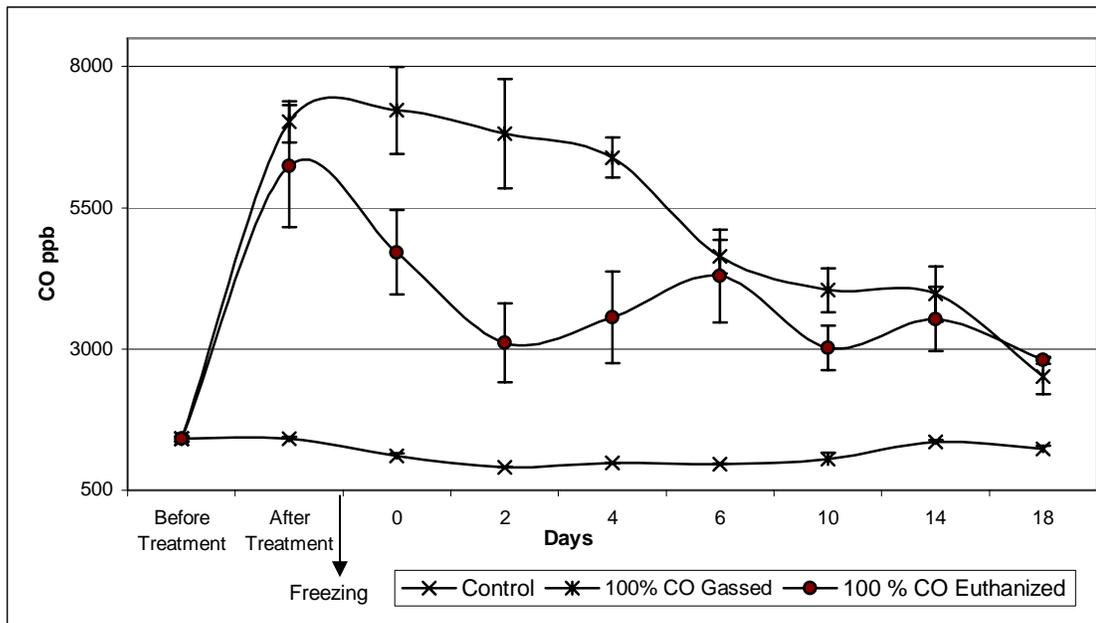


Figure 4-9. Concentration of CO (ppb) in tilapia red muscle after 30 minutes exposure to 100% CO, euthanasia with 100% CO or no treatment.

In Figure 4-9 it can be clearly seen that there was a difference in CO concentration between the treatments after the freezing and thawing process. Control samples had low levels of CO (1408 ppb). It was expected to find some CO in the muscle since endogenous CO is produced during the metabolism of protoheme [36, 49]. This small concentration was maintained for about 10 days, but then increased significantly ($p < 0.05$) in day 14. An increase in CO concentration on extended storage has been reported previously, and is one of the indicators used by the Japanese health authorities that fish has not been treated [36]. If fish has been treated with CO, the level is expected to decline which was the case for the CO treated tilapia in this study.

After treatment a significant ($p < 0.05$) increase in CO concentration was noted for both euthanized and gassed samples (Figure 4-9). CO concentration increased to 6237 ppb and 7020 ppb for the euthanized and the gassed treated fillets respectively. After thawing, initial concentration of CO in the post-mortem gassed fillets remained considerably higher (~6380 ppb) than the level for the euthanized fillets (4712 ppb), but then dropped suddenly after day 4 of storage. The additional amount of CO present in the gassed samples is likely CO trapped in the muscle and thus was not bound to the heme proteins. This is very possible, considering that the initial heme peak wavelengths were similar for both treatments. Thus, the data suggest similar CO saturation of heme proteins, which means the higher level of CO in the CO gassed fillets is due to additional CO trapped in the extracellular matrix. The gas treatment was applied post-mortem by exposing the fillets to 100% CO for 30 min. The exposure is based on surface contact between the muscle and the CO and therefore it is very possible that CO can be trapped in the extracellular muscle matrix. Davenport and coworkers [39] subjected tuna to 100% CO treatment and found that much of the CO is trapped in the extracellular matrix of the muscle, which supports the results seen here with tilapia. Figure 4-9 shows that this excess CO over the CO for the euthanized fish remained in the muscle for four days and then most of it left the muscle since euthanized fish and gassed fillets had similar values at days 6 and beyond ($p < 0.05$). As discussed before, this additional CO in the muscle may be the reason the CO treated fillets had higher heme peak absorbance (i.e., suggesting more CO saturation) throughout the experiment, since extracellular CO would have replenished CO lost from the heme proteins during extraction. This however would

also suggest that the CO gassed fillets should have had higher a^* -values, which was not the case.

The euthanized fillets had higher CO concentration than the control but as discussed above, lower levels than the gassed fillets. Since the CO was transferred to the edible muscle tissue through the respiratory and circulatory system it can be inferred that all the CO present in the muscle was bound to the heme proteins. The amount of heme proteins present in the muscle dictates the amount of CO that will be bound. At day 6 both CO treatments had declined to similar CO levels. However, even at the end of the 18 days storage period, both treatments still had significantly ($p < 0.05$) higher levels than the control.

Figure 4-10 represents the amount of CO found in white muscle. The difference between red and white muscle of the two CO treatments is ~2-2.8 fold. Previous work by Miyazaki and coworkers [44] has also shown that CO levels are higher in red muscle compared to white muscle. This suggests that the heme proteins are the main source of bound CO in the muscle since white muscle contains significantly less heme proteins than red muscle. The white muscle of untreated control has similar levels of CO red muscle (943 ppb). Analysis of the CO levels in white muscle of the treated fish confirms that there is entrapment of CO in the extracellular matrix of the muscle when it was treated post-mortem with 100% CO. The starting values of CO for the gassed fillets were significantly higher than the values for the euthanized samples. The difference between the two is close to the difference seen in the red muscle. This difference is therefore very likely explained by additional CO trapped in the muscle, and not bound to heme proteins. The concentration of CO dropped more suddenly in the gassed white muscle than it did in

the red muscle. The higher heme content in the red muscle may have aided in the stabilization of the CO in the muscle during the first days of storage, while the lower level in the white muscle caused CO to be released sooner from the muscle. After 6 days of storage the values stabilized and were similar to those of the euthanized fish.

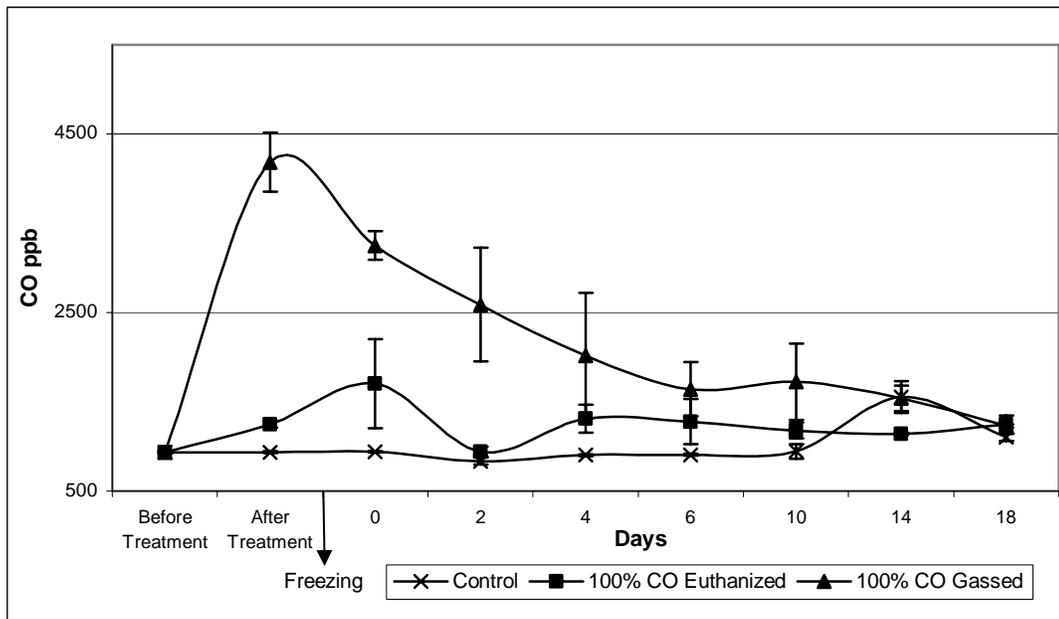


Figure 4-10. Concentration of CO (ppb) in tilapia white muscle after 30 minutes exposure to 100% CO, euthanasia with 100% CO or no treatment.

Muscle pH and Drip Loss

The amount of stress at which the animal is put under before being slaughter can have a great influence in its final pH and thus in its water holding capacity [26]. As the muscle pH decreased post-mortem, the number of negative charges decreases on the muscle proteins and they are moved closer to their isoelectric point. Muscle has its lowest water-holding capacity at the isoelectric point of the myofibrillar proteins [50]. It was expected that the euthanasia of tilapia would be less traumatic for the animal and thus a lower drop in pH and higher water holding capacity would be obtained. The pH data does not indicate that there was any significant ($p < 0.05$) difference between the different

treatments. However, Figure 4-11 shows that fillets from control and euthanized tilapias had a smaller change in pH compared to the gassed fillets. Nevertheless it cannot be inferred that the CO gassing of fillets represented a more stressed process, since the fish used for the gassed fillets were slaughtered in the same way as the control and the pH should therefore have been similar. It can be assumed the level of stress at which the tilapia experienced from every treatment was different from the beginning due to the handling of the live animal. Tilapia were transported live in small coolers from a tilapia farm located 2 hours away from the laboratory. According to Terlouw [26] an animal is under stress when it is confronted with a potentially threatening situation. It is possible that the tilapia used in the CO fillet gassing experiments were under more stress than the other treatments before slaughtering, thus explaining the lower pH values obtained.

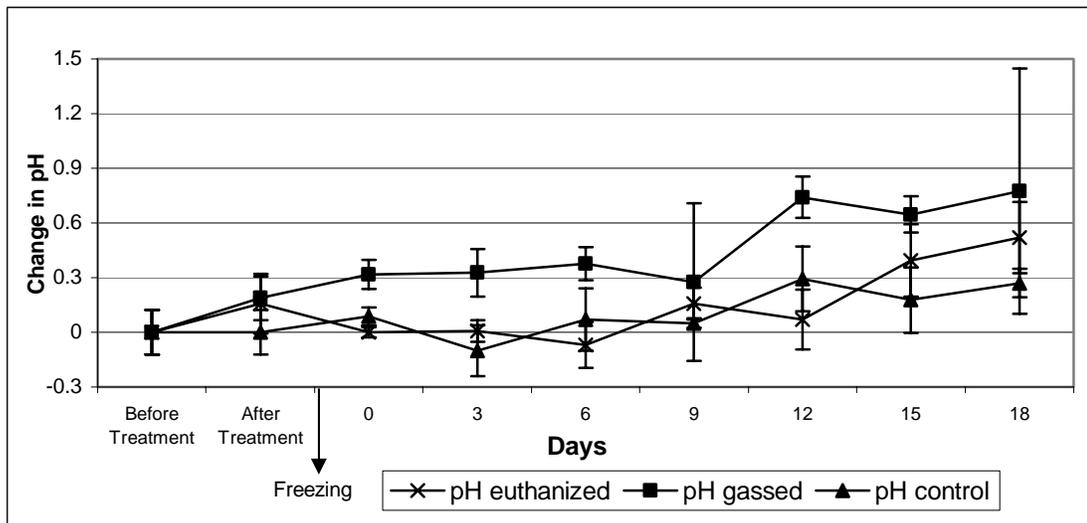


Figure 4-11. Change in pH of fillets gassed with 100% CO, fillets from fish euthanized with 100% CO and untreated tilapia fillets stored at 4°C for 18 days.

Water lost on thawing (thaw loss) was also recorded for the fillets from the three treatments. Even though statistical analysis showed no significant difference among the three treatments, Figure 4-12 shows that the euthanized samples had the highest thaw loss after 1 month of frozen storage. Thawed fillets lost 2.8% of their original weight. The

gassed samples had the lowest thaw loss with a change of 2% from their weight before freezing. These results are contradictory to the pH results, i.e., fillets from the control and euthanized fish had more stable and higher pH, but still higher thaw loss than gassed fillets which had a lower pH, which is contrary to what was expected.

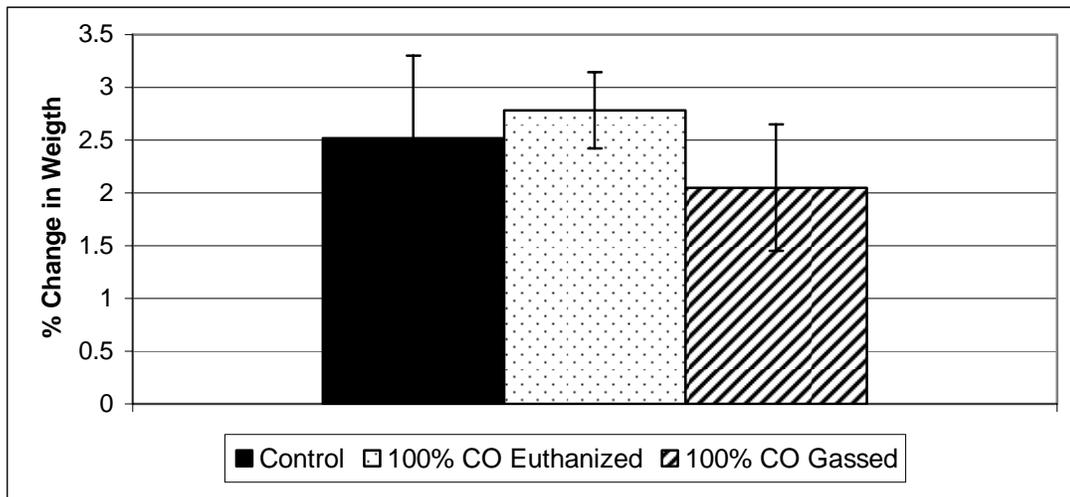


Figure 4-12. Thaw loss of gassed fillets (100% CO for 30 min), fillets from euthanized fish (100% CO) and untreated fillets after 1 month of freezing. Results obtained are based on the change on weight of the fillets.

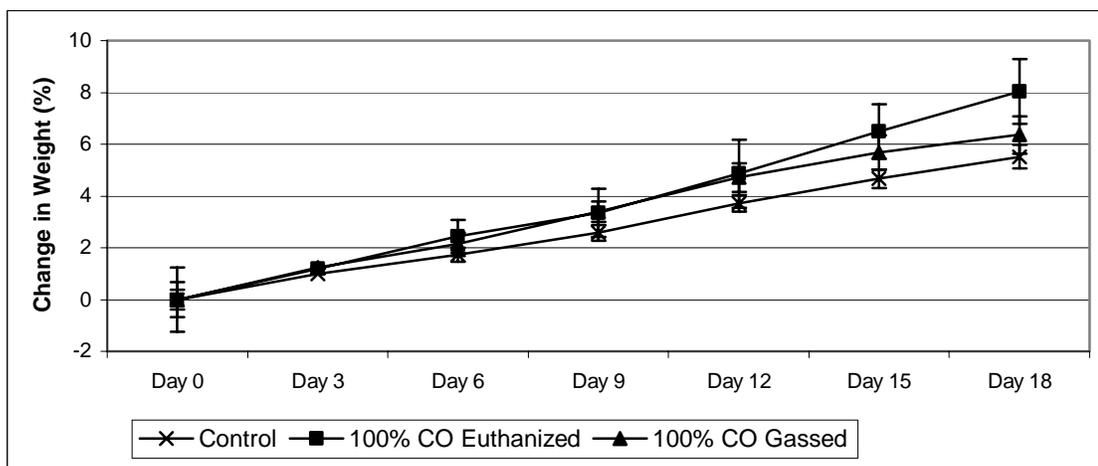


Figure 4-13. Change in drip loss of gassed fillets (100% CO for 30 min), fillets from euthanized fish (100% CO) and untreated fillets during 18 days of storage at 4°C after thawing.

There were no significant differences in the amount drip loss among the three treatments during the 18 day storage at 4°C after thawing (Figures 4-12). Apparently the differences in pH did not affect the drip loss of the fillets.

Study 2: Whole Frozen Tilapia

Effect of Carbon Monoxide Euthanasia on Color

Color was analyzed for fillets obtained from fresh control and euthanized tilapia, and also after defrosting the frozen whole fish after 2 and 4 months (Figure 4-14). The fresh data was obtained to compare the color of the muscle at the fresh state to the color of the muscle after being subjected to frozen storage. The effect of euthanizing the fish on the a*-values can be appreciated as the a*-values of the fillets from the euthanized fish were significant higher ($p < 0.05$) than the a*-values of the control.

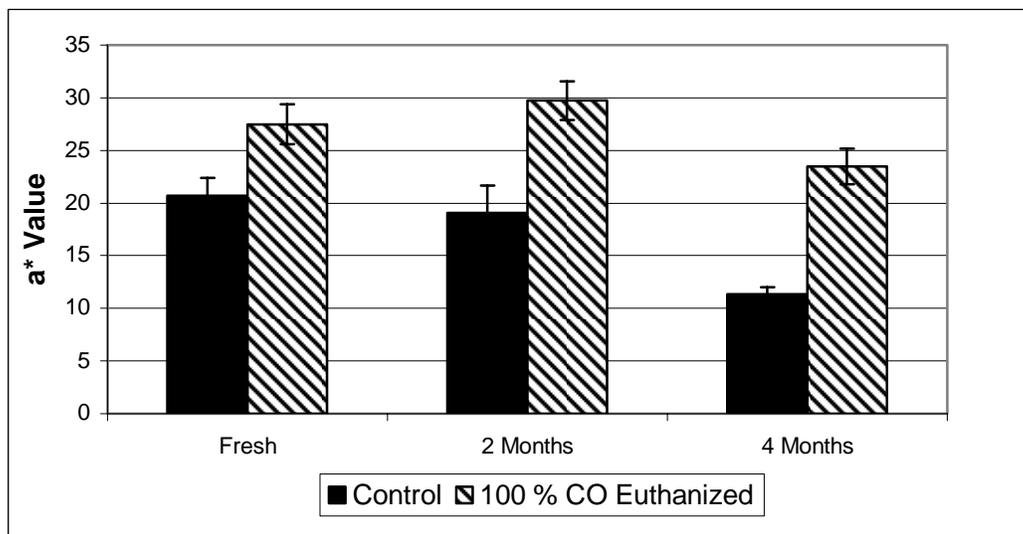


Figure 4-14. Effects of euthanasia with 100% CO and no treatment on a*-values of the red muscle of fresh tilapia and tilapia stored frozen for up to four months.

The highest a*-values for the control samples were obtained for the fresh fish. At the fresh state, the fillets were exposed to air after filleting which leads to oxygen binding to hemoglobin which gives the bright red color characteristic of oxy-hemoglobin. This is

confirmed by the heme peaks wavelengths obtained for the control samples (see next section). The control a^* -values obtained at this point are representative and can be used as reference for a^* -values of a fresh tilapia fillet. In addition, the difference between the euthanized samples and the control are less significant at this point than at the other samples points. The euthanized a^* -values were however significantly higher than the a^* -values of the control. The increase in redness is explained by the formation of the carboxy-hemoglobin complex, as discussed before.

The control a^* -values progressively decreased after freezing. However there was no significant ($p>0.05$) difference between the fresh and after 2 months a^* -values. Meaning that storing the fish whole in a frozen state preserved the color of the fillets. There was a significant decrease ($p<0.05$) in a^* -values after 2 months of frozen storage. This decrease comes from the oxidation of the heme proteins where the characteristic bright red color of the red muscle of the fresh fillets gradually turns into a more brownish color. The a^* -values of the fillets from the euthanized fish were maintained significantly ($p<0.05$) higher throughout the study compared to the control. It can be seen from Figure 4-13 that the euthanized a^* -values were significantly ($p<0.05$) higher at all times than the control values. After 4 months of frozen storage, the euthanized a^* -values were not significantly ($p>0.05$) different from the initial control values, underlining how stable the a^* -values became after the euthanasia with 100% CO. This stability came from the strong binding of CO to the heme proteins. Interestingly an increase in a^* -values was observed after 2 months of frozen storage. This agrees with the data shown in the previous chapter on the fillets. Danyali [34] also noted an increase in a^* -values when CO treated yellowfin tuna steaks were subjected to 30 days of storage at -25°C . The increase in red color of the

tilapia muscle corresponded to an increase in the concentration of CO in the muscle (see later). These results thus suggest that the more CO is bound to the muscle the higher a^* -values would be obtained.

Similar results were found for the white muscle where fillets from the euthanized fish also show significantly ($p < 0.05$) higher a^* -values than control (Figure 4-15). Furthermore, a^* -values for the euthanized fish were maintained significantly ($p < 0.05$) higher at all times compared to the control. After four months of frozen storage, a^* -values of the white muscle from the euthanized fish were similar (no significant difference ($p < 0.05$)) to the initial a^* -values, thus mirroring that seen for the red muscle. The control a^* -values on the other hand decreased significantly after 4 months of frozen storage.

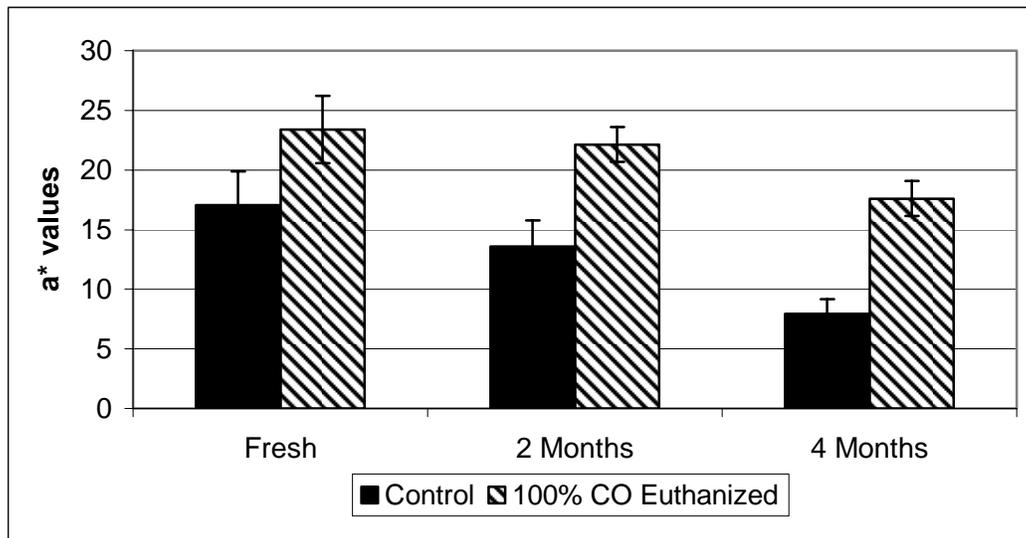


Figure 4-15. Effect of euthanasia with 100% CO and no treatment on a^* -values of the white muscle of fresh tilapia and tilapia stored frozen for up to four months.

The euthanasia process did not have an influence on b^* or L^* -values. No significant differences ($p > 0.05$) were found for the euthanized fillets when compared to the control fillets (Figures 4-16 to 4-19).

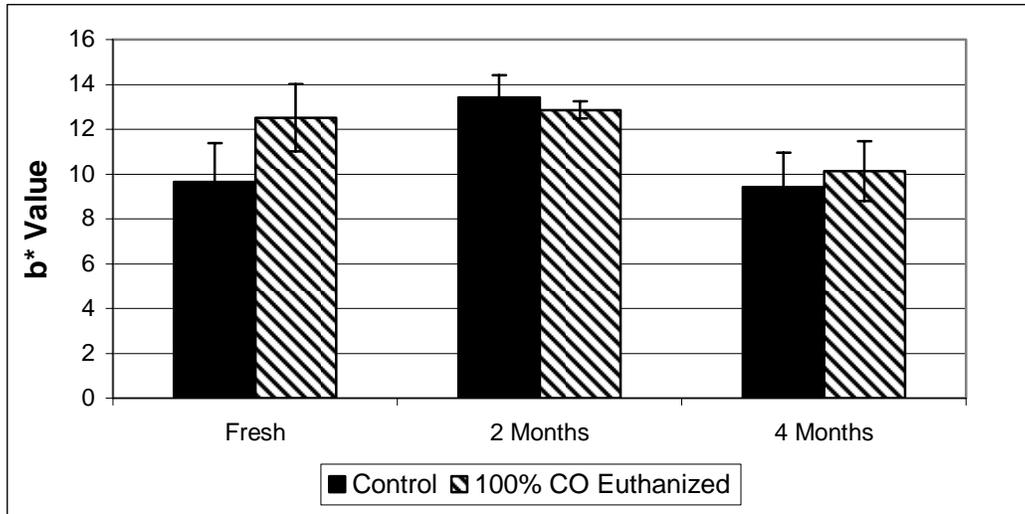


Figure 4-16. Effect of euthanasia with 100% CO and no treatment on b*-values of the red muscle of fresh tilapia and tilapia stored frozen for up to four months.

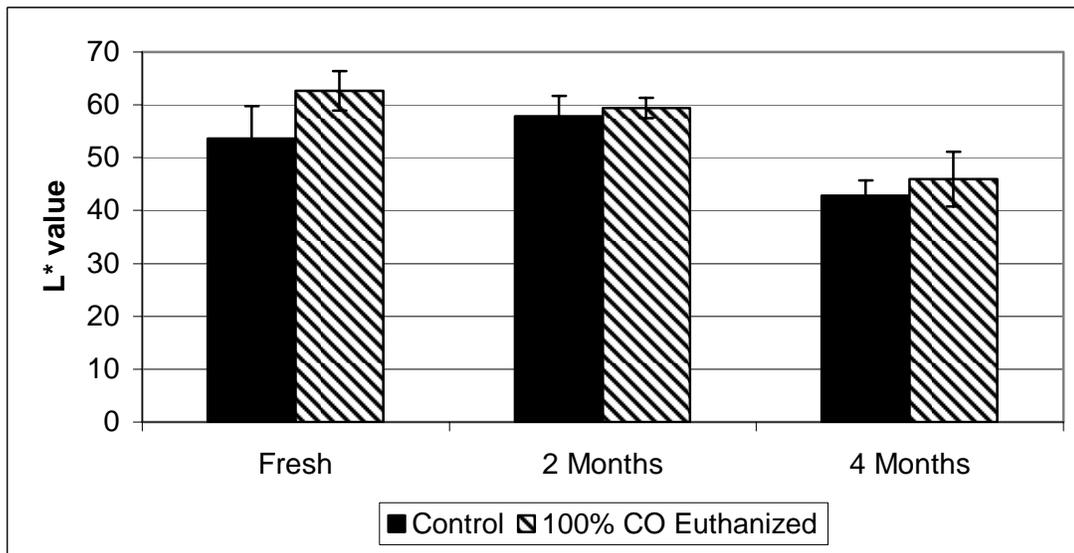


Figure 4-17. Effect of euthanasia with 100% CO and no treatment on L*-values of the red muscle of fresh tilapia and tilapia stored frozen for up to four months.

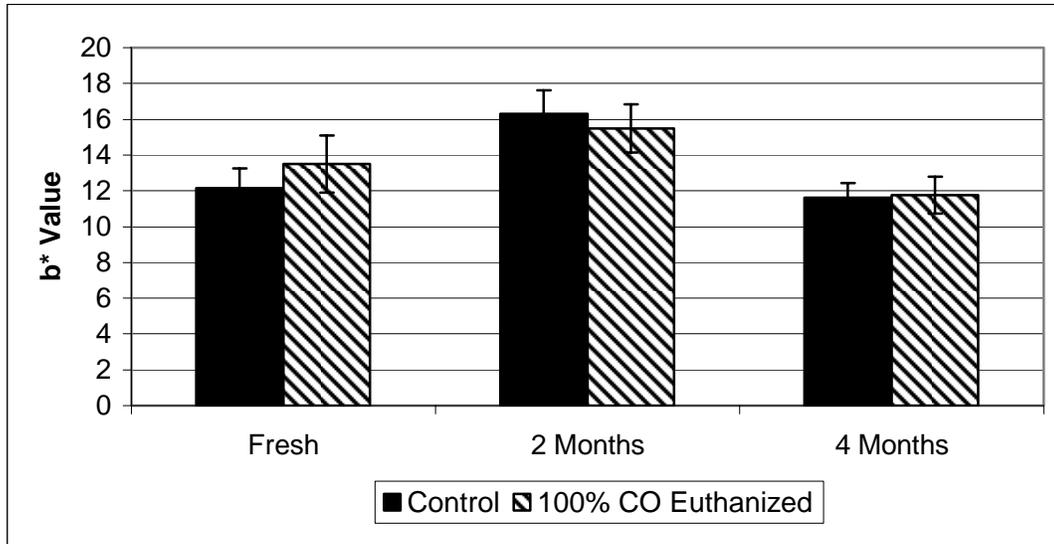


Figure 4-18. Effect of euthanasia with 100% CO and no treatment on b*-values of the white muscle of fresh tilapia and tilapia stored frozen for up to four months.

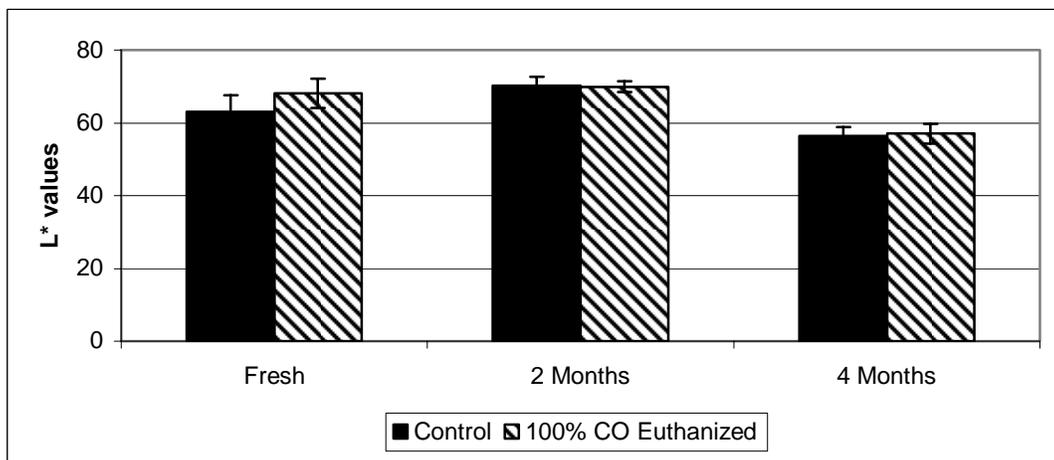


Figure 4-19. Effect of euthanasia with 100% CO and no treatment on L*-values of the white muscle of fresh tilapia and tilapia stored frozen for up to four months

Heme Spectroscopic Analysis

The red color stability throughout the study is caused by the stability of the heme proteins. If the formation of the met-form of the heme proteins is avoided, the red color will be maintained [19]. As shown in the first study on fresh and frozen fillets, the red muscle is where most of the CO is bound due to its high content of heme proteins. Figure 4-18 shows the heme peaks wavelengths obtained of red muscle extracts for the three

samples points. The difference in heme protein ligand binding and stability is clearly different between the euthanized and untreated (control) fish.

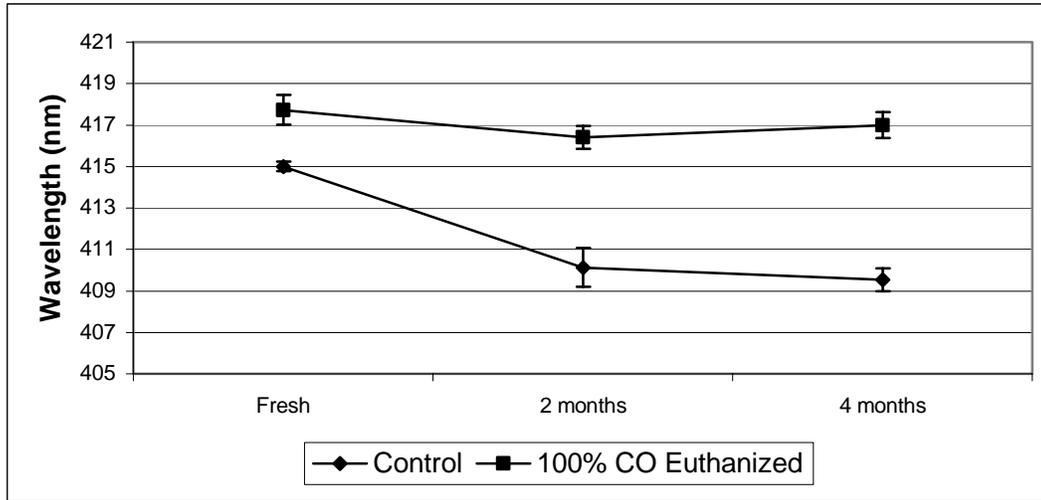


Figure 4-20. Maximum heme peak values for red muscle of euthanized (100% CO) and untreated fresh and frozen whole tilapia

A heme peak wavelength of 415 nm was seen for the fresh control signifying the presence of oxy-hemoglobin/myoglobin (Figure 4-20). This peak was expected since the fillets were exposed to air during the filleting and skinning process (which was part of the sample preparation) which would have allowed oxygen from the air to bind to hemoglobin/hemoglobin. The coupling of oxygen with hemoglobin/myoglobin gives a bright red color, explaining the high a^* - values obtained for the control in the fresh state. The heme peak wavelengths decreased on freezing, suggesting oxidation of the heme proteins as well as loss of oxygen, which agrees well with red color results (Figures 4-20 and 4-14). At the end of the study, a heme peak wavelength of 409 nm was found for the control samples implying that the heme proteins were significantly oxidized and thus met-hemoglobin/myoglobin had formed. The presence of the met- form was also evident in the fillets. As presented and discussed in the section before, a brown color had replaced the bright red color of the fresh fillets.

The euthanized fillets had higher heme peak wavelengths (~418 nm after euthanasia) than control, indicating the presence of carboxy-hemoglobin. These wavelengths demonstrated the intake of CO by the fish during the euthanasia process. The heme proteins in the euthanized fish were also significantly ($p < 0.05$) more stable during the study as they maintained high heme peak wavelengths. After four months of frozen storage, it can be seen that CO was still bound to hemoglobin showing a great stability of the carboxy-hemoglobin complex. This stability is also responsible for the high and stable a^* -values discussed earlier. A substantially higher stability of tilapia carboxy-hemoglobin compared to tilapia oxy-hemoglobin during extended frozen storage has been reported [19], which would explain this high heme protein and color stability of the CO euthanized samples during the 4 month frozen storage.

The same analyses were done on the white muscle (Figure 4-21). As reported in the first study on white muscle, the heme peak wavelengths were higher for the euthanized samples than the control, however not as high as those for the red muscle. The higher wavelengths were also maintained higher for the euthanized samples for the duration of the study. The heme peak wavelengths found in the white muscle of the euthanized fish correspond to wavelength one would expect for oxy-hemoglobin/myoglobin and not carboxy-hemoglobin/myoglobin. The fact that the heme peak wavelengths of the euthanized fish white muscle is higher than that of the control, does however suggest that CO binding took place. The increase in a^* -value for the white muscle of the euthanized fish also supports this (Figure 4-15). These lower wavelengths for the euthanized white muscle compared to the red muscle, suggest that the values represent an average of all three different heme protein derivatives, i.e., met, oxy and carboxy, while the control

value suggest a mixture of met and oxy forms[45]. The heme peak wavelength increase, increased heme protein stability and increased red color of the euthanized white muscle during the four months study therefore must come from partial CO binding to the heme proteins in the white muscle.

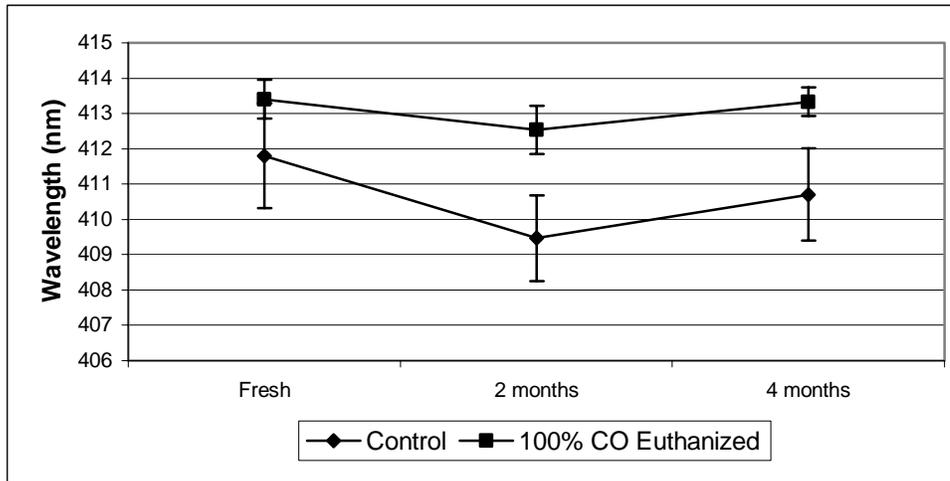


Figure 4-21. Maximum heme peak values for white muscle of euthanized (100% CO) and untreated fresh and frozen whole tilapia.

It is interesting that the heme peaks wavelength for both red and white muscle did not decrease as much as in the second study compared to that seen in the first study. The fact that the fish were frozen whole appeared to preserve the heme proteins better compared to freezing fillets.

Carbon Monoxide Quantification

The amount of carbon monoxide that is found in the muscle is important. It reveals the uptake of CO in the muscle and can also be used to differentiate treated products from untreated ones. The heme spectroscopic analyses and the increased redness (a^* -values) revealed the intake of CO by the live animal, but does not tell us how much CO is in the muscle. Figure 4-22 shows the difference in CO concentration in the red muscle between a 100% CO euthanized and untreated fish. CO concentration in the untreated red muscle

is low compared to the concentration in the euthanized fish. Furthermore, it can be observed that the CO concentration of the control is very stable and does not change significantly during the four months of study.

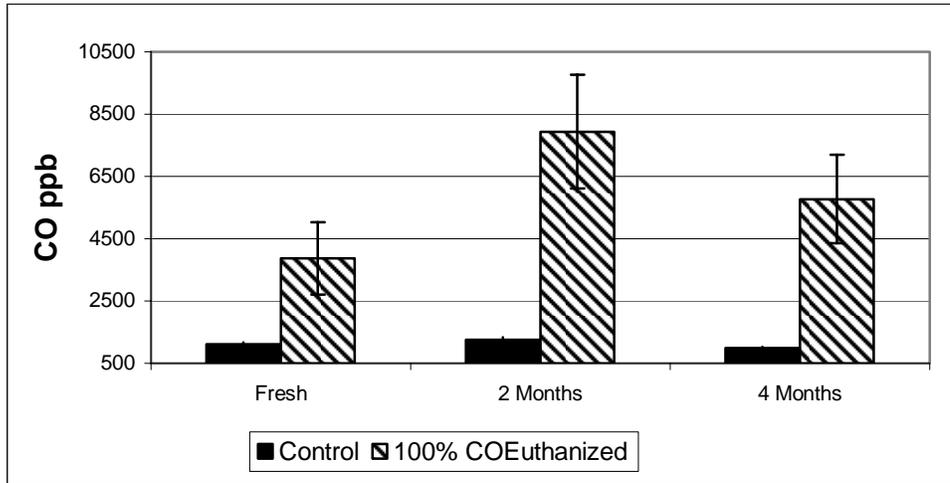


Figure 4-22. Concentration of CO (ppb) in the red muscle of fresh and frozen untreated and euthanized (100% CO) tilapia.

The amount of CO found in the euthanized fillets was considerably higher than the CO concentration in the control fillets and was more variable. Many natural conditions inherent to tilapia may have accentuated these differences. For instance, CO is bound to the heme proteins, explaining the high concentration found in the red muscle compared to the white muscle. Every fillet has different amounts of red muscle influencing the final amount of CO recovered from the muscle. This could explain the higher values found after 2 months of frozen storage. Another possible explanation for the lower values for the fresh fish is that fillets were cut from the euthanized fish very shortly after it was euthanized, which could have led to some CO loss from the muscle. On the other hand, the samples at 2 and 4 months were taken from whole frozen fish filleted after thawing, thus giving the muscle more time to “trap” CO than the muscle of the freshly killed and filleted fish.

The concentration of CO found in the white muscle follows the same trends as that seen for the red muscle (Figure 4-23). The difference between the euthanized and the control muscle were not as large in the white muscle compared to the red muscle. This relates to the lower amount of heme proteins present in the white compare to the red muscle.

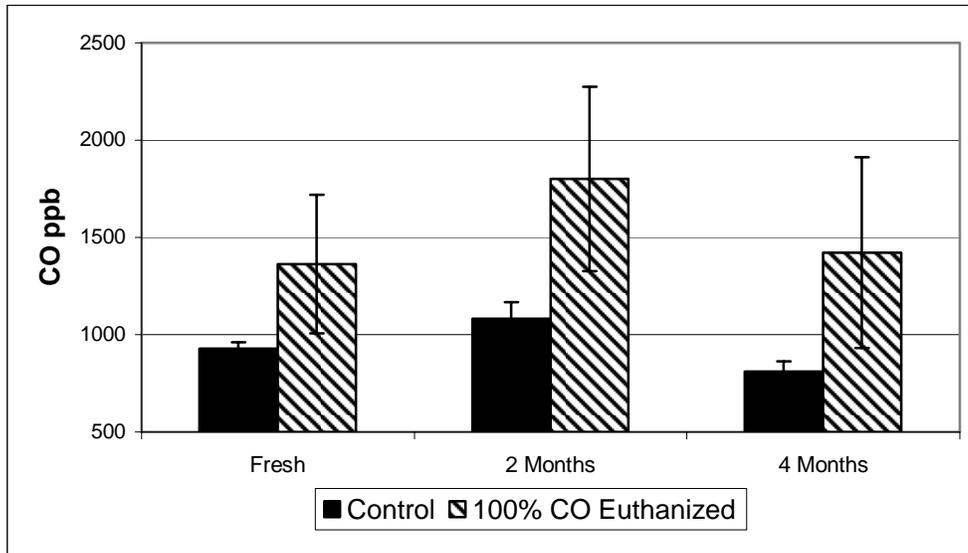


Figure 4-23. Concentration of CO (ppb) in the white muscle of fresh and frozen untreated and euthanized (100% CO) tilapia.

From Figures 4-22 and 4-23 it can be observed that the amount of CO was maintained high during the whole study and that it did not decrease significantly ($p < 0.05$) over time. Since the tilapia was kept frozen whole and vacuum packed it may have aided in keeping the CO levels high at all times. This can be also observed in the high a^* -values obtained throughout the study and in the high heme peak wavelengths discussed earlier.

As discussed previously, an increase in a^* -values after 2 months of frozen storage was seen in the red muscle, thus corresponding to the increase in CO levels, revealing a close relation between the amount of CO present in the muscle and a^* -values (Figures 4-12 and 4-20). However, as seen in Figure 4-13, a^* -values from the white muscle did not

follow the same trend as in red muscle. A higher amount of CO after 2 months of frozen storage was found in the white muscle of the fish (Figure 4-23), similar to that seen for the red muscle, however a^* -values in the white muscle did not increase. This can possibly be explained to the amount of heme proteins present in each muscle. The small amount of heme proteins present in the white muscle could explain these results. Since there are more heme proteins present in the red muscle, more binding could take place and more CO is needed to stabilize all the heme proteins. Meanwhile, in the white muscle the limit of CO intake is much lower and maybe the excess of CO present is trapped in the muscle are not necessarily bound to the heme proteins and thus has no effect on color.

CHAPTER 5 SUMMARY AND CONCLUSIONS

The practice of treating fish with CO in order to avoid the oxidation of the heme proteins and maintain an appealing and very attractive fresh red color is now widespread and increasingly more common. The red color is stabilized by the formation of the carboxy-hemoglobin/myoglobin complex. In the tilapia industry, a common practice is to expose the fillets pre-rigor, while the muscle is still respiring, to CO for a brief period of time. Another less common practice is to incorporate CO into fish tanks while the animal is still alive.

The intake of CO to the muscle by the euthanasia with 100% CO process was confirmed by the heme peaks wavelengths obtained and by the difference in the concentration of CO found in the treated product compared to the untreated ones. The incorporation of CO in the muscle of tilapia by either treatment had a positive effect in the red color of the fillets. After the treatments, the red color was enhanced and maintained for a longer period of time. In addition, the CO treatment affected only the redness (a^* -values); b^* and L^* -values were not significantly affected. Comparing the euthanasia process against the gassing of fillets it was found that both processes had good results preserving the color of the red muscle. The euthanasia with 100% CO had a few advantages compared to the post-mortem 100% CO treatment. A larger increase in a^* -values was seen when the fillets came from a euthanized fish. Also, a better stability through the freezing process was seen for the euthanized samples. Finally, the euthanized method was found to preserve better L values giving the fillets a more “natural” look.

Due to the inherent stress of the whole process, muscle pH and water holding capacity were not significant different among treatments. Nevertheless, it is believed that the euthanasia process will be less stressful to the fish if they were initially without the added stress of transportation and laboratory handling. In addition, the euthanasia with 100% CO represents less product handling since there will be no need of post-mortem CO gassing. This advantage may positively influence the shelf life of the final product since there will be less product handling, implying less probability of contamination. Furthermore since the product is already treated via the gas euthanasia process, less labor required is another advantage over a post-mortem gassing process.

The different level of acceptance and varying regulations between different countries on the use of CO in seafood processing has created a need to test products for CO content. In this study it has been shown that a simple heme analysis could differentiate a treated from a non treated product. In addition, the method used to measure the amount of CO in the muscle is very important and should be considered and specified when CO regulation and CO limits are put in place. It is worth mentioning that the use of CO in seafood processing has to be very closely studied and regulated. Without the proper control, the use of CO could mask potential health hazards or it could make a product look better than it actually is.

In this research study, 100% CO was used to euthanize the fish. It was observed that tilapias remained calm before dying, revealing that the process was not stressful. It was also observed that the use of CO had an anesthetic effect on the animal since they stopped moving and remained calm until euthanasia was completed. This is an important observation since animal welfare has been giving increasingly importance, and more

regulation regarding humane slaughter practices are being required. The euthanasia with 100% CO of fish would be in agreement with the animal welfare act, in my opinion.

APPENDIX A
FIRST STUDY DATA

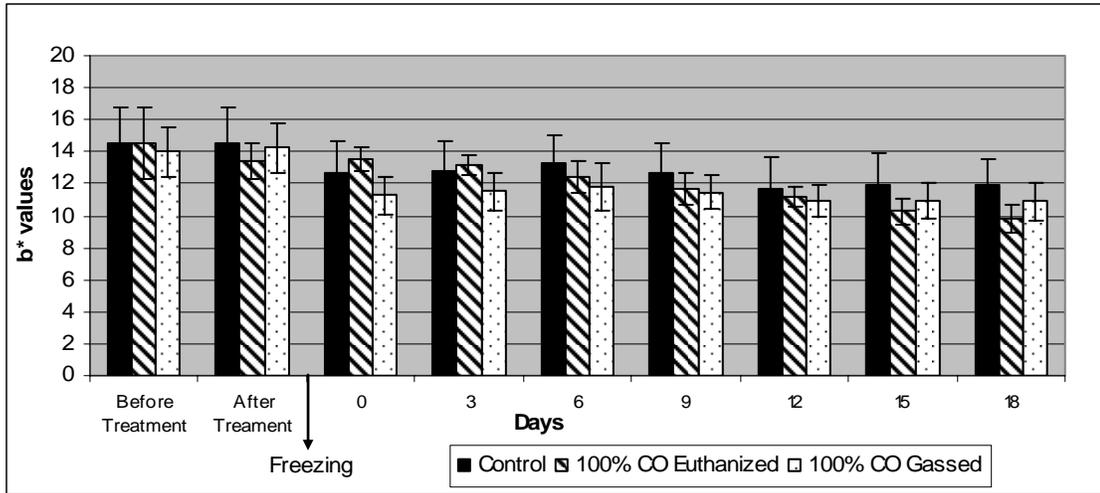


Figure A-1. Effect of CO treatments on the b*-values of the red muscle of tilapia.

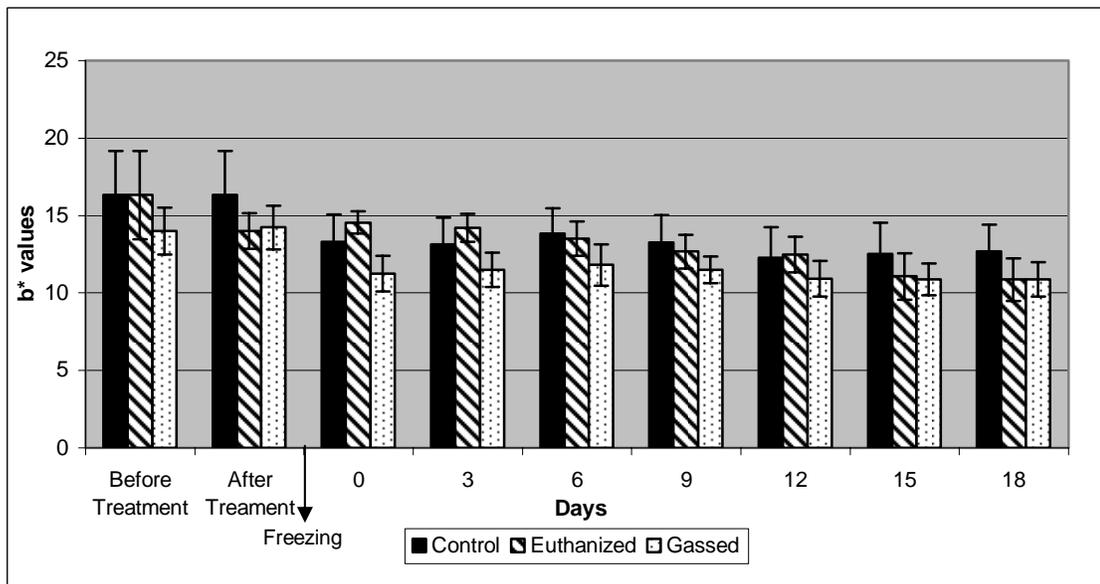


Figure A-2. Effect of CO treatments on the b*-values of the white muscle of tilapia.

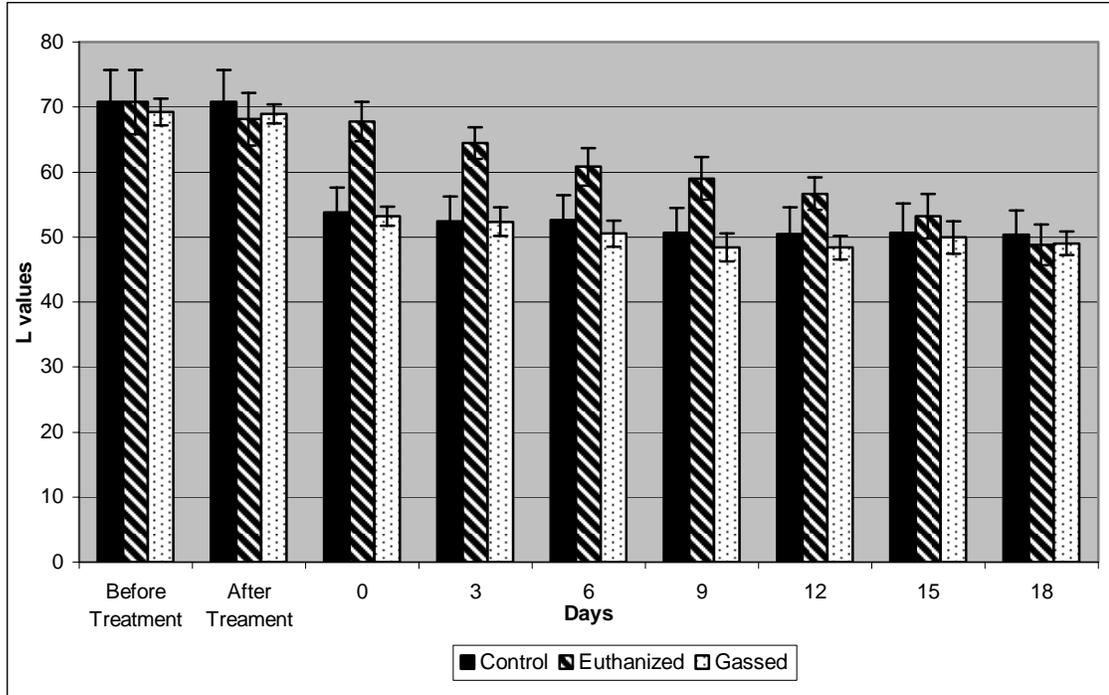


Figure A-3. Effect of CO treatments on the L*-values of the white muscle of tilapia.

APPENDIX B
CO CONCENTRATION IN THE WATER

Table B-1. Concentration of CO in the water used to euthanize tilapias

Time	Ret. Time	Peak Area	[CO] ppb	Time	Ret. Time	Peak Area	[CO] ppb
0 min	0.490	5.400	2166.504	20 min	0.453	1833.290	27082.385
0 min	0.459	5.490	2167.730	20 min	0.453	1821.400	26920.313
0 min	0.456	5.460	2167.321	20 min	0.457	1369.140	20755.579
0 min	N/A	N/A	N/A	20 min	0.460	1348.140	20469.329
Ave	0.468	5.450	2167.185	Ave	0.456	1592.993	23806.901
Stand				Dev	0.003	270.786	3691.074
Dev	0.019	0.046	0.625	25 min	0.453	1846.280	27259.451
5 min	0.465	872.940	13991.900	25 min	0.455	1440.570	21729.238
5 min	0.456	844.440	13603.418	25 min	0.456	1547.700	23189.521
5 min	0.459	633.640	10730.013	25 min	0.458	1515.120	22745.425
5 min	0.455	612.740	10445.126	Ave	0.456	1587.418	23730.909
Ave	0.459	740.940	12192.614	Stand			
Stand				Dev	0.002	178.305	2430.472
Dev	0.004	136.729	1863.752	30 min	0.454	1832.360	27069.708
10 min	0.459	820.970	13283.499	30 min	0.460	1822.440	26934.489
10 min	0.459	802.520	13032.008	30 min	0.454	1334.740	20286.674
10 min	0.462	1109.130	17211.395	30 min	0.458	1318.220	20061.490
10 min	0.464	1104.260	17145.012	Ave	0.457	1576.940	23588.090
Ave	0.461	959.220	15167.979	Stand			
Stand				Dev	0.003	289.313	3943.615
Dev	0.002	170.468	2323.635	35 min	0.453	1814.700	26828.986
15 min	0.466	1676.640	24947.096	35 min	0.453	1796.570	26581.857
15 min	0.452	1665.550	24795.929	35 min	0.452	1518.100	22786.045
15 min	0.457	1583.100	23672.057	35 min	0.450	1511.960	22702.351
15 min	0.454	1559.580	23351.457	Ave	0.452	1660.333	24724.810
Ave	0.457	1621.218	24191.635	Stand			
Stand				Dev	0.001	167.963	2289.492
Dev	0.006	58.564	798.280				

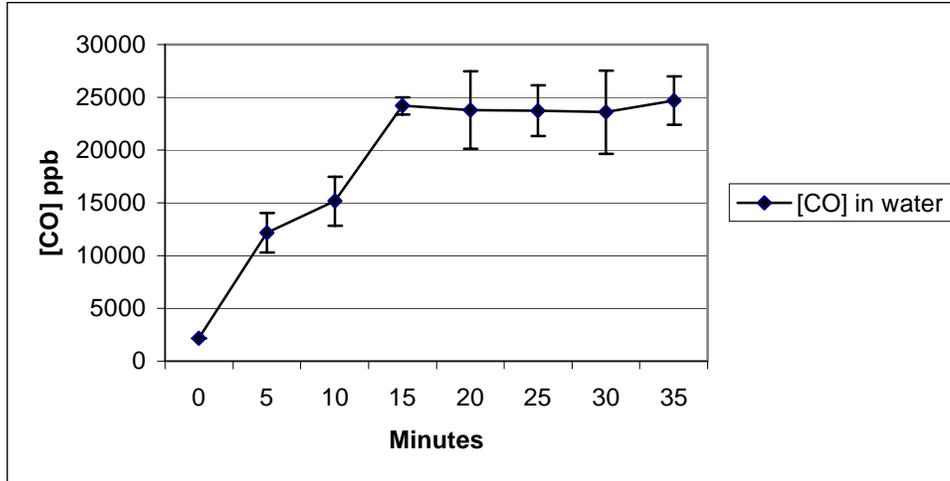


Figure B-1. Change of CO concentration in the water used for the euthanasia process.

Table B-2. Time and amount of CO needed to euthanize tilapia in the tank

Date	Time min	CO used (L)	
1/19/2005	39	182	
1/19/2005	52	110.4	CO did not flow correctly
1/19/2005	32	121.6	
2/16/2005	27	500	Pump not working properly
3/14/2005	28	197.3	
3/14/2005	26	258	
Average	34	228.216	
Std Dev	10.01998	143.591	
Average of Correct Trials	31.25	189.725	
Std Dev of Correct Trials	5.737305	56.034	

APPENDIX C
PICTURES OF THE TILAPIA FILLETS

Representative pictures of the “red” muscle side of a tilapia fillet, and a ‘white’ muscle side of a tilapia fillet are shown below. All the images can be found in the CD D:/Thesismantilla/tilapiaimages (Kept in Dr. Hordur G. Kristinsson’s office)

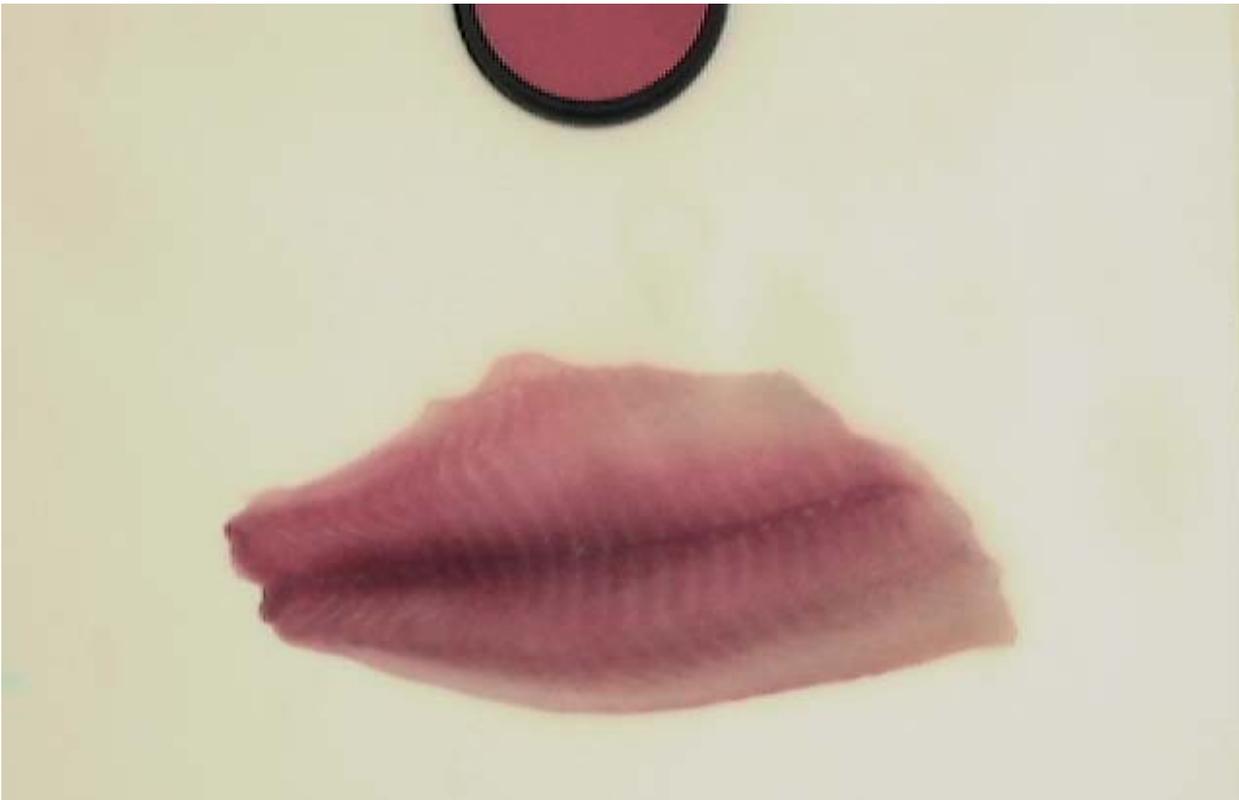


Figure C-1. Picture of a “red” muscle side of a tilapia fillet taken by the CMVS



Figure C-2. Picture of the “white” muscle side of a tilapia fillet taken by the CMVS

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BIOGRAPHICAL SKETCH

David Mantilla was born on February 3, 1979, in Quito, Ecuador. He attended a French high school and graduated from it in July 1997. He came to the U.S. in Fall 1999 to the English Language Institute (ELI) at the University of Florida. He attended Santa Fe Community College and graduated in December 2000. David graduated in December 2002 from the University of Florida with a Bachelor of Science degree in food science and human nutrition. He did two internships as an undergrad, one with Tyson Foods and the other with Darden Restaurants. He started his master's degree in August 2003 under Dr. Hordur G. Kristinsson. While at the University of Florida he was invited to join Phi Tau Sigma, the honorary society of food scientists.