CARBON MONOXIDE DETERMINATION AND PENETRATION IN TREATED FISH PRODUCTS

By

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To my loving parents and especially my boyfriend.

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CARBON MONOXIDE DETERMINATION AND PENETRATION IN TREATED FISH PRODUCTS

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Treating fish muscle with carbon monoxide (CO) or filtered wood smoke (FS) has become a common practice in the industry due to their effectiveness in maintaining red color of muscle. This color stabilization comes from binding of CO to heme proteins and forming stable carboxy heme proteins. The scientific information about this technology is limited.

In this study simple spectrophotometric methods were developed to determine CO in fish muscle, which is based on the fact that heme proteins and their derivatives have characteristic absorption spectra, and that sodium dithionite can deoxygenate oxyHb/Mb and metHb/Mb without influencing COHb/Mb. With this simple one-wavelength spectrophotometric method, any heme protein extract which exhibits a maximum peak wavelength at 415 nm or above can be determined as CO treated. With the two-wavelength spectrophotometric method, after addition of a reducing reagent, a two pigment mixture is produced and absorbance of the pigments is measured at 419 and 430 nm. The percentage of COHb/Mb can be calculated directly from the ratio of the absorbance values at 419 and 430 nm.

A gas chromatographic method was improved to determine CO in fish muscle. Liberating CO directly from the mixture of fish flesh and buffer was more effective than releasing CO from Hb/Mb containing supernatant and the muscle homogenate. There was a significant difference

between various CO liberating methods ($p<0.05$). A solution containing 5% sulfuric acid could release CO from the muscle more effectively compared to the others tested. In addition, various two-wavelength spectrophotometric methods were evaluated for CO determination. Both the Soret range and the visible range have similar sensitivity in COHb/COMb% determination. It is important to combine both spectrophotometric method and GC method together if we aim to determine both the total CO amount and the CO binding status in the muscle.

Finally the fate of CO was investigated during CO treatment and following storage of CO treated samples. We also studied the effect of external factors, such as CO concentration, and internal factors, such as Hb concentration, Hb oxidation status, pH value, ionic strength, lipid content, on the CO penetration rate and CO amount in the muscle. CO could get into the first layer (0-2 mm from surface) after only 15 min treatment, and as CO treatment time went on, CO amount in each layer of the muscle increased. In addition, the one-wavelength and two-wavelength spectrophotometric methods were also used to study CO penetration into the muscle, and results correlated well with the GC results.

CHAPTER 1
INTRODUCTION

Seafoods are a source of high quality protein and nutritionally beneficial lipids, and have become an important muscle food throughout the world. Total world fish production increased steadily from 19.3 million tons in 1950 to more than 100 million tons in 2002. Seafoods are highly perishable and they are susceptible to microbial/chemical spoilage and deterioration which may lead to large economic losses. One of the major factors influencing the market value of seafoods is their color, especially for some dark muscle fish species such as tuna and mahi mahi. Consumers usually regard this red color as an indicator of fish freshness. Therefore the challenges facing commercial fisheries are to stabilize and maintain the red color of the products during processing, transportation and storage.

Heme proteins, most notably myoglobin (Mb) and hemoglobin (Hb), contribute to the color of fresh muscle (Kanner and others 1987). The color status depends on both oxidation of the iron atom in the protein heme group and the type of ligand bound to the iron atom. The surface of muscle is characterized by reduced oxymyoglobin (oxyMb) which provides a desirable red color, while the interior is characterized by reduced deoxymyoglobin (deoxyMb) which gives a purple color. Mb is susceptible to autoxidation forming metmyoglobin (metMb) which gives an undesirable brown color. In order to maintain the red color, it is important to maintain the reduced state of Mb/Hb and prevent the autoxidation of Mb/Hb. Since carbon monoxide (CO) binds to heme proteins with higher affinity than O_2, treating muscle with CO or filtered smoke (FS), which contains a certain amount of CO, can increase the stability of heme proteins and thus maintain a cherry red color for a extended time. COHb has been found to be much more stable against autoxidation compared to oxyHb (Kristinsson and others 2005). Although this positive effect of CO on muscle color has been known for a long time, and was

patented over 100 years ago for meat products (Church 1994), it is not until recently that this process has been employed to maintain the quality of seafood products.

CO treatment has stirred up a considerable controversy. On one hand, treated meat or fish products with CO or FS are advantageous. Firstly, CO/FS treatment has been reported to develop and maintain a desirable red color, reduce lipid oxidation, and lower aerobic bacterial counts which result in extending shelf life (Sorheim and others 1997; Jayasingh and others 2001; Krause and others 2003; Kristinsson and others 2003; Kristinsson and others 2007; Kristinsson and others 2008). Secondly, COHb/Mb is quite stable during frozen storage (Kristinsson and others 2005), which may help fish species that are very sensitive to freezing. Finally, CO can help protect color after irradiation (Kusmider and others 2002) and also CO may be good for cooked or cured meat products (Sorheim and others 2006). On the other hand, CO is a toxic substance and the consumption of CO treated meat may pose a public health hazard. Since CO binding to Hb is over 200 times stronger than oxygen binding to Hb, CO can displace oxygen on Hb even at very low partial pressure and prevent oxygen transportation in blood. Besides, CO treatment can in some cases represent economic fraud as the cherry red color may last well beyond the microbial shelf life of the meat and thus mask spoilage and may hide underlying safety problems such as histamine formation and pathogens, as well as enhance the color of oxidized muscle products.

Despite many positive effects of CO/FS treating on fish or meat products, the adoption of this technology has been relatively limited primarily because of the regulatory limitations. In Canada, it is prohibited to use CO alone on any food products. Also, in the European Union, CO or FS is not allowed as a treatment for fish or meat (Schubring 2004). Norway discontinued using CO due to its entry into the European Union on July 1st, 2004. Japan issued a notice

banning fish that have an initial CO content ≥500 μg/kg, or an initial content ≥200 μg/kg which decreases significantly during two days refrigerated storage (Japan, Food Sanitation Law – Article 6). The United States is much more open to CO/FS treated products compared to many countries. Several patents have been issued about producing and applying filtered smoke to fish or meat in order to retain its red color (Woodruff and Silliker 1985; Yamaoka 1996; Kowalski 1999; Shaklai 2001; Olson and Brinsmade 2004). Currently CO is being used to treat some seafood either as a single gas or in combination with other gases at various concentrations. Packaging meat products in atmospheres containing 5-100% CO has not been used much in the meat industry, but high amount of CO or FS treatment is commonly used for fish containing high levels of Hb/Mb, such as tuna.

FDA has no questions regarding the conclusion of Hawaii International that tasteless smoke is GRAS (generally recognized as safe) for use on tuna before it is frozen (FDA 2000). In 2002, in response to a request from the Pactiv Corporation, FDA issued a GRAS approval of CO application in MAP for fresh cut and ground red meats destined for retail display. Further, FDA announced another GRAS approval about using 0.4% CO for direct case-ready packaging (FDA 2004). Since USDA classified the use of CO in Pactiv system as a processing aid rather than a product ingredient, there are no labeling requirements associated with this CO-MAP system. However, the other FS or CO processed products must be labeled since FS and CO are considered by FDA as ingredients.

Since the CO or FS treatment can provide a positive effect on quality and safety of fish species, which may give consumers seafood products with higher quality and safety, the technology of using CO or FS has gained considerable interest in the meat and fish industries worldwide, and there is definitely a growing market for CO treated products. However, the

scientific information about this technology is rather scarce. Considering the growing use of CO/FS to treat meat or fish products and the regulatory restrictions by many countries, many scientific studies focus on identifying if the product has been treated with CO/FS and on accurately quantifying the CO amount in the treated products. Numerous methods have been reported for the detection of CO or COHb% in blood (Boumba and Vougiouklakis 2005). The two-wavelength spectrophotometric method (Tietz and Fiereck 1973; Rodkey and others 1979; Katsumata and others 1982; Beutler and West 1984; Fechner and Gee 1989; Stonek and others 2004) and the gas chromatographic (GC) method (Guillot and others 1981; Juntarawijit and others 2000) are two methods which are normally used to detect CO or COHb% in blood. Later, these two types of methods were used to determine CO in fish muscle (Smulevich and others 2007; Ishiwata and others1996; Chow and others1998a; Anderson and Wu, 2005). However, the two-wavelength spectrophotometric method developed to determine COMb% (Smulevich and others 2007) was quite complicated with Mb purification and sample preparation procedure, and it is hard to execute in practice. Moreover, these methods have not considered the extent of CO penetration and total absorption of CO into the muscle. Different methods to determine CO in muscle can result in highly different outcomes which make quantification difficult. There is a lack of information on the CO fate during treatment and storage of treated products.

The objectives of this study were firstly to develop a simple one-wavelength spectrophotometric method to identify CO in the muscle, and further to develop a two-wavelength spectrophotometric method to determine the percentage of COHb/Mb in the muscle. Secondly the aim was to improve the current GC method and to evaluate various two-wavelength spectrophotometric methods for CO determination in the muscle, and further to obtain a sound understanding about both the GC and spectrophotometric method. Thirdly the fate of CO during

CO treatment and following storage of CO treated samples, and also the effect of the various factors, such as CO concentration, Hb concentration, Hb oxidation status, pH value, ionic strength, lipid content, on the CO penetration rate and CO amount in the muscle were investigated.

CHAPTER 2
LITERATURE REVIEW

Muscle Pigments and CO Related Colors

Color is a primary sensory parameter of meat or fish products and it plays an important role in their economic value and acceptance. The scientific basis of fish muscle color is complicated and it can be influenced by various factors such as fish species, harvest season, freshness level, muscle type, sex, age, nutrition and environment. There are mainly two sources for the red/pink color of fish muscle: one is from carotenoids such as salmon color, and the other is from heme proteins, mainly hemoglobin (Hb) and myoglobin (Mb), such as tuna color. Carotinoids are yellow to red isoprenoid polyene pigments; many carotenoids have been isolated from various fish species. Fish, in general, can not synthesize carotenoids and those found in their body are either a result of direct accumulation from food or partly modified through metabolic reactions (Matsuno 2001). Heme proteins result in the bright red color of fish muscle, especially for dark muscle fish. This bright red color is an economically important factor, which may have a major effect on the purchase decision of consumers.

Hb is the iron-containing metalloprotein in the red blood cells of vertebrates. Hb transports oxygen from the lungs or gills to the rest of the body, such as muscle where it releases oxygen for cell use. The Hb molecule is an assembly of four globular protein subunits; each subunit is composed of a protein chain tightly associated with a non-protein heme group. Mb is located in the muscle cells and is the primary oxygen-carrying pigment of muscle tissues. Mb is a single-chain globular protein of 153 amino acids, and it is also composed of a protein chain as well as a heme group. The relative concentrations of Mb and Hb may differ depending on animal species, age of animals, muscle type, and post-slaughter treatment. Their combined concentrations will contribute to the color and acceptability of meat products. Both Mb and Hb can contribute

equally to color if they are found at the same concentration (Livingston and Brown 1981). It has been reported that most color changes are believed to be due to reactions of Mb considering Hb is more easily lost during handling and storage while Mb is retained by the cellular structure (Haard 1992). However, it was discovered that Mb concentration was higher in trout whole muscle and mackerel light muscle even after bleeding (Richards and Hultin 2002).

Both Mb and Hb have a heme group consisting of an iron in the center of a porphyrin ring. This iron bonds with the four nitrogens of the prophyrin ring, which all lie in one plane. The fifth bond orbital of iron is bound to a histidine side chain of the globin protein, and the sixth site is available to molecules, which may affect color of the muscle. Since the iron atom is located in the center of the heme group, only some small ligands, such as O_2, can access it via the cleft in the protein structure (Livingston and Brown 1981). The oxidation state and type of ligand bound to the iron atom play a crucial role in the reaction of Mb and meat color changes (Livingston and Brown 1981). Normally, there are three kinds of Mb/Hb derivatives in the flesh: oxyMb/Hb, deoxyMb/Hb and metMb/Hb. Most of the reactions of interconverting ferrous and ferric forms of Mb/Hb contribute to the color change in meats. The surface of fresh flesh is characterized by oxyMb/Hb (Mb/Hb-Fe^{2+}-O_2), which gives a desirable red color. When the intracellular oxygen tension is low (below 0.1 mm Hg), deoxyMb/Hb is formed, which gives a purplish-red color and is responsible for the corresponding color in the interior of muscle. The deoxyMb/Hb is favored to form when oxygen concentration is low or pH is decreased (Stryer 1999). Both oxyHb/Mb and deoxyMb/Hb can be oxidized by one-or two-electron oxidants to metMb/Hb (Mb/Hb-Fe^{3+}-H_2O), which gives a brown color.

Sometimes under severe processing or storage conditions, reactions associated with the unfolding of native Mb/Hb will occur and usually lead to color changes (Livingston and Brown

1981). Generally, fish myoglobin (notably tuna) is at least 2.5 times more sensitive to autoxidation than mammalian myoglobins, and this factor increases under high temperature or low pH (Livingston and Brown 1981). Irradiation has been reported to result in color change of the muscle, and the change of color depends on irradiation dose, animal species, muscle type and packaging type (Nam and Ahn 2003). Usually the light muscle will show pink color while the dark muscle will become brown or gray after irradiation. This pink color is due to CO, which is generated by irradiation, and the formation of COMb (Ahn and Lee 2004; Lee and Ahn 2004).

This undesirable brown color caused by autoxidation of heme proteins has been demonstrated to be promoted by various factors. The pH value of fish muscle decreases below neutrality postmortem. Hb autoxidation was found to occur more rapidly at pH 6.0 compared to pH 7.2 (Richards and Hultin 2000). Autoxidation also increases with increased temperature (Cashon and others 1997). Some fish Hb/Mb are very sensitive to freezing, and they lose their red color after freezing and thawing. Moreover, Hb/Mb oxidation can be accelerated when oxygen pressure is very low or very high. Light in display cases or irradiation process can also increase oxidation of heme proteins. Since reduced oxyMb/Hb represents the desirable red color of fresh meat, it is important to maintain all of the Mb/Hb in the ferrous form if we want to maintain the desirable red color of flesh. One way of preventing or delaying this browning is to rapidly freeze fish and store them at extremely low temperature. However, this process is hard to execute for most fish species. The other way of preventing or delaying browning of fish muscle is to treat it with CO to stabilize the heme proteins against oxidation. CO binds to heme proteins with around 270 times more affinity than oxygen (Stryer 1999). Since COMb/Hb is stable even when denaturation of the protein occurs, it can provide a stable red color in meat (Livingston and Brown 1981). COHb can be maintained longer in its reduced state and is significantly more

stable against autoxidation compared to oxyHb, even at frozen storage (Kristinsson and others 2005). It has been described that the application of CO can result in a significant increase in redness of the fish muscle (Balaban and others 2005; Chow and others 1997; Danyali 2004; Demir and others 2004; Garner and Kritinsson 2004; Hsieh and others 1998; Kristinsson and others 2003; Ross 2000).

Commercial Development of CO or FS Treated Products

Application of CO as a Component in MAP

The positive effect of CO on muscle color has been known for a long time, and was patented over 100 years ago for meat products (Church 1994). One method of CO application is the use of CO as a component of packaging gases of meat or fish under reduced oxygen packaging (ROP) or modified atmosphere packaging (MAP). In 1985, a patent was issued which involved retaining the good color of fresh meat, poultry and fish using carbon monoxide treatment (Woodruff and Silliker 1985). It was found that a gas mixture with CO provides a unique combination of a long microbiological shelf life and a stable, cherry red color of the meat (Sorheim and others 1997). These positive attributes for CO were supported by other research results (Sorheim and others 1999; Jayasingh and others 2001; Krause and others 2003; Kusmider and others 2002; Viana and others 2005; Wilkinson and others 2006; Ferdandez-Lopez and others 2008). It is improbable that the use of CO (< 0.5%) in meat packaging will present a toxic threat to consumers (Sorheim and others 1997). CO acting as a component in MAP has no effect on flavor and minimal effect on sheen and purge loss of cooked beef steaks or pork chops (Wicklund and others 2006; Stetzer and others 2007). This CO amount (< 0.5%) in MAP of meats has been commercially used for retail meat packaging in Norway since 1985, and it was discontinued in July 2004 because of Norway's entry into the European Union, where CO is not approved as a gas component in the packaging (European Parliament and Council Directive

1995). Since MAP may lead to the discoloration of some fish species such as tuna, certain fish species are hardly stored under MAP.

Application of CO as a Single Gas or as a Component of Filtered or "Tasteless" Smoke

Since the flesh color is important for quality evaluation and there is a possibility of influencing flesh color though muscle color pigment, CO or FS, which contains a certain amount of CO, was introduced to treat fish or meat products, specifically fish species with high amount of Hb/Mb such as tuna. The objective of filtered wood smoking processes is not to process smoked products like traditional smoking does, but to extend or preserve the shelf life of fresh and frozen seafood products (Olson 2006). In filtered smoking processes, smoke mainly acts as not a flavoring agent but a preservation agent. Some fish species, such as tuna and mahi mahi, are highly perishable and they are susceptible to microbial and chemical spoilage and deterioration. Usually it takes two to three weeks for these fish species to move from the harvest place to their final market destination, during which can compromise their safety and quality. One way to solve that problem is to employ freezing right after their harvest. However, the red muscle of these fish species is very sensitive to freezing and rapidly turns brown. Therefore, FS or CO was introduced to treat these fish species due to the fact that CO can stabilize the red color during frozen storage.

In 1996, filtered wood smoke was patented to treat raw fish and meat in order to prevent and discoloration without losing freshness (Yamaoka 1996). The filtered wood smoke is generated by burning a smoking material at 250°C to 400°C, and then filtering the smoke, followed by cooling between 0-5^{o}C during treatment of fish or meat (Yamaoka 1996). Later, another relevant patent was granted to Kowalski (1999) about applying tasteless super-purified smoke to treat seafood and meat to preserve the freshness, color, texture and natural flavor, particularly for frozen seafood. The process was unique due to the burning temperature used to

generate the smoke and the filtering process for producing a super-purified smoke. The products treated with this tasteless super-purified smoke are then frozen, stored for up to one year, and quickly thawed with little degradation. The adoption of this technology initiated commercial applications of filtered smoke and CO for color retention in seafoods, especially seafoods in frozen markets. Considering the above patents concern either the exposure of raw meat to a gas mixture containing carbon monoxide or the exposure of meat slurries to carbon monoxide in combination with other steps, Shaklai (2001) patented a simple method of exposing raw meat solely to CO, which includes exposing raw meat to an atmosphere consisting essentially of CO and maintaining the meat in a sealed container to maintain color and freshness. More recently, a patent (Olson and Brinsmade 2004) for seafood preservation process using Clearsmoke^{TM} was issued, and this preservation process is accomplished by treating fish first with filtered smoke, followed by treatment with ozone and an optional freezing step. This process extends the shelf life of the fish products and permits the fish to maintain its freshness and freedom from bacterial decomposition for a longer period of time following catch.

Treating fish muscle with CO or FS has been reported to improve the quality. Carbon monoxide or FS treatment may decrease aerobic bacterial growth (Kristinsson and others 2003; Garner & Kristinsson, 2004; Ludlow and others 2004; Danyali 2004; Kristinsson and others 2006a; Kristinsson and others 2008; Leydon and others 2005; Kristinsson and others 2007), and CO or FS treated fish muscle has a prolonged and increased red color compared to untreated controls (Kristinsson and others 2003; Danyali 2004; Ludlow and others 2004; Garner and Kristinsson 2004; Balaban and others 2005; Kristinsson and others 2006a; Kristinsson and others 2007). It has also been demonstrated that treating fish fillets, such as mahi mahi and Spanish mackerel, with various CO levels or FS may reduce lipid oxidation (Kristinsson and others 2003;

Demir and others 2004; Garner and Kristinsson 2004; Kristinsson and others 2007). CO or FS treatment can result in the reduction in formation of secondary lipid oxidation products during freezing and following cold storage, which may be due to increased stability of heme proteins (Kristinsson and others 2006b). FS also has a positive effect on muscle texture and juiciness (Hawaii International Seafood Inc. 1999).

A few Taiwanese and Japanese researchers also investigated CO treatment of tuna with 100% CO (Ishiwata and others 1996; Chow and others 1998b; Hsieh and others 1998; Chow and Chu 2004), and it was concluded that during frozen storage at -20℃ for 6 months, metHb% and Hunter a* values for CO treated tuna steaks remained almost constant, while MetHb% for untreated control increased gradually up to 60% and a* values decreased from 11 to 5 (Chow and others 1998). After 4 hrs CO treatment, Hunter a* value of tuna steaks increased more than untreated control, and the metHb% in the surface layer (2 mm thickness from the surface) of the steak showed no significant difference between samples with and without CO treatment while that in the middle layer (2 to 4 mm from the surface) and inner layer (4 to 6 mm from the surface) was lower than untreated control (Hsieh and others 1998).

In addition, the application of CO to live fish via euthanasia has been studied and performed by the aquaculture industry (Kristinsson and others 2006c; Mantilla and others 2005; Mantilla and others 2006). It was found that it takes less than 30 min to euthanize fish with this technique while it may take hours to obtain proper CO binding in fish fillets or steaks (Mantilla and others 2005). The euthanasia treatment is very effective and leads to even higher a* values than CO treated fillets (Kristinsson and others 2006c). Because this technology is rapid and can give a more ‘natural’ appearance, it is possibly promising in the future. Since applying CO into

live fish via euthanasia is very recent, limited scientific information is available about this subject.

Regulations of Treating Fish or Meat Products with CO or FS

CO was not a permitted additive in the United States previously. In May 1999, FDA issued Import Bulletin 16B-95 in response to processing tuna with "tasteless smoke" (TS) in Indonesia, Philippines and Taiwan (FDA 1999). This bulletin approved the use of CO or TS treatment on the condition that all the treated products have been labeled as processed foods that have been treated with CO or TS. This approval was more formally announced as a response to a request by Kowalski's company, Hawaii International Seafood, Inc. in 2000 (FDA 2000). FDA considered TS as a preservative and it was required to declare both the name of the ingredient and a separate description of its function by labeling.

Pre-treatment with CO was approved (GRAS) by the Food and Drug Administration (FDA) in the United States for use at a level of 0.4% in meat packaging systems in February 2002 (FDA 2002). As an extension of this approval, in July 2004, FDA issued another GRAS approval on the use of 0.4% CO for fresh beef and pork for direct case-ready packaging in the United States, and it was also declared that CO is a "processing aid" that does not require product labeling declarations (FDA 2004), and it was agreed that 0.4% CO in modified atmosphere package for meats did not have a lasting functional influence in food and there will be no significant amount of CO in the finished food products. FDA's response for use of CO shows that using CO for color development and retention in muscle foods is becoming more acceptable. These FDA approvals have made CO allowed to be used in packaging technology for the US meat industries.

The regulations of CO/FS used to treat meat or fish are quite different in different countries. In Canada, seafood treated with tasteless smoke or with sole CO is not permitted.

However, the use of wood smoke is permitted on seafood, as long as the label indicates the process (Andruczyk 2000). Tasteless smoke can be regarded as a kind of physically modified wood smoke. Health Canada is evaluating safety of the fish treated with tasteless smoke, and Canadian Food Inspection Agency is evaluating sensory characteristics of the treated fish (Andruczyk 2000). One of the concerns is a deceptive appearance of the treated products. These products may be decomposed when they still show the bright red color. The process should be clearly indicated on the label. Sometimes, consumers may be confused about the terms such as "tasteless smoke" or "wood smoke" or "modified smoke". CO is a component of smoke and it is permitted in smoked fish, but there is no provision for the use of CO alone on any food products in Canada.

The European Union regards all the packaging gases as additives, and CO has not been included in the approved gas list (European Parliament and Council Directive 1995). In 2000, the joint Norwegian meat industry applied for continued and permanent acceptance of up to 0.5% CO in gas mixtures of red meat packaging, which was supported by meat industries in many European countries. The European commission concluded that there is no health concern associated with the use of 0.3%-0.5% CO in a gas mixture with CO_2 and N_2 as a modified atmosphere packing gas for fresh meat provided the temperature during storage and transport does not exceed 4°C (European Commission 2001). However, after a vote in June 2003 by the committee on the Environment, Public Health and Consumer Policy of the EU Parliament, it was decided not to accept the use of low-CO atmosphere for meat packaging. Therefore, CO is not permitted for meat packaging in the European Union. In Norway, it was previously permitted to use CO as a packaging gas at concentrations of up to 0.5%, and around 50-60% of retail fresh meat products had been packaged with CO since 1985 (Sorheim and others 2001; Sorheim and

others 1997). However, Norway discontinued using CO due to its entry into the European Union on July 1st, 2004. In addition, since the European Union considers the use of CO or FS in tuna or other fish species the same as their use in meat, both CO and FS are not allowed as a treatment (Schubring 2004). Japan issued a notice banning fish that has an initial CO content ≥500 ug/kg, or an initial content ≥200 ug/kg which decreases significantly during two days refrigerated storage (Japan, Food Sanitation Law – Article 6).

Concerns about CO or FS Treatment

The approval of CO packaging technology in the US market has made treating fish with CO or FS a common practice in the industry, specifically for fish species intended for the frozen market. CO treated products have an increasing market due to their desirable red color and longer shelf life. However, the major concerns are: 1) the possibility that CO can pose a health hazard; 2) CO treated products could mask underlying seafood safety problems such as pathogens and biotoxins; 3) economic fraud caused by misuse of this technology.

CO is a colorless, odorless and tasteless gas, which is mainly generated from incomplete combustion of carbon containing materials. Since CO can bind with high affinity to human Mb/Hb and then interferes with oxygen transportation and delivery, CO is a toxic substance. However, a review by Sorheim (Sorheim and others 1997), which was about technological, hygienic and toxicological properties of CO used in MAP, indicated that 40 ppm CO in the environment would result in CO reaction with around 2.5% of blood Hb in a normal individual (Sorheim and others 1997). Beef treated with a gas mixture containing 1% for 3 days, produced around 0.1 mg CO per kg meat upon cooking (Watts and others 1978). Cooking removed as much as 85% of CO from the meat products (Watts and others 1978). Therefore, human exposure to CO from CO packaging of meat is extremely low, and it is highly improbable that meat treated with gas mixtures containing less than 0.5% will result in a measurable increase in

COHb level in blood (Sorheim and others 1997). In addition, commercial packaging with CO was performed using a preblended gas mixture with 1% or less CO, which will not pose any potential risk in processing plants (Sorheim and others 1997). Davenport and others (2006) investigated absorption of CO into humans after consumption of CO treated tuna, and it was concluded that human may absorb CO from CO treated products but this absorbed CO is quickly removed from the body by exhalation from the lung.

Another concern about CO packaging of meat is that the bright red color may last beyond the point of microbial spoilage. However, it was observed that increased color stability as a result of 0.4% CO treatment was not enough to mask microbial spoilage that occurred with mild temperature abuse (6^{o}C) (Hunt and others 2004). Therefore, low CO levels (0.3-0.5%) are very unlikely to mask product spoilage. Besides, the other indicators such as odor can be used to determine meat qualities. However, high levels of CO treatment are becoming very common and there is little scientific information about the relationship between color and microbial spoilage of meat or fish treated with high levels of CO.

It was reported that the use of high CO concentration, such as 100% CO, can restore the red color of lower grade brown tuna to higher grade (Balaban and others 2006). This kind of color "enhancement" was also applied to mahi mahi, and a week-old mahimahi was shown to look similar or even better than fresh after 100% CO treatment (Balaban and others 2006). In addition, the color of aged tilapia can be enhanced by CO treatment (MarinGomez and others 2007). There are no precice scientific answers for this color enhancement right now, except that there is a metHb/Mb reducing enzyme in muscle which may play an important role in this enhancement.

Determination of CO or COHb/Mb

Due to the increasing amount of commercially CO/FS treated seafood products in the market and the possibility of misuse of this technology which may result in food safety problems, there is a great need to determine if the product has been treated with CO/FS and also quantify CO in the muscle system. Color evaluation has been described as a method to determine if products are CO-treated. Currently, the primary methodology for fish treated with CO is sensory evaluation (unnatural color) in Canada. However, this method has not been verified appropriately to identify CO treated products. According to Kristinsson and others (2006c), the National Marine Fisheries Service (NMFS) has suggested a color threshold of $a^* = 16.2$ for tuna, but either very fresh tuna can have an a^* value above this level, or it is relatively easy to control the a^* value of CO-treated products so they stay below this threshold value. Besides, for determination of CO in blood, most colorimetric methods are limited due to the low solubility of carbon monoxide in aqueous solutions, and also the reaction time is too long, which leads to loss of some carbon monoxide (Lambert and others 1972).

As early as 1959, gas chromatographic (GC) methods have been developed to determine CO in postmortem blood (Domiguez and others 1959), which was modified and improved later (Goldbaum and others 1986; Lewis and others 2004). This method is based on liberating CO from the blood sample to the headspace of a closed system by adding a CO liberating reagent, and then headspace gas was injected into GC for separation and detection. Since gas chromatography (GC) is highly specific for CO (Vreman and others 1984) and can not be affected by both spectral and chemical interference, it is regarded as a more sensitive and accurate CO detection method. Several kinds of detection systems such as flame ionization detection (FID) (Collison and others 1967; Guillot and others 1981; Sundin and Larsson 2001; Kaminski and others 2003), thermal conductivity detection (TCD) (Goldbaum and others 1986;

VanDam and Daenens 1994; Lewis and others 2004), infrared detection and even mass spectrometry (Oritani and others 2000), have been applied to CO analysis. Currently CO is being used to treat certain types of seafoods either as a single gas or in combination with other gases at various concentrations. CO treated products have an increasing presence in the market due to their desirable red color and longer shelf life. However, CO treatment could in some cases represent economic fraud as the cherry red color may last well beyond the microbial shelf life of the meat, and thus, mask spoilage and hide underlying safety problems, as well as enhance the color of oxidized muscle products when used at very high levels. Due to the ban of importation of CO treated tilapia by the Japanese government in 1997, a GC method was developed to quantify CO in treated seafood products (Ishiwata and others 1996; Miyazaki and others 1997; Chow and others 1998a). This GC method is based on CO releasing from the muscle tissues to the headspace of a closed system by adding a CO liberating reagent, such as sulfuric acid. Headspace gas is then injected to the GC, where CO is separated from the other gases in the column, and then reduced to methane by an installed methanizer and finally detected by FID. FID is a common GC detector, but it does not respond to CO. After CO is catalytically reduced to CH_4, it can be detected by FID (Griffin 1979; Guillot and others 1981; Costantino and others 1986; Miyazaki and others 1997; Chow and others 1998a). This GC-FID method was improved by separating CO from O_2 before CO reduction (Kaminski and others 2003), which made it more accurate to determine trace amounts of CO, CO_2 and CH_4. The detection limit of this method was demonstrated to be 2 μg CO/kg fish muscle (Ishiwata and others 1996; Miyazaki and others 1997). Recently, this GC method has been presented to identify and verify the type of CO or filtered smoke treatment employed to the muscle (Crynen and Kristinsson 2007). Anderson and Wu (2005) reported a GC/MS method to quantitatively determine CO in tuna and mahi mahi

muscles. A molecular sieve column was used to allow the separation of CO from nitrogen and oxygen, which eliminated the need to flush the headspace of each sample bottle with helium before CO liberation.

There are several spectrophotometric methods used to determine the total concentration of Mb/Hb and its derivatives. Specifically, absorbance measurements of the heme protein extract can be used to calculate both the total content of the pigments and the concentrations of their derivatives such as reduced and oxidized forms of Mb/Hb, which can be used to evaluate meat quality. A manual multi-wavelength spectrophotometric method was described to determine relative and absolute pigment concentrations of Mb derivatives, oxy-, carboxy-, and met-Mb in fish or meat samples (Wolfe and others 1978). It was found that no interaction of metMb and carbon monoxide was observed and the indicated amount of metMb was independent of the duration and rate of CO bubbling (Wolfe and others 1978). Unfortunately this multi-wavelength spectrophotometric method can not measure deoxyMb and its sensitivity is low, which limits its application.

A two-wavelength spectrophotometric method was developed to determine COHb% in human blood in routine clinical laboratories (Klendshoj and others 1950). The two-wavelength spectrophotometric method is based on the fact that Hb/Mb and their derivatives have characteristic absorption spectra, and that sodium dithionite can deoxygenate oxyHb/Mb and metHb/Mb without influencing COHb/Mb (Tietz and Fiereck 1973). In that way, the sample can be converted into a two-component system, COHb/Mb and deoxyHb/Mb, and analyzed by measuring absorbances at the two wavelengths selected. Although this method has been developed and modified with time (Rodkey and others 1979; Katsumata and others 1982; Beutler and West 1984; Fechner and Gee 1989), its principle remains the same. It was not until recently

that a two-wavelength spectrophotometric method was used to detect COMb% in CO treated muscle tissues (Smulevich and others 2007). Its drawback was a very complex Mb purification and sample preparation procedure, which was not easy to implement in practice. An official food analysis method needs not only to be confirmatory but ideally also simple and low cost to be of value.

The CO-oximeter, a special automated spectrophotometer, is capable of determining hemoglobin and its derivatives in blood by measuring absorbance at selected wavelengths (Yukawa and others 1998). It is popular because of very small blood samples, short measuring time required (<0.1 ml) and easy handling. Although the primary use of the CO-oximeter is to accurately measure Hb derivatives in living persons, it can also be used for the determination of COHb post-mortem (Brehmer and Iten 2003). Compared to two-wavelength methods, it is less influenced by other pigments, and other hemoglobin derivatives can simultaneously be quantified in addition to the concentration of COHb. However, it is still necessary to pre-treat samples in order to determine COHb in postmortem blood samples. Besides, there are still some influences and limitations existing (Mahoney and others 1993; Brunelle and others 1996; Boumba and Vougiouklakis 2005). Since the CO-oximeter is mainly used to detect CO and seldom used for other detection, it is expensive for a small lab if the number of samples for this analysis is not large. This method has not been used to detect CO in muscle up to now.

Usually spectrophotometry is regarded as a basic instrument in the routine clinical laboratory. The spectrophotometric CO detection may meet the need of most routine determinations. The spectrophotometric method is low cost and easy to execute compared to the GC method. Since a GC requires a trained person for proper operation, it may be considered too complicated. However, a GC can accurately determine total amount of CO in the muscle.

Considering the above, the selection of CO determination method should depend on the purpose of analysis, sensitivity requirements and other factors.

CHAPTER 3
RAPID DETECTION OF CARBON MONOXIDE TREATED SEAFOOD PRODUCTS BASED ON SPECTRAL PROPERTIES OF HEME PROTEINS

Introduction

The market value of some seafoods is normally based on their color. The commercial seafood industries have to maintain the desirable color of seafoods during their processing, transportation and storage. CO and FS have been introduced to treat seafoods in order to maintain their bright red color.

The attractive bright red color is due to the presence of oxy-hemoglobin (oxyHb) and oxy-myoglobin (Mb) (Kanner and others 1987). However, these proteins are not stable and with time they will gradually oxidize to metHb and metMb, which result in an undesirable brown color. Carbon monoxide (CO) can react with oxyHb and oxyMb to form the very stable COHb and COMb forms, which give the muscle an attractive red color. Because of this, CO and FS, which contains a moderate amount of CO, has become a popular processing method to help stabilize and maintain the red color of various seafood products. CO stabilizes Hb and Mb against oxidation and denaturation (Kristinsson and others 2005) and can effectively stabilize the red color of fish during fresh and also frozen storage (Kritinsson and others 2008). Seafoods are the most international commodity sold in the US, which means that a large part of the products are harvested far away from the market destination, and thus in frozen form. This technology has therefore been very useful to help maintain color stability during frozen storage, to provide consumers with an attractive product.

CO or FS treated meat or fish have been found to maintain a desirable fresh red color longer than untreated products, be less susceptible to lipid oxidation, and may in some cases have lower aerobic bacterial counts which may result in increased shelf life (Jayasingh and others 2001; Krause and others 2003; Kristinsson and others 2003; Kristinsson and others 2006b;

Kristinsson and others 2007; Kritinsson and others 2008; Sorheim and others 1997). CO however is a toxic molecule, which leaves a negative impression with most people. In addition, CO or FS treatment could in some cases represent economic fraud as the cherry red color may last well beyond the microbial shelf life of the meat or fish and thus mask spoilage. Also the possibility exists that an inferior, potentially hazardous product may be made to look better than it is with this technology. Since there is a growing use of CO or FS treatment and the possibility of misuse of this technology, many countries have some regulatory restrictions regarding CO treatment. Japan issued a notice to ban fish which contain an initial CO ≥500ug/kg (Japan, Food Sanitation Law—Article 6). CO treatment of seafoods is not permitted in the European Union. Norway, where it was commonly used, discontinued the use of CO in meat packaging in July 2004 because of their entry into the European Union. There is no provision for the use of CO alone on any food products in Canada. In the United States, FDA has no questions regarding the conclusion of Hawaii International that tasteless smoke is GRAS (generally recognized as safe) for use on tuna before it is frozen (FDA 2000). CO in meat packaging was approved by the FDA (2002) for use at a level of 0.4% in red meat packaging systems. Recently, FDA also declared that CO is a "processing aid" that does not require product labeling declarations (FDA 2004), and it was agreed that 0.4% CO in modified atmosphere package for meats did not have a lasting functional influence in food and there will be no significant amount of CO in the finished food products. FDA's response for use of CO shows that CO for color development and retention in muscle foods becoming more acceptable.

Considering the safety and quality problems CO or FS treatment may cause, there is a great need to develop a method to simply detect if the fish or meat has been treated with CO or FS. Numerous methods have been reported for the detection of CO or COHb% in blood

(Boumba and Vougiouklakis 2005). The two-wavelength spectrophotometric method (Tietz and Fiereck 1973; Rodkey and others 1979; Katsumata and others 1982; Beutler and West 1984; Fechner and Gee 1989; Stonek and others 2004) and a gas chromatographic (GC) method (Guillot and others 1981; Juntarawijit and others 2000) are two types of methods, which are normally used to detect CO or COHb% in blood. Later, these two types of methods were used to determine CO in fish muscle (Wolfe and others 1978; Smulevich and others 2007; Ishiwata and others 1996; Chow and others 1998; Anderson and Wu 2005). Compared to the GC method, the spectrophotometric method is low cost and easy to execute. The objective of this study was to develop a one-wavelength spectrophotometric method to determine if the fish products have been treated with CO or FS. This one-wavelength method, based on the spectral characteristics of heme proteins, is rapid, simple and low cost, and can be used as a screening method for determining the presence of CO in fish products.

Materials and Methods

Washed Tilapia Muscle (WTM) Model System

Fresh tilapia fillets were obtained from a local store. Dark muscle was removed and the remaining white muscle was minced. Then the minced muscle was washed with distilled deionized water twice (1:3 muscle to water) by stirring with a plastic rod for 2 min. The mixture was allowed to stand for 15 min, and then dewatered on a nylon screen. The muscle was then homogenized in 20 mM sodium phosphate buffer (pH 7.0) for 1 min by using a homogenizer (Biospec products Inc., Bartlesville, OK) and left to sit for 15 min. The system was then centrifuged at 10,000 g for 20 min by an Eppendorf 5702 centrifuge (Eppendorf North America Inc., New York, NY) and then the washed muscle vacuum packed and stored at -80°C until needed. The final moisture content of the samples was ~82%.

Preparation of OxyHb, COHb, DeoxyHb and MetHb

Tilapia was obtained locally from a tilapia aquaculture farm. The fish was placed on ice (pectoral side facing up) for 1 min and blood was drawn though the caudal vein. OxyHb was isolated and purified from the red blood cells according to Richards and Hultin (2000). Purity of oxyHb was verified by SDS-PAGE, and the results showed that the samples were more than 99% Hb. Samples were stored in 500 µl aliquots at -80°C and thawed under 20°C running water before they were used. COHb was prepared from oxyHb based on Tietz and Fiereck (1973). DeoxyHb was prepared by adding sodium dithionite to diluted oxyHb (~0.25 mM) and standing at room temperature for 5 min (the final concentration of sodium dithionite was ~2 mg/ml). The ferric metHb was made by adding 200 µl Hb stock solution to 1.8 ml buffer (pH 6.5) with 2 mM sodium phosphate (dibasic) and 1 mM sodium citrate, and then adding 0.43 mg potassium ferricyanide. The mixture was inverted a few times, followed by centrifugal filtration to remove the potassium ferricyanide. Hb was kept on ice during all experiments. Hb levels were determined using the Bradford method (Chang 1998). The Coomassie plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) was used with bovine Hb as standard.

Addition of Tilapia Hb to WTM (WTM-Hb) and CO Treatment

WTM was thawed overnight at 4°C. An appropriate volume of the oxyHb stock (around 25.15 mM Hb) was added to disposable polystyrene weigh dishes (Fisher Scientific, Fair Lawn, NJ) containing 5 g washed tilapia muscle so that the final concentration was 5 µmol Hb/kg muscle. The WTM-Hb system was mixed and spread on the weigh dishes (~2 mm thickness). All the weigh dishes with WTM-Hb were placed in a gastight bag, which was vacuumed, flushed with pure CO and stored at 4°C for 24 hr. A treatment time of 24 hr was selected according to processing times used commercially. Controls were exposed to air for 24 hr in oxygen permeable

bags. After CO treatment, samples were taken for both Hb recovery analysis and spectrophotometric analysis.

Hb Recovery Analysis

The CO treated and untreated (control) WTM-Hb system were dissolved in 20 mM sodium phosphate buffer at two different pH values (pH 6.0 vs. pH 8.0), homogenized for 1 min, and then centrifuged for 15 min at 4,000 rpm. The supernatant and precipitate were both collected. Total protein of the supernatant (Hb extract) was determined by the Bradford method (Chang 1998) and relative percentage of Hb in the Hb extract was quantified by running SDS-PAGE (4-20% Tris-HCl precast gel, BioRad, Hercules, CA). The SDS-PAGE gels were scanned and analyzed using ImageJ software. Hb recovery was calculated as:

$$[Hb] = \frac{[totalprotein] \times Hb\%}{[totalHb]}$$

Sample Analysis by the Spectrophotometric Method

WTM-Hb system (5g) were added to a 10 ml buffer containing 20mM sodium phosphate (pH 8.0), homogenized for 10 seconds using a homogenizer (Biospec products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 10 min with an Eppendorf 5702 centrifuge (Eppendorf North America Inc., New York, NY), and then the supernatant (Hb extract) was taken and scanned between 350-700 nm with a spectrophotometer (Agilent Technologies, Palo Alto, CA). All the samples were kept on ice during the experiments.

Validation of the Spectrophotometric Method

Fresh, never frozen, headed, gutted, filleted and skinned three fish species, yellowfin tuna, mahi mahi and snapper were obtained from a local seafood supplier (Northwest Seafood Inc., Gainesville, FL). Tuna was cut into ~1 cm thick pieces and mahi mahi fillets were cut into 5 pieces, with one piece being one sample. For snapper, one fillet was one sample. Then all the

samples were placed into a gastight bag (dark muscle side facing up), and vacuum packed to expel air and then treated with 20% and 100% CO by flushing and filling bags with CO gas. At the same time, the controls were placed in bags with high air permeability. The bags were stored at 4°C for 12 hr and 24 hr. Samples were then removed from the bags and stored in bags of high oxygen permeability at 4°C for 9 days. Every third day, a small amount of dark lateral muscle (in the case of tuna a piece was taken from the loin ordinary muscle) was taken from each sample, minced and added to 10 ml 20 mM phosphate buffer (pH 8.0), homogenized for 10 seconds, and then centrifuged at 4,000 rpm for 10 min. The supernatant spectra were recorded with a UV-visible spectrophotometer. Five repeated samples were taken for analysis at each time point.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine if there was a significant difference ($p<0.05$) between various CO treatments for some commercially important fish species commonly treated with CO. Comparison of means was done by using the least significant difference (LSD) test. All statistical analysis was done by using Microsoft Office Excel 2003.

Color Analysis

A color machine vision system developed by Luzuriaga (1999) was used to analyze a* values of the muscle, which provides information about the redness of the muscle. After various CO (vs. air as control) treatments, three types of fish, as described above, were stored in bags of high air permeability at 4°C for 9 days. Every third day, the dark lateral muscle (the loin ordinary muscle for tuna) was analyzed for color.

Results and Discussions

Development of the Spectrophotometric Method

A wide range of ligands, such as NO, O_2 and CO, may form complexes with either Fe^{2+} or Fe^{3+} in heme proteins. CO binding to heme proteins differs from O_2 binding in terms of the

ability to obtain an ionic character of the ligand due to variation in dipole moment and iron ligand binding geometry of the complexes (Moller and Skibsted 2006). Spectrophotometry has been used for the determination of Hb and its derivatives from human blood for a long time. It is based on the fact that different ligand binding states of heme proteins show a characteristic spectrum. The absorbance maxima are 413-414 nm, 576-578 nm and 540-542 nm for oxyHb/Mb, 418-420 nm, 568-572 nm and 538-540 nm for carboxyHb/Mb, and 430-431 nm, 555-556 nm for deoxyHb/Mb (Tietz and Fiereck 1973; Smulevich and others 2007; Klendshoj and others 1950; Kristinsson and others 2006c).

Heme proteins exhibit a large peak between 400-450 nm, and two small peaks between 500-600 nm (Figure 3-1). When heme proteins fully bind to oxygen, they exhibit a maximum absorbance peak wavelength, heme peak wavelength, at 413-414 nm (Figure 3-1), while when they fully bind to CO, they exhibit a maximum absorbance peak wavelength at 418-419 nm (Figure 3-1). When heme proteins are fully oxidized, this peak shifts down to 405-408 nm (Figure 3-1). Therefore, it is possible to use the heme peak wavelength of extracted heme proteins to indicate the presence or absence of CO in the muscle tissue. The heme peak wavelength at or below 412 nm, therefore, indicates presence of metHb/Mb and the wavelength at or above 415 nm represents the presence of COHb/Mb.

It was of interest to determine conditions where heme proteins, still bound to CO, could be extracted from CO treated samples. Heme proteins are highly soluble at low ionic strength and at a range of pH values. It was important to separate heme proteins from the other muscle proteins, especially those causing turbidity, which can interfere with the spectroscopy results. Two pH values were chosen for Hb extraction, pH 6.0 and 8.0. As shown in Figure 3-2, extraction was more effectively achieved at pH 8.0 compared to pH 6.0, as many muscle proteins will aggregate

and be centrifuged out at pH 6.0 and at a low ionic strength. At lower pH values, Hb becomes less stable while it is exceptionally stable at pH 8 (Kristinsson and others 2005). The oxidative stability of Hb was greatly enhanced at pH 8 vs. pH 7 and 6 (Kritinsson and others 2003). Although more muscle proteins from the WTM were present in the Hb extract at pH 8.0, a higher recovery of Hb from the WTM-Hb system, 91.56±1.51%, was achieved compared to 78.51± 4.24% at pH 6.0.

Table 3-1 shows that extracts from CO treated samples contained heme proteins with heme peak wavelengths of 417-418 nm. CO binding to heme proteins can stabilize the red color of muscle, and protect heme proteins from oxidation (Kristinsson and others 2003). Generally as temperature is increased heme proteins become more susceptible to oxidation (Kristinsson and others 2005). It was of interest to study the temperature sensitivity of the extraction process. It was found that tilapia COHb maintains stability at both 20°C and 0°C for several hours, and it was even more stable at 20°C than at 0°C (Table 3-1). This is likely because tilapia is a warm water fish. This indicates that the tilapia Hb extraction can take place at ambient temperatures and is not very time sensitive, which allows more flexibility for sampling and analysis.

Validation of the Spectrophotometric Method

This spectrophotometric method was validated by treating fillets and steaks of various fish species which are frequently commercially treated with CO. Before CO treatment, all fish species tested demonstrated a maximum absorbance peak of oxyHb/Mb at 413-414 nm. However, after CO treatment, the maximum absorbance peak shifted to 417-419 nm (Figure 3-1), which is the characteristic absorbance peak of COHb/Mb. There was also a significant increase in redness (Figures 3-6, 3-7, 3-8), especially for the 24 hr 100% CO treatment. The increase in redness is due to the binding of CO to reduced heme iron (Fe^{2+}) in muscle Hb/Mb

(Kristinsson and others 2007). During refrigerated storage of these CO treated samples, their a* values decreased (Figures 3-6, 3-7, 3-8), and their heme peak wavelength shifted to a lower wavelength, which is the evidence of CO releasing from heme proteins, but did not change much compared to the untreated controls. Even after 9 days storage, the maximum absorbance peak wavelength was still above 415 nm, and even above 417 for snapper and mahi mahi (Figures 3-4, 3-5). However, during refrigerated storage of untreated control samples, their a* values declined very quickly (Figures 3-6, 3-7, 3-8), and their heme peak wavelength shifted to a lower wavelength quickly, which indicated the oxidation of heme proteins. It was concluded that there is a close relationship between the change of a* values and the shift of heme peak wavelength (Figures 3-3, 3-4, 3-5, 3-6, 3-7, 3-8). The a* value increases or decreases when the heme peak wavelength shifts upward or downward. The decline of red color has been found to be followed by the decline of CO binding to heme proteins (Danyali 2004; Garner and Kristinsson 2004). CO treatment, therefore, significantly increased muscle redness and stabilized the red color, and even after 9 days storage, there still was CO bound to Hb based on the fact that the peak wavelength was still above 415 nm. However, untreated control samples had an undesirable brown color, which is indication of the oxidation of the heme proteins, based on the fact that their peak wavelengths shifted to values below 410 nm at the third day of storage (Figures 3-3, 3-4, 3-5).

The maximum absorbance peak wavelength depends on various factors. Firstly, CO percentage in the gas mixture has an effect on CO binding to the heme proteins in the muscle, and thus, different maximum peak wavelength may be achieved. Higher CO percentage in the gas mixture may promote CO binding to heme proteins, which may result in a higher upward shift of the maximum peak wavelength. Red color increase and stability was directly proportional to the percentage of CO used to treat fish as assessed by Hb/Mb UV-visible spectra,

which agree with the results of Kristinsson and others (2003). Figures 3-3, 3-4, 3-5 show the maximum absorbance peak wavelengths based on UV-visible spectra after treatment with different CO concentration for three fish species. The 100% CO treatment results in a higher absorbance peak wavelength than the 20% CO treatment, therefore more CO is binding to the heme proteins for 100% CO treated muscle compared to 20% CO treatment. For all the 24hr 100% CO treated samples, especially for snapper and mahi mahi, COHb could still be detected even after 9 days of refrigerated storage (Figures 3-3, 3-4, 3-5). The 24hr 100% CO treatment also led to the highest increase in redness (Figures 3-6, 3-7, 3-8). Secondly, heme protein concentration has an influence on the maximum absorbance peak wavelength. It was found that there is a higher maximum absorbance peak wavelength for CO treated mahi mahi and snapper compared to CO treated tuna during their refrigerated storage (Figures 3-3, 3-4, 3-5). It was reported that a higher CO level was found in fish flesh with higher Mb concentrations (Chow and others 1997). Thirdly, CO treatment time is another important issue. The depth of color development and duration of color retention was affected by prior CO concentration and length of product exposure to CO (Ross 2000). It can be seen (Figures 3-3, 3-4, 3-5) that with the same CO concentration, 24 hr treatment will lead to a higher maximum absorbance peak wavelength than 12 hr treatment. Longer treatment may be required for lower CO concentrations compared to higher concentrations (Ross 2000). Finally, CO treatment temperature will influence CO binding to heme proteins. Tuna steaks treated at 20°C gave a higher a* value and also had higher CO levels than those at 4°C, which indicates more CO binding to heme proteins (Kristinsson and others 2006c). Tuna heme proteins are adapted to relatively warm temperatures and thus may have a greater structural flexibility and higher CO binding capability at 20°C compared to 4°C (Kristinsson and others 2006c).

The same CO treatment may lead to a different heme peak wavelength and color stability for different types of fish. Right after CO treatment, all CO treated tuna samples could be positively identified as CO treated based on the heme peak wavelength. As the refrigerated storage progressed, all CO treated samples retained their red color longer than untreated controls. After 9 days storage, 24 hr 100% CO treated tuna still had a desirable red color, and their a* values were similar to what they had before treatment (Figure 3-6), and at that time COHb/COMb could still be detected (Figure 3-3). However, for 20% CO treated tuna samples, it could not be conclusively determined if COHb/COMb were present at day 3 and beyond (Figure 3-3), and their a* values reached their pretreatment a* values after only 3 days storage. This decline in heme peak wavelength and a* values were associated with oxidation (browning) of the 20% CO treated samples (Figure 3-6). These results indicate that 20% CO treatment for 12 or 24 hr is not enough for tuna if they need to maintain the red color for longer than 3 days. Statistical analysis showed no significant difference ($p<0.05$) between 12 hr 20% CO and 24 hr 20% CO treatment during storage, and also no significant difference ($p<0.05$) between 12 hr 100% CO and 24 hr 100% CO treatment. However, there was a significant difference ($p<0.05$) between 20% CO and 100% CO treatment for tuna samples.

All CO treated snapper fillets could be positively identified as CO treated products even after 9 days of cold storage using the spectroscopic method (Figure 3-4). Statistical analysis showed that there was no significant difference ($p<0.05$) among the four CO treatment methods during 9 days storage. Compared to untreated mahi mahi fillets, which turned brown at the first days of cold storage, 100% CO treated samples had a desirable red color even at day 9, and at that time COMb/Hb could still be detected (Figures 3-5, 3-8). However, presence of COMb/Hb could not be conclusively detected for the 12 hr 20% CO and 24 hr 20% CO treatment at day 6

and day 9, respectively. This indicated that a 20% CO treatment is not enough for mahi mahi if they need to maintain the red color for 6-9 days. Statistical analysis of mahi mahi showed that during cold storage, there was no significant difference ($p<0.05$) between the 12 hr 20% CO and 24 hr 20% CO treatment, and there was also no significant difference ($p<0.05$) between 12 hr 100% CO and 24 hr 100% CO treatment. However, there was a significant difference ($p<0.05$) between 20% CO and 100% CO treatments.

In this study, the freshest fish possible was used. The freshness of fish can have a large influence on the CO treatment and CO binding. CO treatment is particularly helpful to maintain the red color of fish if it is treated very fresh since the fish would contain a sizable amount of reduced oxyMb/Hb. However, if the fish is not fresh, it may have elevated levels of metMb/Hb, which CO can not bind (Kristinsson and others 2006c). Therefore, in order to maintain the red color of fish, they should be treated with CO as fresh as possible. It was demonstrated that CO treatment also gives an increase in redness for white muscle, although it contains much lower amount of heme proteins than dark muscle (Kristinsson and others 2006c). Only around half of the time is needed for white muscle to get maximum CO binding compared to dark muscle (Kritinsson and others 2006c).

Conclusion

A simple spectrophotometric method was developed to determine the presence of CO in the muscle tissue. This method involves a simple extraction of heme proteins from the fish muscle using a slightly alkaline low ionic strength solution and centrifugation. Then the UV-visible spectrum of the supernatant is obtained by a spectrophotometer. Using the model system the proper conditions to extract COHb and maintain its stability were found. The overall procedure requires only 15 min. Higher COHb recovery and stability were found when Hb was extracted at pH 8.0 compared to pH 6.0. This method can be used to determine various forms of

Hb, which may reveal if the food products have been treated with CO based on COHb/Mb peak wavelength. It was concluded that any heme protein extraction which exhibit a maximum peak wavelength at 415 nm or above can be determined as CO treated. As a result of this study, it is possible to rapidly detect seafoods treated with CO or FS, which will be an important tool for the industry as well as inspection agencies.However, this one-wavelength spectrophotometric method can only apply to fresh fish, and when levels of metMb/Hb increase on prolonged storage and the average heme peak wavelength of fish extracts fall below 415 nm, the method can no longer conclusively say the fish was CO treated.

Table 3-1. Heme peak absorbance wavelength of Hb extracts as a function of storage time at 20°C compared to 0°C.

	20°C		0°C	
Time (hr)	3.7 µmol Hb/kg WTM	13.4 µmol Hb/kg WTM	3.7 µmol Hb/kg WTM	13.4 µmol Hb/kg WTM
0	417.0±0.0 nm	418.0±0.0 nm	417.0±0.0 nm	418.0±0.0 nm
1	417.0±0.0 nm	418.0±0.0 nm	416.0±0.0 nm	416.5±0.5 nm
2	415.5±0.5 nm	417.0±0.0 nm	415.0±0.0 nm	416.0±0.0 nm
3	414.5±0.5 nm	416.0±0.0 nm	414.5±0.5 nm	416.0±0.0 nm
4	413.5±0.5 nm	416.0±0.0 nm	414.0±0.0 nm	414.0±0.0 nm
5	413.0±0.0 nm	415.0±0.0 nm	413.0±0.0 nm	414.5±0.5 nm

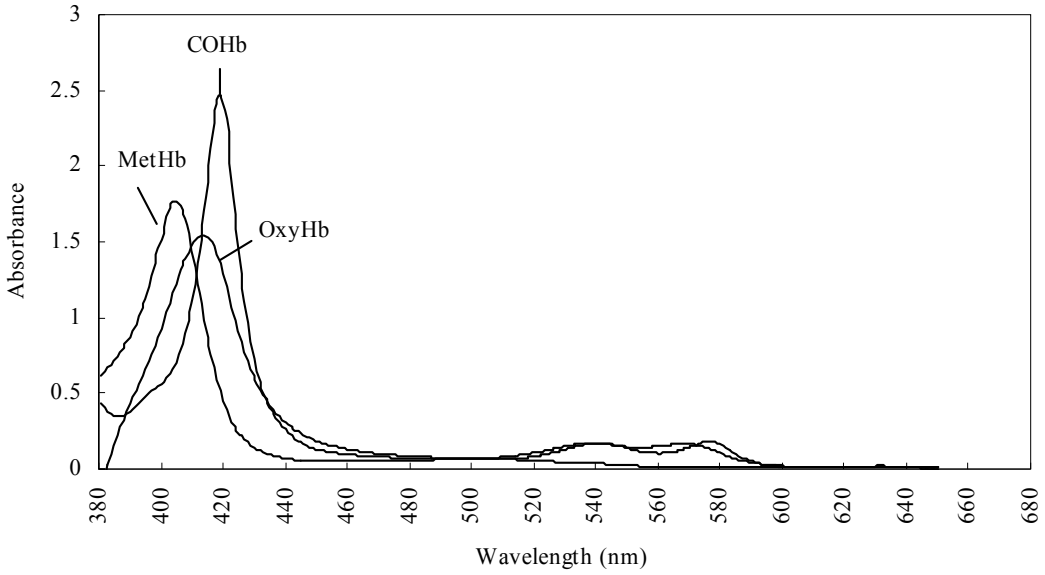


Figure 3-1. UV-visible spectra of tilapia Hb extracts. Tilapia Hb, bound to different ligands, was extracted with 20 mM sodium phosphate buffer (pH 8.0).

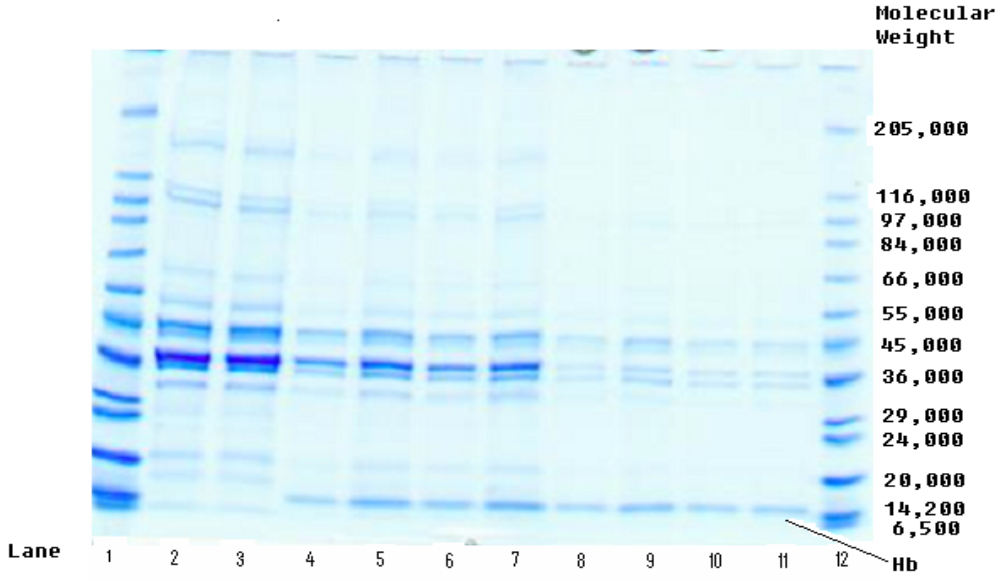


Figure 3-2. SDS-PAGE results for both supernatant and precipitate of Hb extracted from WTM at pH 6.0 vs. pH 8.0. (lane 1 & 12: protein standard marker (Sigma-Aldrich Inc, St. Louis, MO); lane 2: precipitate at pH 8.0; lane 3: precipitate at pH 6.0; lane 4-7: supernatant at pH 8.0; lane 8-11: supernatant at pH 6.0).

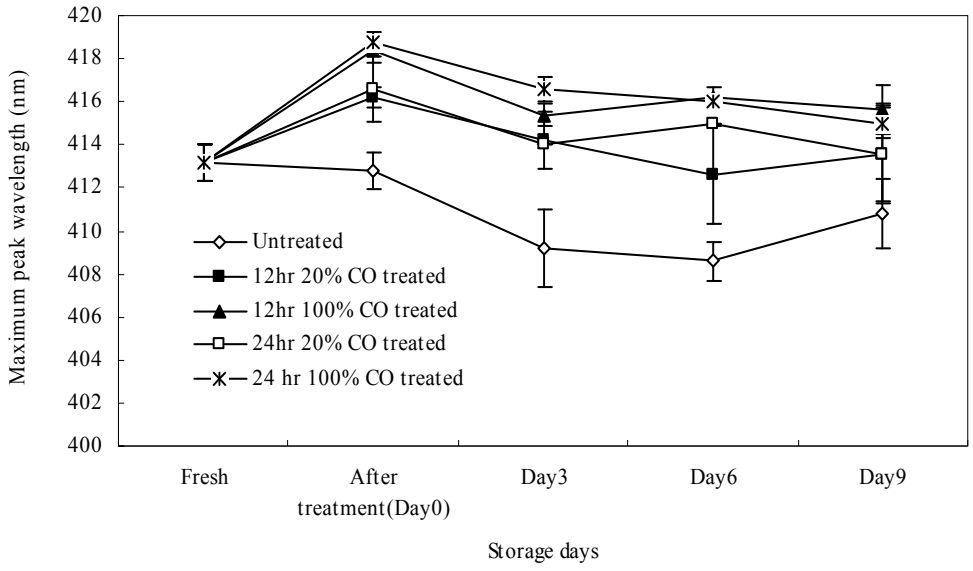


Figure 3-3. Maximum absorbance peak wavelength of tuna muscle extracts as a function of storage time (4°C). Tuna steaks were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

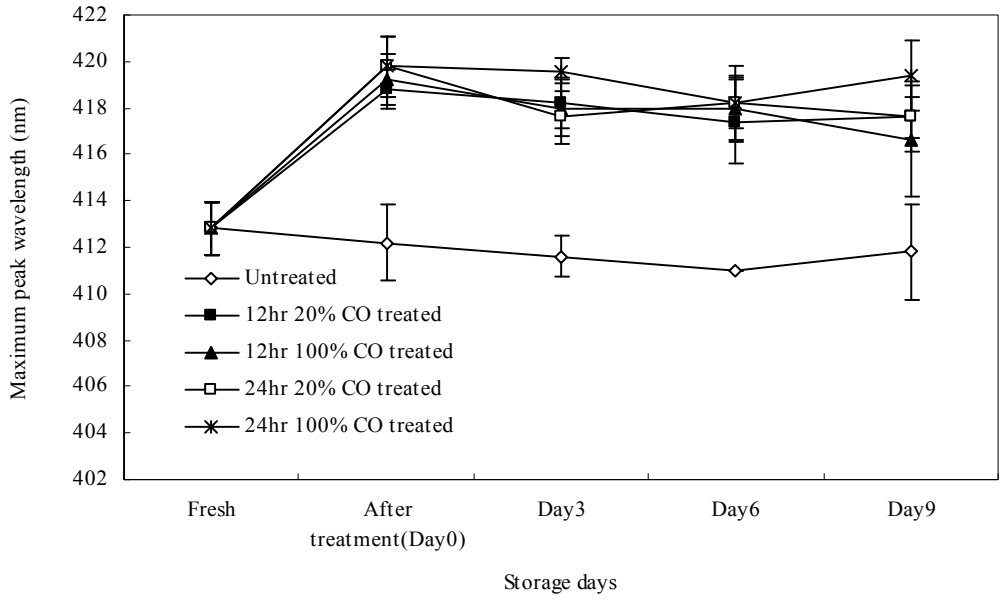


Figure 3-4. Maximum absorbance peak wavelength of snapper dark muscle extracts as a function of storage time (4°C). Snapper fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

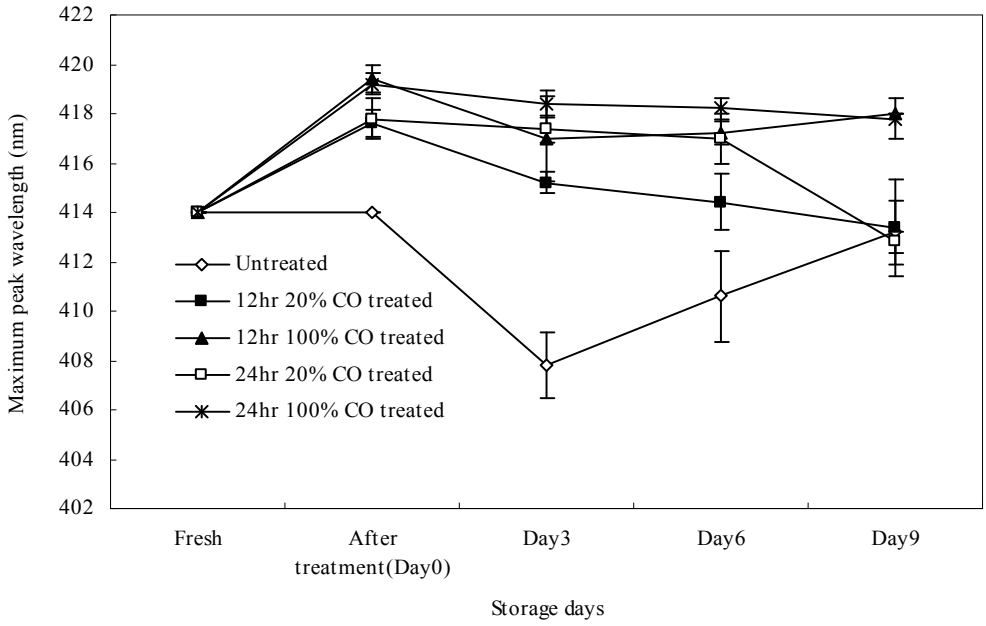


Figure 3-5. Maximum absorbance peak wavelength of mahi mahi dark muscle extracts as a function of storage time (4°C). Fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

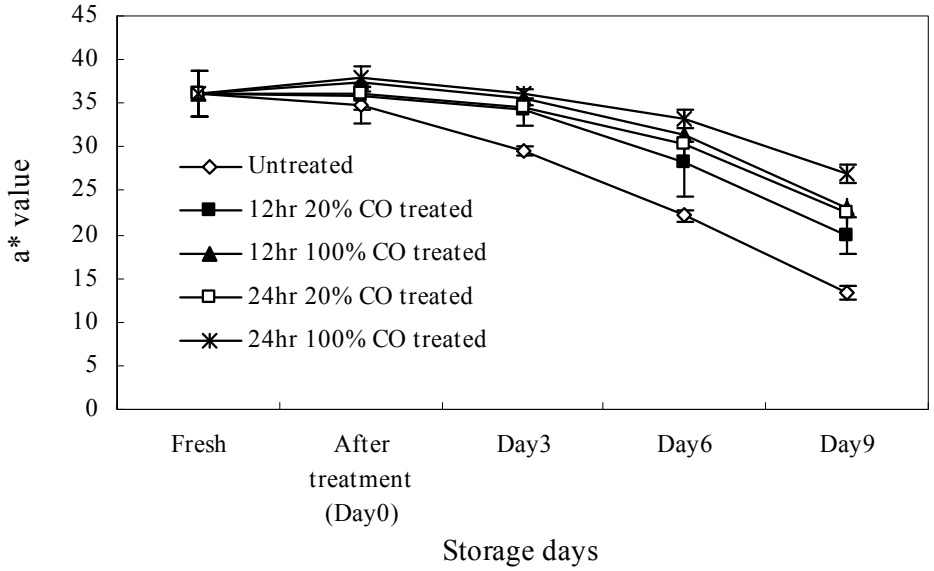


Figure 3-6. Effect of different CO gas treatments on a* value increase and stability of tuna steaks as a function of storage time (4°C). Tuna steaks were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

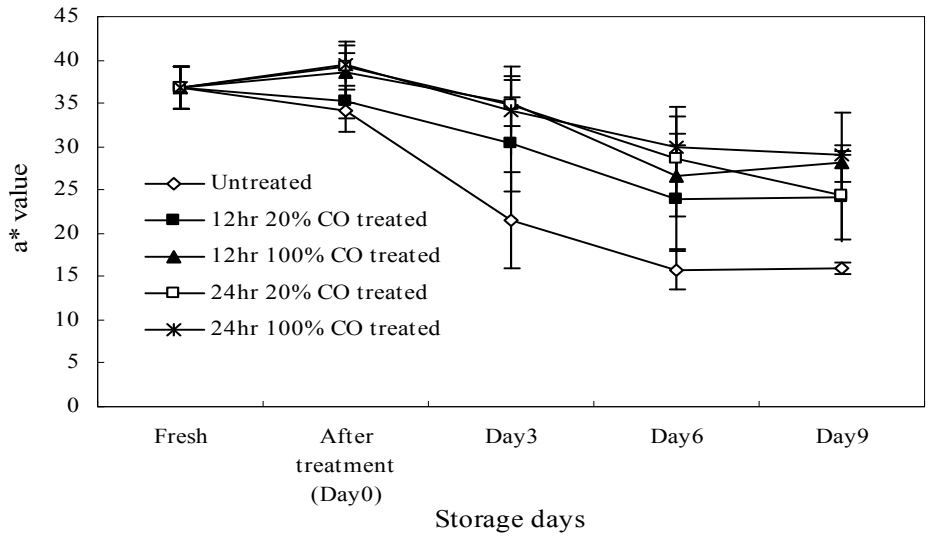


Figure 3-7. Effect of different CO gas treatments on a* value increase and stability of snapper fillets as a function of storage time (4°C). Snapper fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

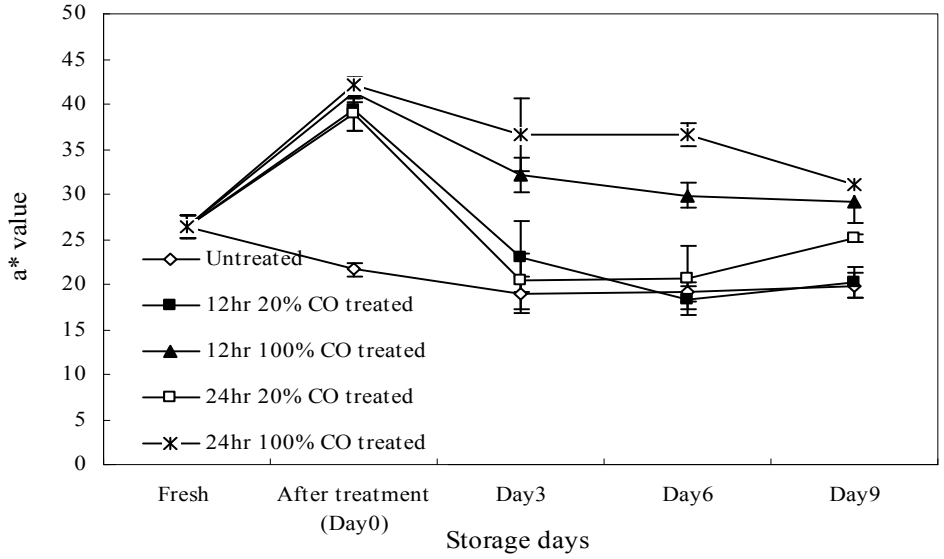


Figure 3-8. Effect of different CO gas treatments on a* value increase and stability of mahi mahi fillets as a function of storage time (4°C). Mahi mahi fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

CHAPTER 4
RAPID SPECTROPHOTOMETRIC METHOD TO QUANTIFY CARBON MONOXIDE IN FISH MUSCLE

Introduction

One of the major factors influencing the market value of fish species is their color, and consumers usually regard the bright red color as an indicator of fish freshness. Heme proteins, mostly notably Mb and Hb, contribute to the color of fresh muscle (Kanner and others 1987). These proteins have an iron-containing heme group, which is capable of binding various gas ligands, such as H_2O, O_2, NO and CO. The color status depends on both oxidation of the iron atom in the protein heme group and the type of ligand bound to the iron atom. The surface of muscle is characterized by reduced oxyHb/Mb, which provides a desirable red color, while the interior is characterized by reduced deoxyHb/Mb, which gives a purple color. Hb/Mb is susceptible to autoxidation to form metHb/Mb, which gives an undesirable brown color. In order to maintain the red color, it is important to maintain the reduced state of Hb/Mb and prevent the autoxidation of Hb/Mb. Since CO binds to Hb/Mb with about 270 times more affinity than oxygen does, treating muscle with CO or FS, which contains moderate levels of CO, can increase stability of heme proteins and thus maintain a cherry red color for a long time. It has been reported that CO/FS treated products maintain a desirable fresh red color longer than untreated fish or meat, are less prone to lipid oxidation, and in some cases may have lower aerobic bacterial counts which may result in extended shelf life (Sorheim and others 1997; Jayasingh and others 2001; Krause and others 2003; Kristinsson and others 2003; Kristinsson and others 2007; Kristinsson and others 2008).

Traditional old processing methods of maintaining red color in certain fish involved placing a piece of fish above a smoking fire which contains CO due to incomplete combustion. In 1985, a patent was issued which involved retaining the good color of fresh meat, poultry and

fish using CO treatment (Woodruff and Silliker 1985). In 1996, filtered wood smoke was patented to treat fish and meat, which is generated by burning a smoking material at 250°C to 400°C and then passing it through a filter (Yamaoka 1996), which is later followed by other relative patents (Kowalski 1999). Shaklai (2001) patented a simple method of exposing raw meat solely to CO, which includes exposing raw meat to an atmosphere consisting essentially of CO and maintaining the meat in a sealed container to maintain color and freshness. Recently (Olson and Brinsmade 2004), a patent (US patent 6777012) on seafood preservation processing using clearsmoke was issued. According to previous federal regulations in the United States, CO is an unapproved food additive. However, FDA has no questions regarding the conclusion of Hawaii International Ltd. that tasteless smoke is GRAS (generally recognized as safe) for use on tuna (FDA 2000) and FDA also has no questions about Pactiv's conclusion that CO is GRAS under the intended conditions of use (FDA 2002). Later a similar response was issued for the Precept Foods petition about CO as a component of a MAP system (FDA 2004). FDA's response for use of CO shows that using CO for color development and retention in muscle foods is becoming more acceptable. Many countries have regulatory restrictions for the use of this technology. For example, Japan issued a notice to ban fish, which contain an initial CO ≥500μg/kg (Japan, Food Sanitation Law—Article 6). In the European Union, CO treatment of seafoods is not permitted, and Norway, where it was commonly used, discontinued the use of CO in meat packaging in July 2004 because of their entry into the European Union. There is no provision for the use of CO alone on any food products in Canada.

Currently CO is being used to treat certain types of seafoods either as a single gas or in combination with other gases at various concentrations. CO treated products have an increasing presence in the market due to their desirable red color and longer shelf life. However, CO

treatment could in some cases represent economic fraud as the cherry red color may last well beyond the microbial shelf life of the meat and thus mask spoilage and may hide underlying safety problems, as well as enhance the color of oxidized muscle products when used at very high levels. Therefore, there is increasingly more research interest in the determination of the amount of CO in muscle tissue. The most common and widely used methods are a two-wavelength spectrophotometric method (Tietz and Fiereck 1973; Rodkey and others 1979; Katsumata and others; Beutler and West 1984; Fechner and Gee 1989; Stonek and others 2004) and gas chromatography (GC) (Juntarawijit and others 2000; Ishiwata and others 1996). However, only a few papers have reported on COHb/Mb determination in muscle tissues. GC-FID (Ishiwata and others 1996; Chow and others 1998a) and GC-MS (Anderson and Wu 2005) have been used to detect COHb/Mb in CO treated muscle system. Compared to the GC method, the spectrophotometric method is low cost and easy to execute. It was not until recently that a simple spectrophotometric method was used to detect COMb% in CO treated muscle tissues (Smulevich and others 2007), but with the drawback of a very complex Mb purification and sample preparation procedure, it is not easy to implement in practice. An official food analysis method needs not only to be confirmatory but ideally also simple and low cost to be of value. Therefore, the objective of this study was to investigate and further refine a simple and rapid spectrophotometric method to determine the percentage of COHb/Mb in fish muscle, which may be used as a tool for laboratories, industry as well as regulatory agencies.

Materials and Methods

Washed Tilapia Muscle (WTM) Model System

Fresh tilapia fillets were obtained from a local store. Dark muscle was removed and the remaining white muscle was minced. Then the minced muscle was washed with distilled deionized water twice (1:3 muscle to water) by stirring with a plastic rod for 2 min. The mixture

was allowed to stand for 15 min, and then dewatered on a nylon screen. The muscle was then homogenized in 20 mM sodium phosphate buffer (pH 7) for 1 min by using a homogenizer (Biospec Products Inc., Bartlesville, OK) and left to sit for 15 min. The system was then centrifuged at 10,000 g for 20 min using a centrifuge 5702 (Eppendorf North America Inc., New York, NY) and then the washed muscle was vacuum packed and stored at -80°C until needed. The final moisture content of the samples was ~82%.

Preparation of OxyHb, COHb, DeoxyHb and MetHb

Tilapia was obtained locally from a tilapia aquaculture farm. It was placed on ice (pectoral side facing up) for 1 min and blood was drawn though the caudal vein. OxyHb was isolated and purified from the red blood cells according to Richards and Hultin (2000). Purity of oxyHb was verified by SDS-PAGE, and the results showed that the samples were more than 99% Hb. Samples were stored in 500 µl aliquots at -80°C and thawed under 20°C running water before they were used. COHb was prepared from oxyHb based on Tietz and Fiereck (1973). DeoxyHb was prepared by adding sodium dithionite to diluted oxyHb (~0.25 mM) and standing at room temperature for 5 min (the final concentration of sodium dithionite was ~2 mg/ml). The ferric MetHb was made by adding 200 µl Hb stock solution to 1.8 ml buffer (pH 6.5) with 2 mM sodium phosphate (dibasic) and 1 mM sodium citrate, and then adding 0.43 mg potassium ferricyanide. The mixture was inverted a few times, followed by centrifugal filtration to remove the potassium ferricyanide. Hb was kept on ice during all experiments. Hb levels were determined using the Bradford method (Chang 1998). The Coomassie plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) was used with bovine Hb as the standard.

Addition of Tilapia Hb to WTM (WTM-Hb) and CO Treatment

WTM was thawed overnight at 4°C. An appropriate volume of the oxyHb stock solution (around 25.15 mM Hb) was added to disposable polystyrene weigh dishes (Fisher Scientific, Fair

Lawn, NJ) containing 5 g washed tilapia muscle so that the final concentration was 5 μmol Hb/kg muscle. The WTM-Hb system was mixed and spread on the weigh dishes (~2 mm thickness). All the weigh dishes with WTM-Hb were placed in a gastight bag, which was vacuumed, flushed with pure CO and stored at 4°C for 24 hr. A treatment time of 24 hr was selected according to processing times used commercially. Controls were exposed to air for 24 hr in oxygen permeable bags. After treatment, samples were taken for both spectrophotometric and GC analysis.

Sample Analysis by the Spectrophotometric Method

The CO treated tilapia fish samples (~1 g) or WTM-Hb system (5 g) was added to 15 ml of sodium phosphate buffer at pH 8.5 (4°C), homogenized for 10 seconds using an homogenizer (Biospec Products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 10 min with a centrifuge 5702 (Eppendorf North America Inc., New York, NY). The supernatant was scanned between 350-700 nm by an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA) and then sodium dithionite was added to give a final concentration of ~2 mg/ml. After standing at room temperature for 5 min, the absorbance of the supernatant was measured at 419 nm and 430 nm with a spectrophotometer as described above.

COHb% Calculations

Percent COHb was calculated as follows. The absorbances at wavelength 419 nm and 430 nm are obtained by the following equations:

$$A_{419} = [\varepsilon_{419}^{deoxyHb}(1-x) + \varepsilon_{419}^{COHb}x] \times l \times c$$

$$A_{430} = [\varepsilon_{430}^{deoxyHb}(1-x) + \varepsilon_{430}^{COHb}x] \times l \times c$$

After addition of sodium dithionite, all oxyHb/Mb and MetHb/Mb will be converted to deoxyHb/Mb without affecting COHb/Mb. This leaves only two heme protein derivatives,

deoxyHb/Mb and COHb/Mb in the sample. In the above equations, x represents the fraction of COHb/COMb in the extraction. A, ε, c and l indicate the absorbance, molar absorptivity, total Hb/Mb concentration and the length of the light path respectively.

$$x = \frac{(A_{430} \times \varepsilon_{419}^{deoxyHb}) - (A_{419} \times \varepsilon_{430}^{deoxyHb})}{[A_{419} \times (\varepsilon_{430}^{COHb} - \varepsilon_{430}^{deoxyHb})] - [A_{430} \times (\varepsilon_{419}^{COHb} - \varepsilon_{419}^{deoxyHb})]}$$

$$x = \frac{1 - (A_r \times E_1)}{A_r \times (E_2 - E_1) - E_3 + 1}$$

Here A_r is the ratio A_{419}/A_{430} for the sample, $A_r = A_{419} / A_{430}$. And $E_1 = \varepsilon_{430}^{deoxyHb} / \varepsilon_{419}^{deoxyHb}$, $E_2 = \varepsilon_{430}^{COHb} / \varepsilon_{419}^{deoxyHb}$, and $E_3 = \varepsilon_{419}^{COHb} / \varepsilon_{419}^{deoxyHb}$. E_1, E_2 and E_3 are constants, which represent a ratio of one molar absorptivity to another. Since at the certain wavelength, $\varepsilon = A/(cl)$, these three constants can be defined as $E_1 = A_{430}^{deoxyHb} / A_{419}^{deoxyHb}$, $E_2 = A_{430}^{COHb} / A_{419}^{deoxyHb}$ and $E_3 = A_{419}^{COHb} / A_{419}^{deoxyHb}$.

Comparison of Expected COHb% with Detected COHb% by the Two-wavelength Spectrophotometric Method

A diluted solution of oxyHb/COHb (~0.25mM) was made by adding an appropriate volume of the oxyHb stock/COHb stock (around 25.15 mM Hb) to 10 ml of 20 mM sodium phosphate buffer at pH 6.5 and pH 8.5 (4°C). Then the diluted oxyHb and COHb were mixed at various ratios in a cuvette (Fisher Scientific, Fair Lawn, NJ), which are expected COHb%. Sodium dithionite was added to the cuvettes to give a finial concentration of 2 mg/ml. The cuvette was covered with Parafilm, and then inverted gently several times to allow for good mixing. After standing at room temperature for 5 min, the sample absorbances were read at 419 nm and 430 nm by using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). E_1, E_2 and E_3 can be obtained by the absorbances at 419 nm and 430 nm of deoxyHb (0% COHb) and fully CO bound COHb (100% COHb). And COHb% of samples can be calculated based on their A_{419}/A_{430} and COHb% calculations described above.

COHb% Standard Curve by the Gas Chromatography Method

The diluted COHb and oxyHb (as described previously) were mixed at various ratios with the total volume 10 ml in a 65 ml vial (Fisher Scientific, Fair Lawn, NJ). After adding 4 drops octanol and 10 ml 10% H_2SO_4, the vial was sealed quickly with a fluorosilicon septum and shaken for 1 min. Then they were placed at room temperature for 10 min. A 200 µl headspace gas sample was taken for GC injection.

Sample Analysis by the Gas Chromatography Method

The CO treated WTM-Hb system was added into 10 ml of 20 mM sodium phosphate buffer at pH 8.5 (4°C), homogenized for 10 seconds by using an homogenizer (Biospec Products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 10 min by an Eppendorf 5702 centrifuge (Eppendorf North America Inc., New York, NY). The supernatant was taken and placed in a 65 ml vial (Fisher Scientific, Fair Lawn, NJ) and buffer added to bring the total volume to 10 ml. After addition of 4 drops of 1-octanol and 10 ml 10% H_2SO_4, the vial was quickly sealed with a fluorosilicon septum and shaken for 1 min, and then left to stand at room temperature for 10 min. Then 200 µl of headspace gas was taken for GC injection.

Gas Chromatography Conditions for CO Analysis

An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA) was used equipped with a Porapak Q column, 80/100 mesh, 6 FT x 1/8 IN (Supelco, Bellefonte, PA), and a nickel catalyst (Agilent Technologies, Palo Alto, CA). A flame ionization detector (FID) (Agilent Technologies, Palo Alto, CA) was used for analysis. The samples were manually injected using a gastight syringe. The oven temperature was set at 35°C for 2 min. Nitrogen was used as carrier gas and held at a constant flow of 30 ml/min. The injector temperature, nickel catalyst temperature and FID temperature were held at 100°C, 375°C and 200°C respectively.

Validation of the Spectrophotometric Method

Fresh, never frozen, skinned fillets of tilapia were obtained from a local market. One fillet was one sample. All the samples were placed into a gastight bag (dark muscle side facing up), and vacuum packed to expel air and then treated with 20% vs. 100% CO by flushing and filling bags with CO gas. At the same time, the controls were placed in bags with high air permeability. The bags were stored at 4°C for 12 hr and 24 hr. Samples were then removed from the bags and stored in bags of high oxygen permeability at 4°C for 9 days. Every third day, a small amount of dark lateral muscle (~1 g) was taken for spectrophotometric analysis as described above. Five repeated samples were taken for analysis at each time point.

Color Analysis

A color machine vision system developed by Luzuriaga (1999) was used to analyze a* values of the muscle, which provides information about the redness of the muscle. After various CO treatments as described above, all the tilapia samples were stored in bags of high air permeability at 4°C for 9 days. Every third day, the dark lateral muscle was analyzed for color.

Statistical Analysis

Analysis of variance (ANOVA) and Student-Newman-Keuls test were used to determine significant difference between percentages of COHb/Mb of tilapia dark muscle and between a* values of tilapia dark muscle during storage of CO treated tilapia. The SAS system for windows 9.0 was used.

Results and Discussions

Development of the Two-wavelength Spectrophotometric Method

As early as the 1950's (Klendshoj 1950), a two-wavelength spectrophotometric method has been studied to determine COHb% in human blood in a routine clinical laboratory. This two-wavelength spectrophotometric method is the oldest and simplest way to determine

COHb/COMb% in fresh blood samples, and is based on the fact that the reducing agent such as sodium dithionite can reduce both metHb/Mb and oxyHb/Mb but never affects carboxyHb/Mb (Tietz and Fiereck 1973). Although this method has been developed and modified as time went on (Rodkey and others 1979; Katsumata and others 1982; Beutler and West 1984; Fechner and Gee 1989; Stonek and others 2004), its principle remains the same. Since there is a growing use of CO to treat fish muscles in order to maintain their desirable red color, there is a great need to determine COHb/Mb% in fish muscle. It was not until recently, this two-wavelength spectrophotometeric method was used to determine COHb/Mb% in tuna (Smulevich and others 2007). After the addition of a reducing reagent, sodium dithionite, there are only two compounds, COHb/Mb and deoxyHb/Mb, in the solution. The most important consideration for this method is the selection of two wavelengths, which may be used to obtain the absorbance response.

Figures 4-1 and 4-2 show the spectral curves of oxyHb and COHb before and after addition of reducing agent. It is clear that COHb shows no change after addition of reducing agent, which means sodium dithionite has no influence on COHb. However, there are two noticeable changes in the spectra of oxyHb before and after addition of reducing agent. Firstly, a change occurs in the lower wavelength range (Figure 4-1), where oxyHb shows a peak at 414 nm before addition of reducing agent while the peak shifts to 430 nm after addition of reducing agent. Secondly, there is a change in the higher wavelength range (Figure 4-2), where oxyHb shows two peaks at 541 nm and 576 nm before addition of reducing agent and the absorbance of these two peaks is reduced and only one peak at 556 nm appears after addition of reducing agent. Both the lower wavelength range (400-450 nm) and the higher wavelength range (500-600 nm) may be selected to pursue two wavelength spectrophotometric detection of COHb/Mb

percentage. The absorbance in the visible region (500-600 nm) needs high Hb concentration in order to obtain a satisfactory sensitivity, but high Hb concentration may cause turbidity and have a big influence on other heme protein derivatives or pigments. The readings in the Soret region (400-450 nm) require relatively low Hb concentration, which reduces Hb precipitation and turbidity. The absorbance of Hb and its derivatives in the Soret region (400-450 nm) is much more sensitive than that in the visible region (500-600 nm) (Small and others 1971), and it can be seen (Figures 4-1, 4-2) that the absorbance of the Hb and its derivatives in the range of 400-450 nm are about 10 times higher than that in the range of 500-600 nm. In addition, at 419 nm the absorbance of COHb is around double that of deoxyHb, and at 430 nm the absorbance of deoxyHb is also around double that of COHb. Measurements at 419 and 430 nm are very sensitive to changes in the ratio of COHb and deoxyHb in this two-pigment system. Therefore, measuring absorbances at 419 and 430 nm after addition of the reducing reagent, is a more accurate way to determine COHb/Mb% rather than measuring absorbances in the range of 500-600 nm.

Both 100% oxyHb (0% COHb) and 100% COHb were scanned between 350-700 nm before and after addition of sodium dithionite. According to the absorbance at 419 nm and 430 nm of deoxyHb (0% COHb) and fully CO bound COHb (100% COHb), the three constants, E_1, E_2 and E_3, were determined as E_1=1.25±0.01, E_2= 0.62±0.02 and E_3= 2.05±0.06. COHb% increased with A_{419}/A_{430} value rising. The ratio of A_{419}/A_{430} for 100% COHb was 3.51±0.12 and 2.65±0.01 for 0% COHb. Any mixture of the two will have an A_{419}/A_{430} value somewhere between these two values. Since the absorbance response and wavelength accuracy may vary with different spectrophotometers, it is desirable to determine these characteristics for the instrument being used. It was reported that the A_{420}/A_{431} ratio was 2.65±0.01 for a fully bound

COMb from tuna, while in the absence of CO, the ratio was 0.86±0.01 (Smulevich and others 2007).

Validation of the Two-wavelength Spectrophotometric Method

Based on Figures 4-3 and 4-4, it can be seen that there was an excellent correlation (r = 0.998) between the expected COHb% and detected COHb% by the spectrophotometric method used, and even at low concentrations the curve was still quite linear. According to our findings the detection limit can get up to ~3.33 COHb% (Figures 4-3, 4-4). The reaction of Hb with sodium dithionite is very complex and some side reactions may occur (Dalziel and Obrien 1957). The absorbance ratio may change slightly after addition of sodium dithionite, so in order to ensure uniformity it is recommended to measure absorbance 5 min after addition of sodium dithionite.

It was reported that pH has some effect on A_r and at high pH A_r increases with an apparent increase in the percentage of COHb (Beutler and West 1984). However, in this study, it was found that at pH 8.5 (Figure 4-4), there was also an excellent correlation (r = 0.998) between the expected COHb% and detected COHb% by the spectrophotometric method. Previous studies, reported that it was necessary to take special precautions to shield samples from air (Rodkey and others 1979; Smulevich and others 2007). However in our study with tilapia Hb, it was observed that protecting the solutions from air was not necessary, which agrees with the result of Beutler and West (1984).

Figure 4-6 shows the comparison of COHb% results by the two-wavelength spectrophotometric method and by the GC method. Right after CO treatment, the CO treated WTM-Hb system were stored at 4°C for up to 9 hours, and every third hour, COHb% was determined by spectrophotometric method and by the GC method, which is based on the COHb% GC standard curve (Figure 4-5). Each sampling point (Figure 4-6) represented triplicate

samples and all results are described as means ± standard deviation. It can be seen that CO was released with storage and there was an excellent correlation (r = 0.9992) between the two procedures (Figure 4-6b). The results indicate that the GC method gave values that were significantly lower ($p<0.05$) than the spectrophotometric results (Figure 4-6a). This is because these two methods are based on different principles. For the spectrophotometric method, the ratio of A_{419} to A_{430} was measured and used to directly calculate COHb%, and in that case, Hb concentration is not an influencing factor, so it is not necessary that all the Hb is in the supernatant after extraction. However, for the GC method, as much as possible COHb should be in the supernatant after extraction in order to get an accurate percentage of COHb. However, during extraction and following centrifugation, some Hb will precipitate with the myofibrillar proteins. Particularly some unstable myofibrillar proteins will be denatured during the extraction procedure, and these denatured proteins may interact with Hb, and reduce their solubility even after multiple extractions with water or low ionic strength buffer. Therefore there may be some COHb left in the precipitate after extraction and centrifugation, which may result in the lower value of COHb% seen with the GC procedure.

Tilapia fillets were treated with either 20% or 100% CO for 12 hr or 24 hr to validate this method on a real fish system. After treatment, all CO treated fillets showed a significant increase ($p<0.05$) in COHb/Mb% (Figure 4-7). Heme peak wavelength and redness (a*-values) can also be used as indicators of CO/FS treatment. Heme peak wavelength above 414 nm would indicate samples containing CO. All treatments led to an increase in the heme peak wavelength (Figure 4-8), which showed the peak wavelength of COHb/Mb. Also, a* values have a significant increase ($p<0.05$) after 100% CO treatment and 24hr 20% CO treatment (Figure 4-9). But there was no significant difference ($p>0.05$) between a* values of untreated control and 12hr 20% CO treated

samples (Figure 4-9). COHb/Mb% had a significant decline ($p<0.05$) during storage (Figure 4-7), and correspondingly, a* values and heme peak wavelength had a significant decrease ($p<0.05$) during storage of CO treated samples (Figures 4-8, 4-9). During the first three days refrigerated storage after CO treatment, COHb/Mb% decreased very quickly, and after that, COHb/Mb% had slightly changed. It was also observed that there was a slight increase of COHb/Mb% and heme peak wavelength for untreated samples during refrigerated storage (Figures 4-7, 4-8), which agrees with the results of Ishiwata and others (1996). CO has been known to be formed during metabolism of protoheme, which indicates that biological materials contain endogenous CO and the CO concentration may increase during the storage (Ishiwata and others 1996). A similar connection between COHb/Mb%, heme peak wavelength and redness was found for dark muscle of tilapia fillets (Figures 4-7, 4-8, 4-9). The increase in redness is related to the amount of CO bound to the heme proteins in muscle, and the more CO is bound, the more stabilized the heme proteins are to either oxidation or denaturation. This explains the color stabilization of CO treatment. In addition to the samples treated in this study, frozen tilapia (labeled as CO treated), was bought from a local store, and was found to have 32.6±1.71% COHb/Mb% with the two wavelength spectrophotometric method.

Heme protein extracts from a muscle system usually contain both Hb and Mb, and their proportions vary with different muscle types and different species. Dark muscle fish species have higher Hb levels than white muscle fish species (Venugopal and Shahidi 1996). Hb was the predominant heme protein in mackerel light muscle (6 μmol Hb/kg) and whole trout muscle (11μmol Hb/kg) (Richards and Hultin 2002). Hb may be lost during bleeding (Richards and others 1998), but recent reports show a significant retention of Hb even after bleeding (Richards and Hultin 2002; Richards and others 1998). In rainbow trout whole muscle and Atlantic

mackerel light muscle, Mb content was reported to be much lower than Hb content (Richards and Hultin 2002). In breast muscle from chicken broilers only Hb was detected while Mb was undetectable (Kranen and others 1999). In the chicken broilers thigh muscle, more than 80% of the total heme protein was detected as Hb (Kranen and others 1999). In the Bicep femoris of Holstein calves the percentage of Hb was 95% compared to 5% for Mb. The high Hb concentration in the fish and bird muscle tissue is due to the fact that muscle fibers of fish and bird are small and the smaller diameter allows better diffusion of oxygen from capillaries into muscle cells, which makes intracellular Mb less needed (Richards and others 2005). In addition, Hb may be partially dissociated into monomers during extraction processing (Kranen and others 1999). Therefore, both Hb and Mb will be present in the muscle systems. It is possible and could be more accurate to use heme protein extract from the muscle system to make 100% oxyHb/Mb and 100% COHb/Mb and further determine the constants E_1, E_2, and E_3. However significant work is needed to separate and purify heme proteins from other components of the muscle system (Smulevich and others 2007), and especially the turbidity in the extract will influence the spectral results. Compared to Mb, Hb can be easily separated and purified from the blood. Each type of oxygenated heme protein has the same peak wavelength and the absorbance is nearly equivalent based on spectrophotometric results (Richards and others 2005). Therefore, it is reasonable and valid to use Hb as a standard to analyze the percentage of CO bound heme proteins.

The spectral curves of human COHb and dexoxyHb are quite similar to those of COHb and deoxyHb from other animals, such as rat, mice and dogs (Rodkey and others 1979). The molar absorptivity values for the three Hb forms at 420 and 432 nm were compared for humans, rat and mice, where it was found that the differences in the molar absorptivity value at a given

wavelength does not exceed about 2-4% of the value. However, the observed differences emphasize the importance of measuring the absorbance values using the same spectrophometer and with blood of the proper species (Rodkey and others 1979). In this study, tilapia Hb was used to determine the constants E_1, E_2, and E_3, which was used to calculate CO saturation of heme proteins in the tilapia muscle system. Therefore, in order to analyze the COHb/Mb% in the muscle tissue of other species, different constants E_1, E_2, and E_3 should be made with Hb from the blood of the corresponding species.

There are some drawbacks of the spectrophotometric method. Heme proteins, which have been homogenized, may contain other pigments arising from their breakdown, which may make this method unsuitable in some cases. The turbidity of extracts, which may come from the heme proteins degradation or the presence of other proteins, can also cause some problems when analyzing the spectra. During the heme protein extraction and analysis, there may be some CO released from heme proteins and heme proteins degradation, which will influence the results.

CO dissolution in the muscle tissue and binding to heme proteins are complex processes. COHb spectra can only represent the COHb complex, but there is also a significant amount of CO, which is dissolved in the muscle tissue and can not be detected by spectra. During refrigerated storage, more and more CO may be released from the muscle tissue, which may skew the balance between CO dissolution, CO binding and CO release. Thus, more and more CO leaves Hb/Mb and dissolves in the muscle tissue and then releases from the muscle tissue. In fact, among all CO in the muscle tissue, a large amount of CO is dissolved in the muscle tissue, while only a small amount of CO binds to heme proteins, which depends on the amount of Hb/Mb concentration in the muscle tissue. The spectrophotometric method can not determine the exact total amount of CO in the muscle system, which may be more important than Hb/Mb

bound CO for example when samples need to pass below a certain regulatory limit to enter the market. Therefore, in future studies, we aim at determining the total amount of CO in the CO treated products by using GC, to build up a relationship between COHb/Mb% and total CO amount in the muscle tissue, and further evaluate the various available analytical methods for CO determination in the muscle system.

Conclusion

A simple spectrophotometric method can be used to give a good estimate of the COHb/Mb% in the muscle tissue. This method is based on that sodium dithionite can deoxygenate oxyHb/Mb and metHb/Mb without influencing COHb/Mb. Therefore, a two-pigment mixture was produced and the percentage of COHb/Mb can be obtained by calculating directly from the ratio A_{419}/A_{430}. This two-wavelength spectrophotometric method can detect COHb/Mb% even as low as 3.33% in the muscle tissues. There is an excellent correlation (r=0.9989) between the expected COHb% and the detected COHb% by the two-wavelength method. The high correlation (r=0.9992) of the two-wavelength method with the highly sensitive GC method shows the results are accurate and reproducible. During the procedure, protecting the solutions against exposure to air is not necessary. Absorption values were stable and reproducible, and were found not to be particularly sensitive to the change in pH, within a pH range typical of fish products. MetHb/Mb is reduced by sodium dithionite and it is not a source of interference, but the other Hb derivatives or pigments may possibly cause interference. The spectrophotometric method described here can therefore be recommended as a simple, rapid and low cost laboratory procedure for determining the presence of CO and percentage of COHb in a fish muscle system.

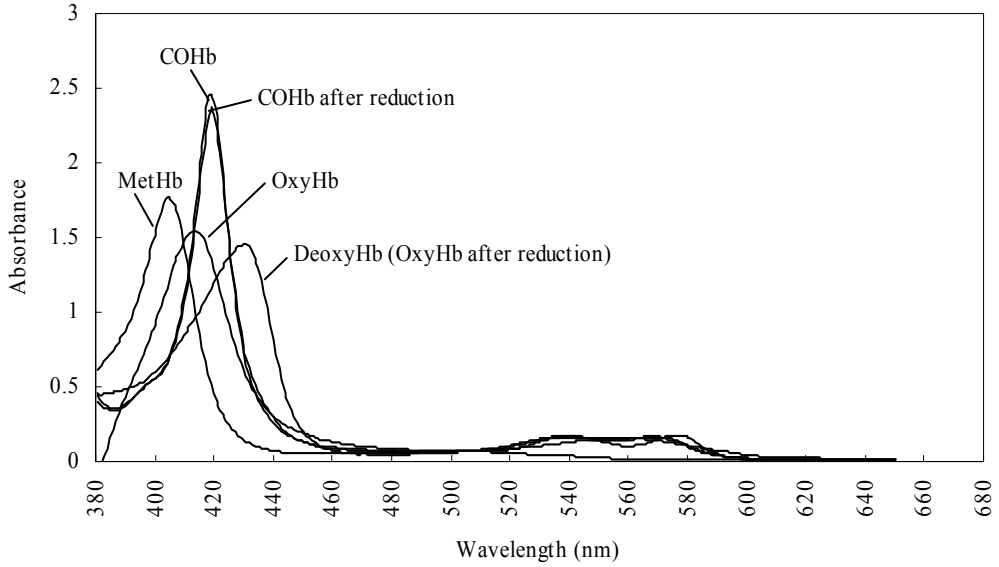


Figure 4-1. UV-visible spectra of tilapia Hb bound to different ligands. OxyHb was extracted from tilapia, and diluted to ~ 0.25 mM by 20 mM sodium phosphate buffer (pH 8.5). COHb, deoxyHb and metHb, were made from oxyHb.

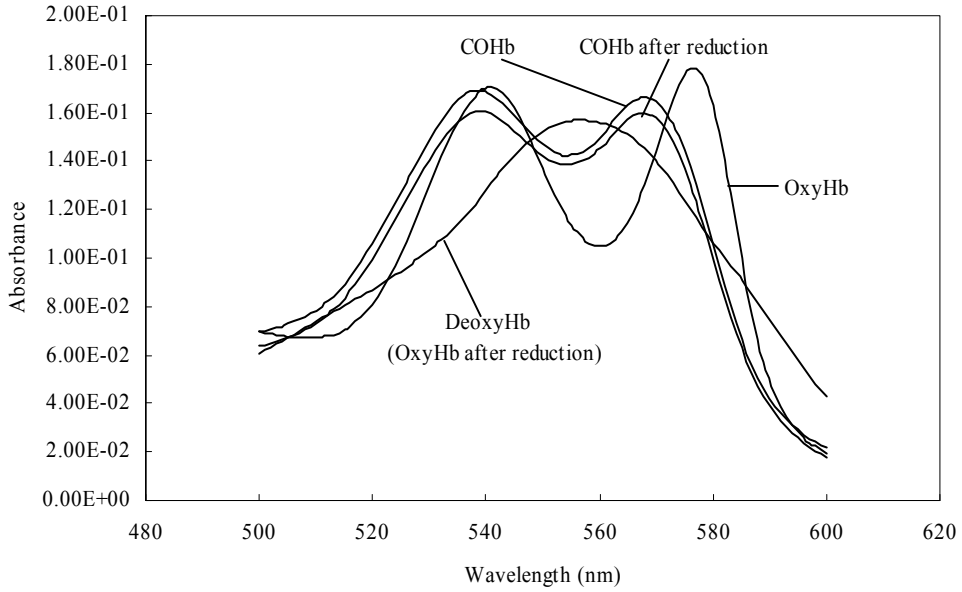


Figure 4-2. UV-visible spectra (500-600 nm) of tilapia Hb bound to different ligands. OxyHb was extracted from tilapia, and diluted to ~ 0.25 mM by 20 mM sodium phosphate buffer (pH 8.5). COHb, deoxyHb and metHb, were made from oxyHb.

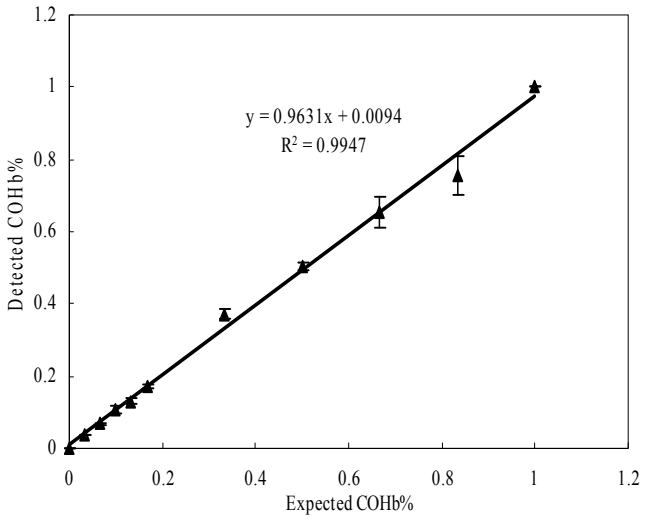


Figure 4-3. Comparison of expected COHb% and detected COHb% by the two-wavelength spectrophotometric method (pH 6.5). OxyHb stock was diluted by 20 mM sodium phosphate butter (pH 6.5) to ~0.25 mM. COHb (~0.25 mM) was made by this oxyHb (pH 6.5). OxyHb and COHb were mixed at various ratios, which are the expected COHb%, and then sodium dithionite was added to the mixtures, and the absorbances at 419 nm and 430 nm were read, which were used to calculate detected COHb%.

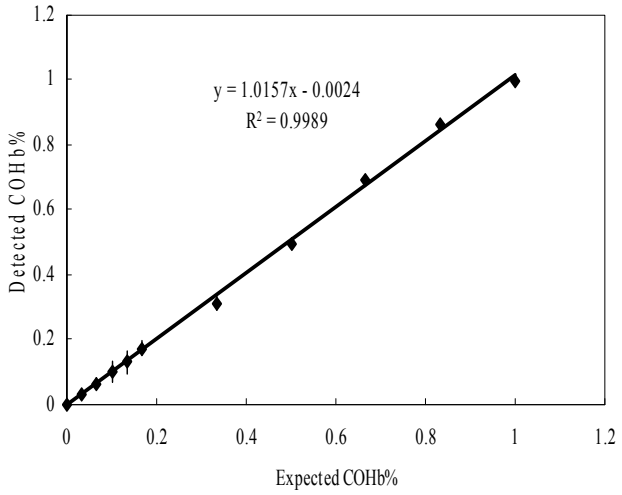


Figure 4-4. Comparison of expected COHb% and detected COHb% by the two-wavelength spectrophotometric method (pH 8.5). OxyHb stock was diluted by 20 mM sodium phosphate butter (pH 8.5) to ~0.25 mM. COHb (~0.25 mM) was made by this oxyHb (pH 8.5). OxyHb and COHb were mixed at various ratios, which are the expected COHb%, and then sodium dithionite was added to the mixtures, and the absorbances at 419 nm and 430 nm were read, which were used to calculate detected COHb%.

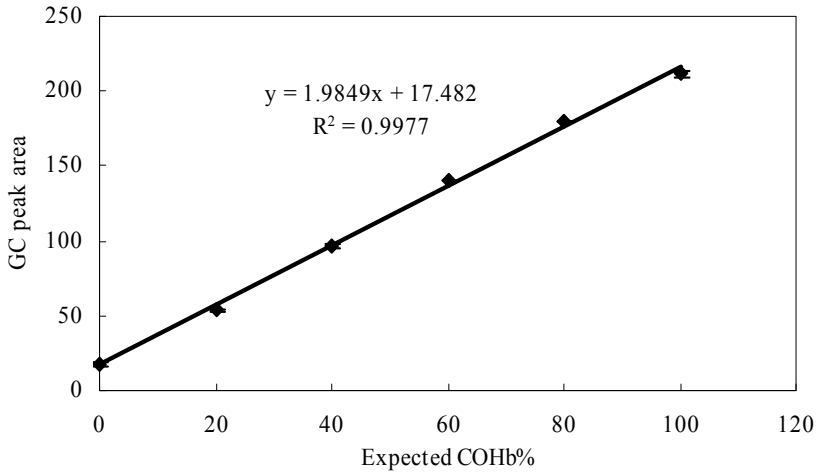
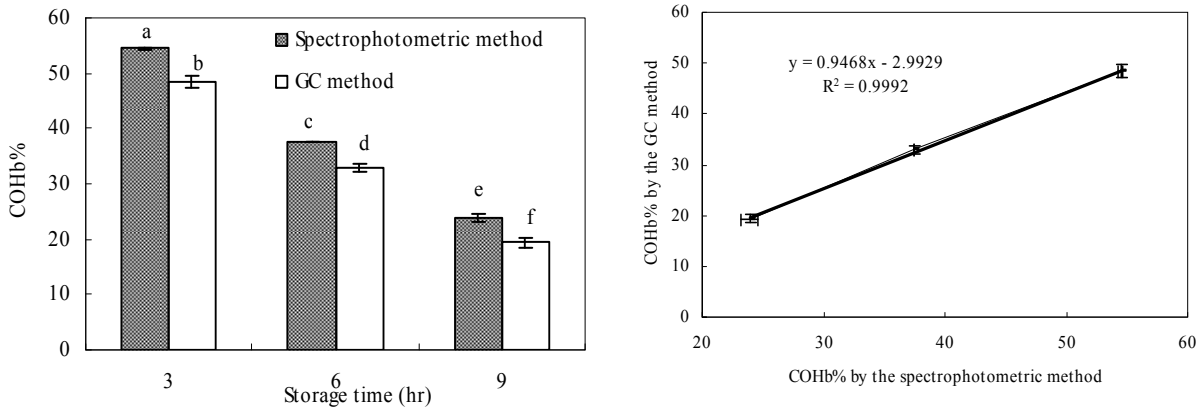


Figure 4-5. Standard curve of COHb% by gas chromatography. Diluted oxyHb and COHb were mixed at various ratios with the total volume 10 ml in a 65 ml vial. CO releasing reagent was added to the sample and it left to stand at room temperature for 1.5 hr before taking a headspace gas sample for GC analysis.



(a) (b)

Figure 4-6. Comparison of results by the two-wavelength spectrophotometric method for COHb% with those by GC method during refrigerated storage of CO treated WTM-Hb. WTM-Hb system was treated with 100% CO for 24 at 4 ℃ and then placed in oxygen permeable bags for up to 9hr. Different small letter indicate a signicatant difference ($p<0.05$).

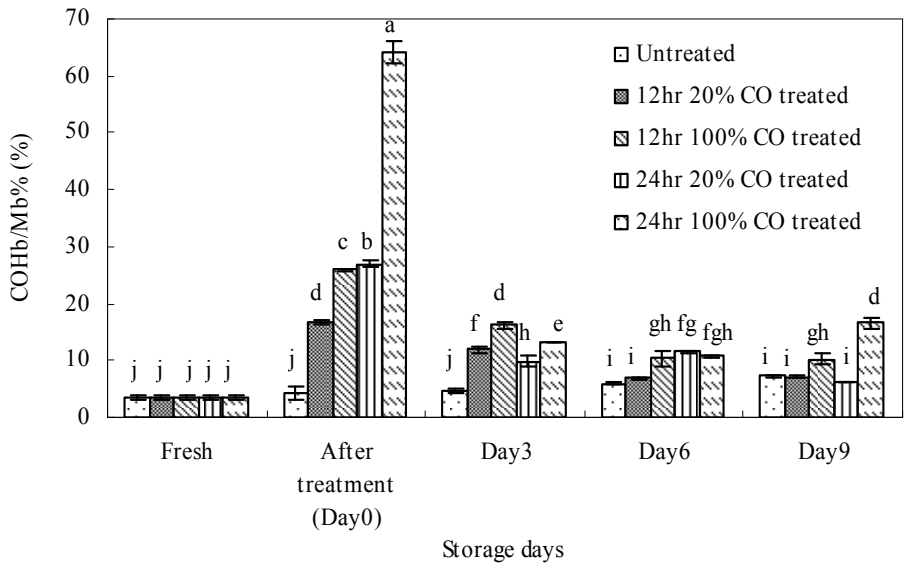


Figure 4-7. Effect of different CO gas treatments on COHb/Mb% in tilapia dark muscle as a function of storage time (4°C). Tilapia fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days. Different small letter indicate a signicatant difference ($p<0.05$).

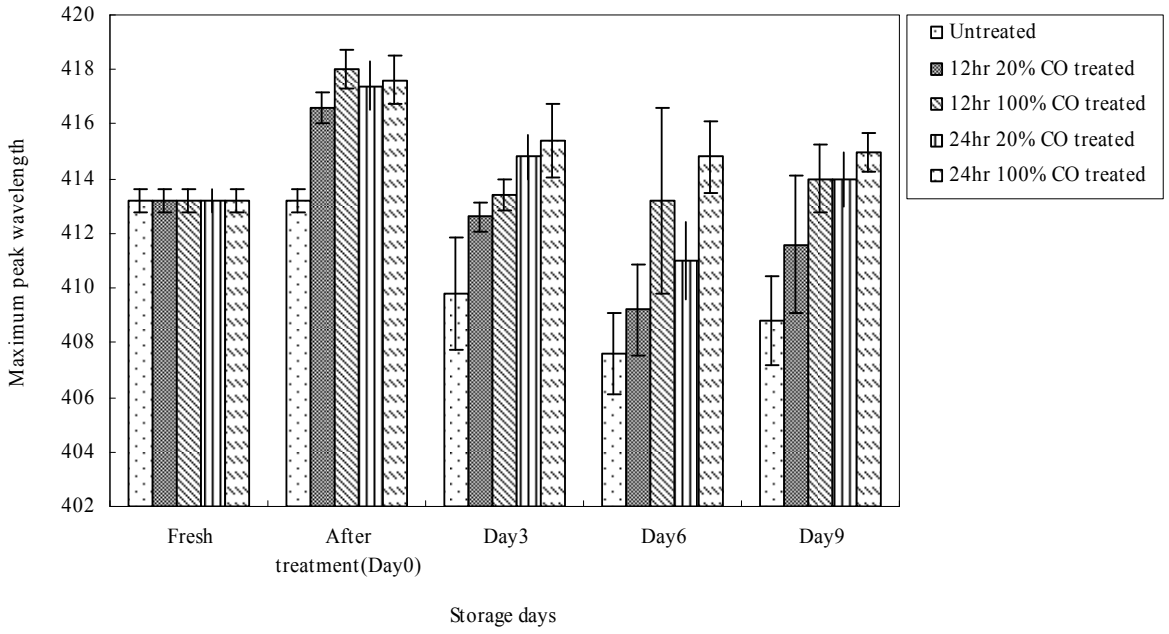


Figure 4-8. Heme peak wavelength of tilapia dark muscle extracts as a function of storage time (4°C). Tilapia fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days.

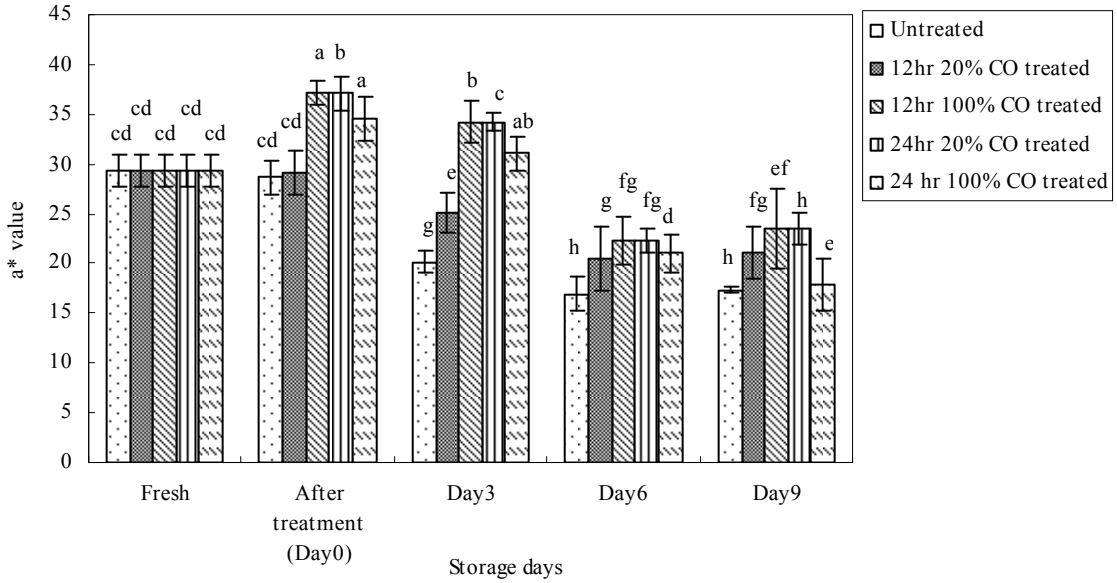


Figure 4-9. Effect of different CO gas treatments on a* value of tilapia fillets as a function of storage time (4°C). Tilapia fillets were treated with either 20% or 100% CO for 12 or 24 h at 4°C and then placed in oxygen permeable bags for up to 9 days. Different small letter indicate a signicatant difference ($p<0.05$).

CHAPTER 5
IMPROVEMENT OF A GAS CHROMATOGRAPHIC METHOD AND EVALUATION OF VARIOUS ANALYTICAL METHODS FOR DETERMINING CARBON MONOXIDE IN FISH MUSCLE

Introduction

The popularity of seafood products treated with CO or FS, containing CO, has increased significantly in the US market over the past decade mainly due to their bright red color preferred by many consumers. Considering the effects of CO enriched atmospheres on the preservation and shelf-life extension of meats and fish, and the possibility of CO related food safety issue, more and more interest focuses on the identification of the treated products and further quantification of CO in the muscle tissue.

Color evaluation has been used as a method to determine if seafood products have been treated with CO. However, this method has been suggested not to be appropriate to identify CO treated products. According to Kristinsson and others (2006), the National Marine Fisheries Service (NMFS) has suggested a color threshold of $a^* = 16.2$ for tuna, but very fresh tuna can have a^* value above this level, and it is also relatively easy to control the a^* value of CO-treated products so they are below this threshold.

The most used analytical methods for CO determination is GC and spectrophotometric methods. Since 1959, GC has been developed to determine CO in postmortem blood (Domiguez and others 1959), which was modified and improved later (Goldbaum and others 1986; Lewis and others 2004). This GC method is based on liberating CO from the blood sample to the headspace of a closed system by adding a CO liberating reagent, and then headspace gas is injected into GC for separation and detection. Since GC is highly specific for CO (Vreman and others 1984) and can not be affected by both spectral and chemical interference, it is regarded as a more sensitive and accurate CO detection method. Several kinds of detection systems such as

flame ionization detection (FID) (Collison and others 1968; Guillot and others 1981; Sundin and Larsson 2002; Kaminski and others 2003), thermal conductivity detection (TCD) (Goldbaum and others 1986; Van Dam and others 1994; Lewis and others 2004), infrared detection and even mass spectrometry (Oritani and others 2000), have been applied to analyze CO. These days CO is being introduced to treat certain types of seafoods either as a single gas or in combination with other gases at various concentrations, and this technology has become a common practice in the seafood industry. A GC method was developed to quantify CO in treated seafood products (Miyazaki and others 1997; Chow and others 1998; Ishiwata and others 1996). This method involves first releasing CO from the muscle tissues to the headspace of a closed system by adding a CO liberating reagent. Headspace gas is then injected to the GC, where CO is separated from the other gases in the column, and then reduced to methane by an installed methanizer and finally detected by FID. FID is a common GC detector, but it does not have a response for CO. After CO is catalytically reduced to CH_4, it can be detected by FID (Griffin 1979; Guillot and others 1981; Costantino and others 1986; Miyazaki and others 1997; Chow and others 1998). This GC-FID method was improved by separating CO from O_2 before CO reduction (Kaminski and others 2003), which made it more accurate to determine trace amount of CO, CO_2 and CH_4. The detection limit of this method was demonstrated to be 2 μg CO/kg fish muscle (Ishiwata and others 1996; Miyazaki and others 1997). Recently, this GC method has been presented to identify and verify the type of CO or filtered smoke treatment employed in muscle (Crynen and Kristinsson 2007).

Several spectrophotometric methods have been used to determine the concentration of Mb/Hb and its derivatives. A manual multi-wavelength spectrophotometric method was described to determine relative and absolute pigment concentrations of Mb derivatives, oxy-,

carboxy-, and met-Mb in fish or meat samples (Wolfe and others 1978). It was found that no interaction of metMb and carbon monoxide was observed and the indicated amount of metMb was independent of the duration and rate of CO bubbling (Wolfe and others 1978). Unfortunately this multi-wavelength spectrophotometric method can not measure deoxyMb and its sensitivity is low, which limits its application. A two-wavelength spectrophotometric method has been developed to determine COHb% in human blood in a routine clinical laboratory (Klendshoj and others 1950), which was developed and modified later (Rodkey and others 1979; Katsumata and others 1982; Beutler and West 1984; Fechner and Gee 1989). This two-wavelength spectrophotometric method is based on the fact that Hb/Mb and their derivatives have characteristic absorption spectra, and that sodium dithionite can deoxygenate oxyHb/Mb and metHb/Mb without influencing COHb/Mb (Tietz and Fiereck 1973). In that way, the sample can be converted into a two-component system, COHb/Mb and deoxyHb/Mb, and analyzed based on absorbances at the two wavelengths selected. It was not until recently that two-wavelength spectrophotometric method was used to detect COMb% in CO treated muscle tissues (Smulevich and others 2007), but with the drawback of a very complex Mb purification and sample preparation procedure, it is not easy to implement in practice. An official food analysis method needs not only to be confirmatory but ideally also simple and low cost to be of value.

Nowadays spectrophotometry and GC are two main methods used to determine CO in the muscle. Usually spectrophotometry is regarded as a basic instruments in the routine clinical laboratory due to its low cost and easy to execute. However, GC can accurately determine total amount of CO in muscle. In this study, we are aiming at improving the current GC method for CO determination in the muscle. We will also investigate and compare the various two-

wavelength spectrophotometric methods for COHb/Mb determination in order to obtain a sound understanding of these methods.

Materials and Methods

Chemicals and Supplies

1-octanol (98%) was purchased from Acros Organics (Pittsburgh, PA). Sulfuric acid was diluted from a 50% (v/v) solution (RICCA Chemical Company, Arlington, TX). Carbon monoxide gas, 99.5%, was purchased from Airgas (Airgas, Gainesville, FL). CO, 10 and 100 ppm, was purchased from Agilent Technology (Wilmington, DE). Gastight vials with septum-fitted caps (65 ml) were purchased from Fisher Scientific (Fair Lawn, NJ). Standard gas sample loops for GC injection were purchased from Agilent Technology.

Washed Tilapia Muscle (WTM) Model System

Fresh tilapia fillets were obtained from a local store. Dark muscle was removed and the remaining white muscle was minced. Then the minced muscle was washed with distilled deionized water twice (1:3 muscle to water) by stirring with a plastic rod for 2 min. The mixture was allowed to stand for 15 min, and then dewatered on a nylon screen. The muscle was then homogenized in 20 mM sodium phosphate buffer (pH 7) for 1 min using a homogenizer (Biospec Products Inc., Bartlesville, OK) and left to sit for 15 min. The system was then centrifuged at 10,000 g for 20 min with a centrifuge 5702 (Eppendorf North America Inc., New York, NY) and the washed muscle was vacuum packed and stored at -80°C until needed. The final moisture content of the samples was ~82%.

OxyHb Preparation

Tilapia was obtained locally from a tilapia aquaculture farm. It was placed on ice (pectoral side facing up) for 1 min and blood was drawn though the caudal vein. OxyHb was isolated and purified from the red blood cells according to Richards and Hultin (2000). Purity of oxyHb was

verified by SDS-PAGE, and the results showed that the samples were more than 99% Hb. Samples were stored in 500 µl aliquots at -80°C and thawed under 20°C running water before they were used. COHb was prepared from oxyHb based on Tietz and Fiereck (1973). Hb levels were determined using the Bradford method (Chang 1998). The Coomassie plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) was used with bovine Hb as standard.

Addition of Tilapia Hb to WTM (WTM-Hb) and CO Treatment

WTM was thawed overnight at 4°C. An appropriate volume of the oxyHb stock solution (around 25.15 mM Hb) was added to disposable polystyrene weigh dishes (Fisher Scientific, Fair Lawn, NJ) containing 5 g washed tilapia muscle so that the final concentration was 5 µmol Hb/kg muscle. The WTM-Hb system was mixed and spread well on the weigh dishes (~2 mm thickness). All the weigh dishes with WTM-Hb were placed in a gastight bag, which was vacuumed, flushed with pure CO and stored at 4°C for a certain time of treatment. After treatment, samples were taken for preparation of GC analysis.

Gas Chromatography System

An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA) was used equipped with a Porapak Q column, 80/100 mesh, 6 FT x 1/8 IN (Supelco, Bellefonte, PA), followed by a nickel catalyst (Agilent Technologies, Palo Alto, CA). A flame ionization detector (FID) (Agilent Technologies, Palo Alto, CA) was used for analysis. The samples were manually injected using a gastight syringe. The oven temperature was set at 35°C for 2 min. Helium was used as a carrier gas and held at a constant flow of 30 ml/min. The injector temperature, nickel catalyst temperature and FID temperature were held at 100°C, 375°C and 200°C respectively. The gas flow to the detector was 400 ml/min for air and 40 ml/min for hydrogen.

CO Standard Curve by GC and CO Mass Calculation

A CO calibration curve was established by injecting CO with various standard gas sample loops, 250, 500, and 1000 ml, and high pressured gas mixture with 10 and 100 ppm CO. CO was introduced directly from its cylinder into the gas sample loop, then into the column with the function of the gas sample valve. The actual CO mass was estimated by the ideal gas law equation: $PV=nRT$, where P is the barometric pressure, V is the volume of CO gas, n is the number of moles of CO, R is the universal gas constant, and T is the temperature in Kelvin.

CO Recovery Analysis

5g WTM-Hb with Hb concentration 5 μmol/kg, 10 ml of 20 mM sodium phosphate buffer at pH 8.0, 10 ml of 5% H_2SO_4, and 4 drops of 1-octanol were added to a 65 ml gastight vial. Then the vial was capped. Then 4, 12, 20, 40, 60 and 80 μl headspace gas was removed from the headspace of the vial followed by adding corresponding amount of CO by using a gastight syringe. This was shaken manually for 1 min and then left to stand at room temperature for 10 min. Then 100 μl headspace gas from the vial was injected into a GC for CO analysis.

Sample Analysis by the Gas Chromatography Method

The CO treated WTM-Hb system was added into 10 ml of 20 mM sodium phosphate buffer at pH 8.0 (4°C), homogenized for 10 seconds using a homogenizer (Biospec Products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 10 min with an Eppendorf 5702 centrifuge (Eppendorf North America Inc., New York, NY). The supernatant was taken and placed in a 65 ml gastight vial. After addition of 4 drops of 1-octanol and 10 ml 5% H_2SO_4, the vial was quickly capped and shaken for 1 min, and then left to stand at room temperature for 10 min. Then 200 μl of headspace gas was taken for GC injection. In order to analyze CO loss during the sample preparation, the mixture of WTM-Hb and buffer, before and after

homogenation, were also taken respectively and placed in a 65 ml gastight vial for CO liberating and following by CO analysis. In order to investigate the various CO liberating methods, the samples were also employed by 5 min water bath heating, 10 min water bath heating, adding 100 μl $K_3Fe(CN)_6$, 200 μl $K_3Fe(CN)_6$, 10 ml 20% H_2SO_4,10 ml 10% H_2SO_4, and 10 ml 5% H_2SO_4, respectively.

Comparison and Evaluation of Various Two-Wavelength Spectrophotometric Methods

A diluted solution of oxyHb/COHb (~0.25 mM) was made by adding an appropriate volume of oxyHb stock/COHb stock (around 25.15 mM Hb) to 10 ml of 20 mM sodium phosphate buffer at pH 6.5 (4°C). Then the diluted oxyHb and COHb were mixed at various ratios in a cuvette (Fisher Scientific, Fair Lawn, NJ), which are the expected COHb%. Sodium dithionite was added to the cuvettes to give a final concentration of 2 mg/ml. The cuvette was covered with Parafilm, and then inverted gently several times to allow for good mixing. After standing at room temperature for 5 min, the sample absorbances were read at 419, 425, 431, 538, 556, and 568 nm using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). For every pair of two wavelengths, the detected COHb% can be calculated based on Chapter 4.

Statistical Analysis

Analysis of variance (ANOVA) and Student-Newman-Keuls test were used to determine significant difference between CO release from various sample sources and between various CO liberating methods. The SAS system for windows 9.0 was used.

Results and Discussion

As early as 50 years ago, GC was developed to determine CO in postmortem blood (Domiguez and others 1959). In the 1990's, the GC method was used to determine CO in fish muscle (Miyazaki and others 1997). That method is based on CO releasing from fish muscle to a

closed system followed by taking headspace gas for GC separation and detection. Figure 5-1 shows the chromatogram of CO liberated from tilapia Hb. It was observed that CO and CO_2 are two major components in the headspace gas, and CO can be separated and detected. The total analysis time was less than 2 min, and neither N_2 nor O_2 can be detected by using this GC system. Due to the high sensitivity of CO in the nickel catalyst system, it was possible to determine low levels of CO with high accuracy. According to application note 228-92 of Hewlett Packard, detection limit of this system could reach up to 50 ppb for CO. The standard curve (Figure 5-2) showed a great linear relationship between CO amount and peak area ($y = 5092.8x + 11.422$, $r^2 = 0.9985$), which ranged from 0.005 to 1.0 μl CO per injection. Therefore, with this technique, it is possible to determine low levels of CO. In order to investigate the absorption of CO by the mixture of fish muscle and buffer, various amount of CO were injected into the headspace of a vial with this mixture inside. After shaking for 1 min and standing for 10 min, the headspace gas was analyzed by GC. It was found that CO recovery from the mixture of fish muscle and buffer was very high, and there was no significant difference ($p>0.05$) between CO recoveries of various amounts of CO added to the mixture (Table 5-1).

Ishiwata and others (1996) and Chow and others (1998) also reported a similar GC method for CO quantification in fish muscle with CO liberated from Mb/Hb extraction instead of directly from fish muscle. Figure 5-3 shows the CO amount from various sample sources taken from each step in preparing the Hb extract. There is a significant difference between CO release from any two sample sources ($p<0.05$). CO may release from the WTM-Hb system after addition of liberating agent, but at that time, detected CO amount was far below the amount which was released from the mixture of WTM-Hb and buffer (Figure 5-3). When sulfuric acid, as a liberating agent, was added into the muscle, it may immediately and directly react with Hb and

part of Hb was denatured and some CO released suddenly from the muscle before the vial was capped, which may result in some CO loss and the detected CO amount lower than the actual value. However, when sulfuric acid was added to the mixture of the muscle and buffer, the buffer at a certain level prevented the immediate interaction between sulfuric acid and the muscle system. After the vial was sealed, followed by vigorously shaking, Hb reacted with sulfuric acid adequately and CO was fully released. Homogenation can cause CO loss according to the results in Figure 5-3. Some researchers (Ishiwata and others 1996; Chow and others 1998) regarded Hb extract, supernatant of Hb homogenate after centrifugation, as a sample source to liberate CO. Our results demonstrated that the detected CO amount released from Hb extract is around as half that from the mixture of the muscle and buffer (Figure 5-3). Homogenization may increase Hb aggregation and precipitation, and after centrifugation, possibly some Hb bound to CO was precipitated, which may decrease the detected CO amount. Anderson and others (2005) also reported that fish flesh homogenate revealed higher amount of CO than myoglobin containing supernatant. Homogenation may physically damage the protein, increase the surface area of the meat exposed to the atmosphere, and/or damage cellular compartments allowing for the release of enzymes and other chemicals (Anderson and others 2005).

Sulfuric acid at various concentrations (Miyazaki and others 1997; Chow and others 1998; Ishiwata and others 1996; Sundin and Larsson 2002; Van Dam and Daenens 1994; Lewis and others 2004), potassium ferricyanide (Dominguez and others 1959; Collison and others 1968; Guillot and others 1981; Costantino and others 1986; Oritani and others 2000) and heating for various times (Nam and Ahn 2002) have been shown to act as CO liberating agents for muscle or blood samples. In this study it was found that there is a significant difference between potassium ferricyanide, sulfuric acid and heating in their CO releasing capability ($p<0.05$). It has been

reported (Collison and others 1968) that potassium ferricyanide can be used as a CO liberating reagent with an RSD (relative standard deviation) of 1.8% for normal human blood. However, in our study with a muscle system, potassium ferricyanide is far less effective than sulfuric acid and heating for CO liberation (Figure 5-4) even though potassium ferricyanide gave a low RSD of 0.65%-4.64% (Table 5-2). There was no significant difference between 100 μl and 200 μl potassium ferricyanide for its ability to release CO ($p>0.05$). In addition, there was no significant different between 5 min and 10 min heating for CO liberation ($p>0.05$). Our results showed that sulfuric acid is more effective for CO release than heating and potassium ferricyanide (Figure 5-3). In addition, sulfuric acid gave the lowest coefficient of variation (0.76%) compared with heating and potassium ferricyanide. Regarding the CO liberating capability, there was no significant difference between 5% and 10% sulfuric acid ($p>0.05$). Considering that 5% sulfuric acid showed a lower RSD of 0.76% than 10% sulfuric acid (Table 5-2), 5% sulfuric acid can be determined as the most effective CO liberating reagent according to these results. Moreover, no significant increase of CO amount extracted was identified if samples were kept at room temperature (after shaking) longer than 10 min.

The two wavelength spectrophotometric method has been used to detect the percentage of COHb in blood for a long time. Recently, this method was used to determine the percentage of COHb/Mb in fish muscle (Smulevich and others 2005). Heme proteins with different ligand binding status reveal different characteristic absorbances in both Soret range (400-450 nm) and visible range (500-600 nm). In particular, the specific absorbances are obtained for oxyHb/Mb at 413-414 nm, 576-578 nm and 540-542 nm; for carboxyHb/Mb at 418-420 nm, 568-572 nm and 538-540 nm; and for deoxyHb/Mb at 430-431 nm, 555-556 nm (Tietz and Fiereck 1973; Smulevich and others 2007; Klendshoj and others 1950; Kristinsson and others 2006). The

Hb/Mb extract sample is then converted into a two-pigment system, usually COHb/Mb and deoxyHb/Mb, by addition of a reducing agent such as sodium dithionite and analyzed by measuring the absorbances at the two different wavelengths selected. The most important issue for this method is the selection of two wavelengths, which will be used to obtain the absorbances and further calculated to gain CO saturation of heme proteins. The wavelength, either in the Soret range (Beutler and West 1984; Rodkey and others 1979; Smulevich and others 2007), or in the visible range (Stonek and others 2004; Tietz and Fiereck 1973; Katsumata and others 1982; Wolfe and others 1978; Klendshoj and others 1949), or at the isosbestic point (Katsumata and others 1982) has been selected for measuring the proportion of COHb/Mb.

A two-wavelength spectrophotometric method for the percentage of COHb/Mb in fish muscle was developed in Chapter 4. Based on that, the following equation,

$$COHb\% = \frac{[1-(A_r \times E_1)]\times 100}{A_r \times (E_2 - E_1) - E_3 + 1}$$

will be used to calculate COHb%. In this study, both 100% oxyHb (0% COHb) and 100% COHb were scanned between 350-700 nm after addition of sodium dithionite. The specific absorbances of COHb/Mb at 419 and 430 nm, and the specific absorbances of deoxyHb/Mb at 538, 556 and 568 nm and the absorbance of their isosbestic point at 425 nm were measured after samples were reduced. For every pair of wavelengths selected, the three constants, E_1, E_2 and E_3, were determined and shown in Table 5-3. COHb and OxyHb were mixed with various ratios and the mixture was analyzed in order to establish a relation between expected COHb% and detected COHb%. For every pair of wavelengths selected, there was an excellent linear correlation between the expected COHb% and detected COHb% ($r^2 > 0.99$), and even at low concentrations (Figure 5-5).

The absorbance of Hb and its derivatives in the Soret region (400-450 nm) is much more sensitive than that in the visible region (500-600 nm) (Small and others 1971). The absorbance of Hb and its derivatives in the range of 400-450 nm are about 10 times higher than that in the range of 500-600 nm. The absorbance in the visible region (500-600 nm) needs high Hb concentrations in order to obtain a satisfactory sensitivity, but high Hb concentration may cause turbidity and have a big influence on other heme protein derivatives or pigments. The readings in the Soret region (400-450 nm) require relatively low Hb concentration, which reduces Hb precipitation and turbidity. It has been discovered that there is a good linear correlation between expected COHb% and detected COHb% at selected wavelengths at 419 and 430 nm (Chapter 4). After investigating the effect of different pair of wavelengths on the efficiency of this two wavelength spectrophotometric method, it was found that the wavelengths not only in Soret range but also in visible range can be selected for determination of COHb% and both get high efficiency.

Spectrophotometry and GC are two main methods which can be used to determine CO in fish muscle. Since the spectrophotometric method is quick, simple and low cost, it could be a good screening tool to identify if the fish or meat products have been treated with CO and even determine the percentage of Hb saturated by CO, which indicates the CO status in the muscle system. This spectrophotometric method may be used to measure CO, even at very low concentrations, but the limit of detection (LOD) was 290 times higher and RSD between samples was about seven times larger than LOD and RSD respectively by the GC method (Sundin and Larsson 2002). But unfortunately it can not quantify the total CO in fish muscle. During our quantification of CO in the muscle by GC, it was observed that even though there are large amounts of CO in the muscle system, which are enough to saturate all the heme proteins there,

only part of CO was binding to heme proteins while a certain amount CO was dissolved in the muscle (data not shown). This amount of residue CO dissolved in the muscle is significant and should be counted when we try to determine if foods have been treated with CO. In contrast, as an analytical method, GC can detect the total CO amount in the muscle with higher accuracy and lower detection limit compared with spectrophotometry. But GC can not provide any information about CO status in the muscle. Therefore, for the analysis of CO in muscle, we can obtain a sound understanding of both bound and unbound CO in the muscle system only if we combine these two analytical methods together. These two methods are all important, and they are complementary to each other and can not be replaced by each other.

Conclusion

Our improved GC method can be used to determine CO in fish muscle effectively. It was found that a CO standard curve by gas chromatography showed excellent linearity ($r^2 = 0.9985$). CO recovery from the mixture of fish muscle and buffer was 86.38-98.85%. There was a significant difference between CO liberated from various sample sources ($p<0.05$). Liberating CO directly from the mixture of fish flesh and buffer was more effective than releasing CO from Hb/Mb containing supernatant and the muscle homogenate. The extraction steps such as homogenization and centrifugation may cause CO loss, which may result in an erroneously low CO value. It was also determined there was a significant difference between various CO liberating methods ($p<0.05$). Sulfuric acid (5%) can release CO from the muscle more effectively compared to the others tested. In addition, it was concluded that both the Soret range and the visible range have similar sensitivity in COHb/COMb% determination even though the absorbance in the Soret range is around 10 times higher than that in the visible range. By using the wavelengths from both ranges, there is an excellent linear correlation ($r^2 \geq 0.992$) between the expected and the detected COHb/COMb%. Finally, it is important to combine both

spectrophotometric method and GC method together if we aim to determine both the total CO amount and the CO binding status in the muscle. This study will have direct implications for regulatory agencies interested in testing suspected seafood products and aid in the control of CO treated products.

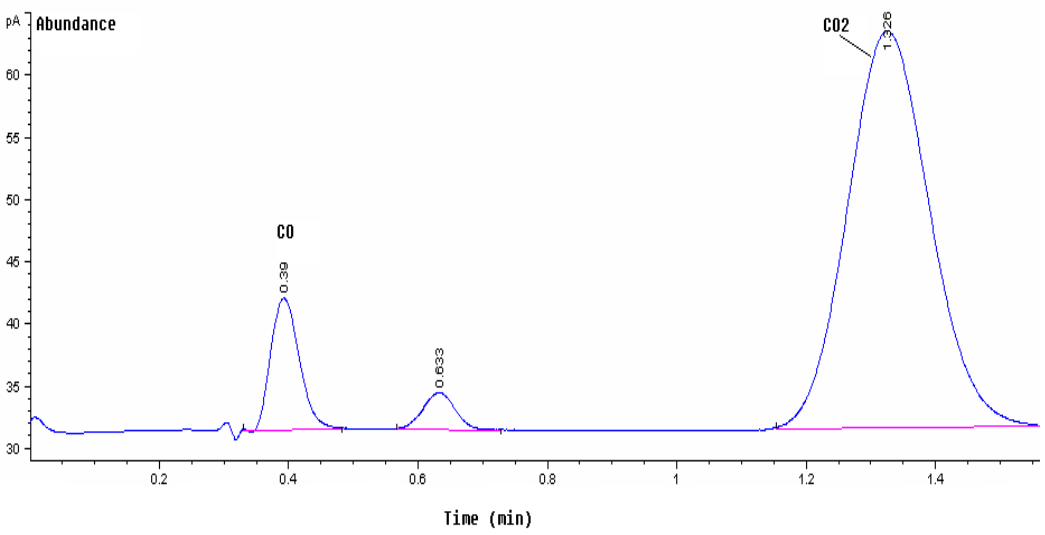


Figure 5-1. Gas chromatogram of headspace samples showing the CO peak and the CO_2 peak. CO was released from the WTM-Hb system to the headspace of the gastight vial, and the headspace gas was collected and injected into a GC equipped with a methanizer and FID detector.

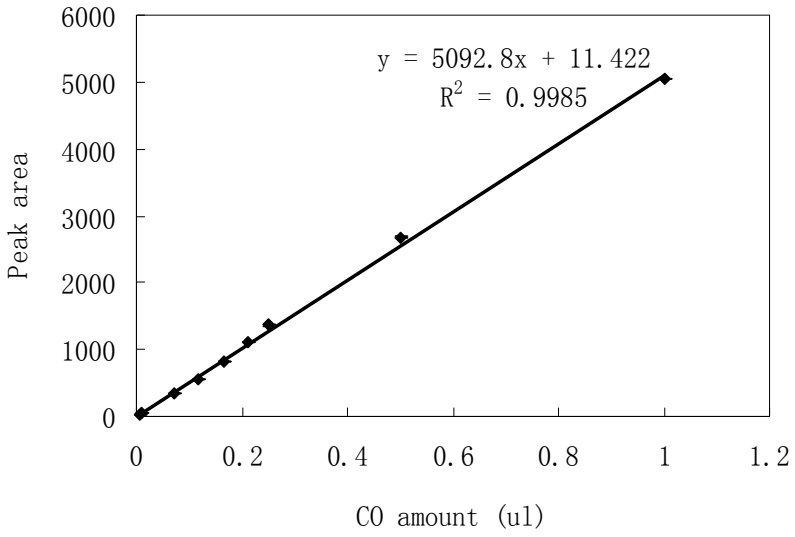


Figure 5-2. Standard curve of CO obtained by gas chromatography. Standard CO gas at different concentrations and a gas sampling loop were used for injection.

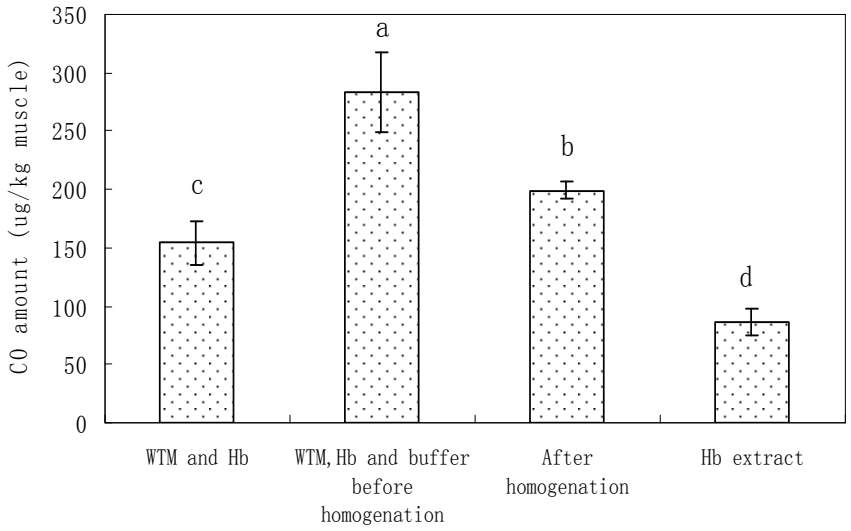


Figure 5-3. Carbon monoxide amount released from various sample sources. CO was released from the mixture of WTM and Hb, the mixture of WTM and Hb and buffer before homogenization and after homogenization, the Hb extract, respectively to the headspace of a closed system. The headspace gas was taken for GC analysis. Different small letters indicate a significant difference ($p<0.05$).

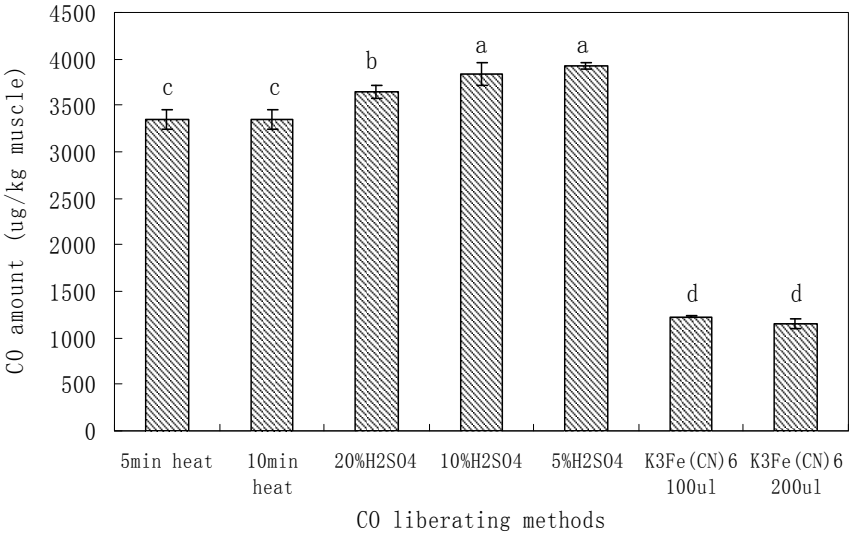


Figure 5-4. Comparison of various CO liberating methods. Different CO liberating methods were used to release CO from the treated muscle system followed by measuring the released CO by GC. Different small letters indicate a significant difference ($p<0.05$).

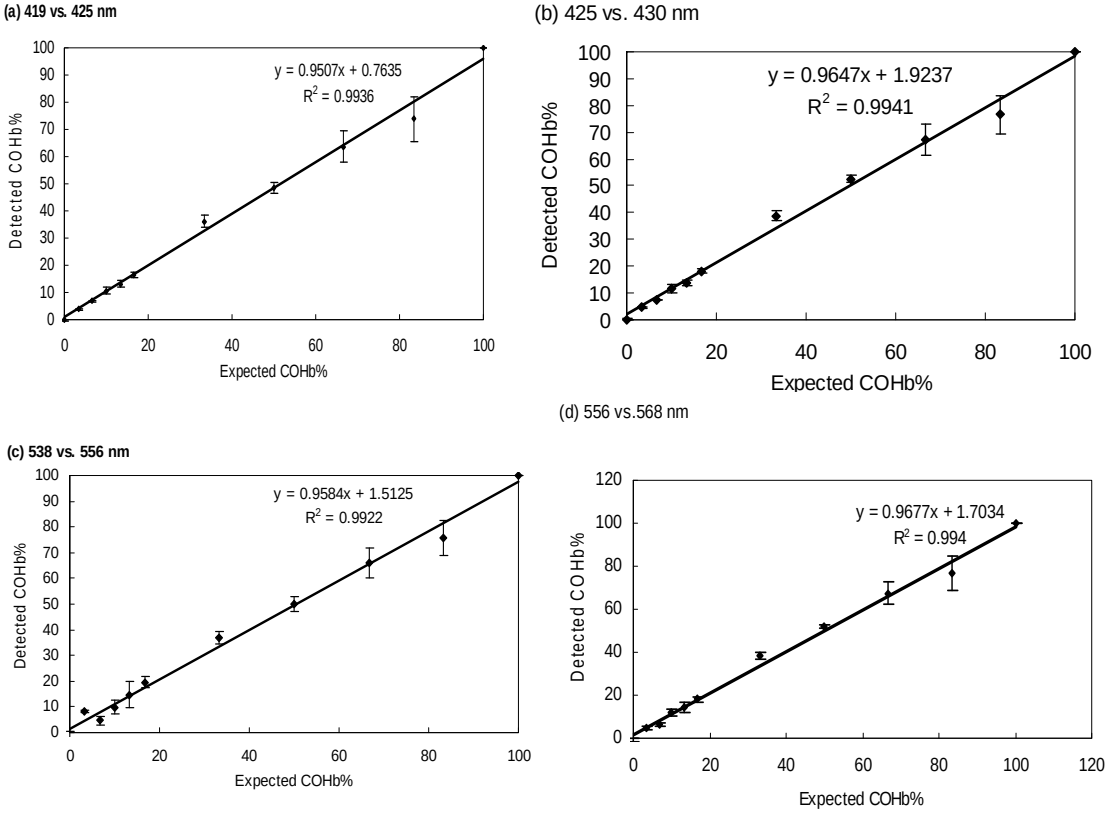


Figure 5-5. Comparison of expected COHb% and detected COHb% by the two-wavelength spectrophotometric method. OxyHb stock was diluted with 20 mM sodium phosphate butter (pH 6.5) to ~0.25 mM. COHb (~0.25 mM) was made by this oxyHb. OxyHb and COHb were mixed at various ratios, which are the expected COHb%, and then sodium dithionite was added to the mixtures, and the absorbances at 419, 425, 431, 538, 556, and 568 nm were read, which were used to calculate detected COHb%.

Table 5-1. Recovery of added CO gas in a mixture of 5 g WTM-Hb, 10 ml sodium phosphate buffer (20 mM) at pH 8.0, 10 ml 5% H_2SO_4, and 4 drops of octanol. Different small letters indicate a significant difference ($p<0.05$).

Added amount of CO	Recovery of CO (mean±SD) (%)
4 µl	92.75±12.51 a
12 µl	86.38±4.49 a
20 µl	94.63±1.54 a
40 µl	89.08±0.40 a
60 µl	98.85±0.92 a
80 µl	98.46±3.74 a

Table 5-2. Coefficient of variation (RSD) of various CO liberating methods. Different CO liberating methods were used to release CO from the treated muscle system and the released CO was measured by GC. The mean, SD and RSD are from triplicate samples.

CO liberating methods	CO amount mean (µg/kg)	SD	RSD(%) n=3
5 min heat	3346.97	108.40	3.24
10 min heat	3347.20	104.29	3.12
5% H_2SO_4	3923.83	29.71	0.76
10% H_2SO_4	3833.10	118.42	3.09
20% H_2SO_4	3642.53	66.87	1.84
$K_3Fe(CN)_6$ 100 µl	1229.59	8.00	0.65
$K_3Fe(CN)_6$ 200 µl	1145.06	53.13	4.64

Table 5-3. Constants, E_1, E_2 and E_3, based on the various two-wavelength selected.

Wavelength 1	Wavelength 2	E_1	E_2	E_3
419 nm	425 nm	1.18	1.18	1.74
425 nm	430 nm	1.06	0.55	1.00
538 nm	556 nm	1.32	1.23	1.39
556 nm	568 nm	0.90	1.04	0.93

CHAPTER 6
STUDY OF CARBON MONOXIDE PENETRATION AND LOCATION IN TREATED FISH MUSCLE

Introduction

Since flesh color is important for quality evaluation and there is a possibility of influencing flesh color though its heme proteins, CO or FS was introduced in the 1990's to treat fish muscle. This technology is based on the strong affinity of CO for heme proteins which may maintain the stable red color and avoid the occurring brown color.

One way of CO application is the use of CO as a component of packaging gases for meat or fish in reduced oxygen packaging (ROP) or modified atmosphere packaging (MAP). It was found that a gas mixture with CO provides a unique combination of a long microbiological shelf life and a stable, cherry red color of the meat (Sorheim and others 1997). These positive effects of CO were also supported by the other research results (Sorheim and others 1999; Jayasingh and others 2001; Kusmider and others 2002; Krause and others 2003; Viana and others 2005; Wilkinson and others 2006; Ferdandez-Lopez and others 2008). This CO amount (< 0.5%) in MAP of meats has been commercially used for retail meat packaging in Norway since 1985, and it was discontinued to use in July 2004 when Norway entered into the European Union, where CO is not approved as a gas component in packaging (European Parliament and Council Directive 1995). In 2002, FDA (Food and Drug Administration) accepted the use of CO in a MAP system at a level of 0.4% as GRAS (generally recognized as safe) (FDA 2002). FDA extended their permission for using CO in MAP (FDA 2004). Since MAP may lead to the discoloration of some fish species such as tuna, fish species are hardly stored under MAP.

The other way of CO application is to use CO as a single gas, or FS treatment on fish, specifically fish species with high amount of Hb/Mb such as tuna. Usually it takes two to three weeks for these fish species from harvest to their final market destination, during which their

safety and quality may have been greatly compromised, and these fish species are highly perishable. Therefore, FS or CO was introduced to treat these fish species due to the fact that CO can stabilize the red color during frozen storage. Until now, several patents have been issued about applying filtered smoke into fish or meat in order to maintain its red color (Woodruff and Silliker 1985; Yamaoka 1996; Kowalski 1999; Shaklai 2001). The objective of the filtered wood smoking processes is not to produce smoked fishery products as the traditional smoking processes do, but to extend or preserve the shelf life of fresh and frozen seafood products (Olson 2006). In filtered smoking processes, smoke mainly acts as not a flavoring agent but a preservation agent.

Treating fish muscle with CO or FS has been reported to improve its quality. CO or FS treated fish muscle has a prolonged and increased red color compared to untreated controls (Kristinsson and others 2003; Garner and Kristinsson 2004; Ludlow and others 2004; Balaban and others 2005; Kristinsson and others 2006b; Kristinsson and others 2007). CO or FS treatment may decrease the aerobic bacterial growth (Kristinsson and others 2003; Garner and Kristinsson 2004; Ludlow and others 2004; Leydon and others 2005; Kristinsson and others 2006a; Kristinsson and others 2007; Kristinsson and others 2008), and reduce lipid oxidation (kristinsson and others 2003; Garner and Kristinsson 2004; Demir and others 2004; Kristinsson and others 2007). Based on the studies of using CO as a single gas to treat fish species, it was concluded during frozen storage at -20°C for 6 months, metHb% and Hunter a* values for CO treated tuna steak remained almost constant, while MetHb% for untreated control increased gradually up to 60% and a* values decreased from 11 to 5 (Chow and others 1998). After 4 hrs CO treatment, Hunter a* value of tuna steaks increased and was higher than that of untreated control, and the metHb% in the surface layer (2 mm thickness from the surface) of the steak

showed no significant difference between with and without CO treatment while CO in the middle layer (2 to 4 mm from the surface) and inner layer (4 to 6 mm from the surface) was lower than CO in untreated control (Hsieh and others 1998).

Since the CO or FS treatment can provide a positive effect on quality and safety of fish species as well as CO packaging technology has been approved and several patents regarding FS application have also been issued by FDA, the technology of using CO or FS has gained considerable interest in the meat and fish industries worldwide. However, limited scientific information is available about the fate of CO in the muscle during CO treatment and storage of treated products.

Some muscle conditions, such as pH values, ionic strength and lipid content, may influence CO penetration into the muscle. Fish muscle may have different pH levels, depending on fish species, muscle type, harvesting method, time of storage and type of processing. Many fish hemoglobins are much more sensitive to a pH drop than their mammalian counterparts due to differences in their structure (Riggs 1970). The oxidized forms of hemoglobin are accelerated as pH is decreased, whereas hemoglobin is stable, specifically stabilized to oxidation, when pH is increased (Shikama 1998; Baron and Andersen 2002). Changing the oxidation status of hemoglobin is expected to have an impact on CO binding. Fish muscle has a physiological ionic strength around 150 mM. However, many processed fish will have an elevated ionic strength, which may influence heme proteins conformation and further influence their binding to CO. Lipid content is another muscle condition which may influence CO penetration in the muscle. There are two major types of muscle lipids, phospholipids and triacylglycerols. Phospholipid content is relatively constant in fish muscle, whereas triacylglycerol content is highly dependent on fish species and their environmental conditions and can vary significantly. The total lipid

content can vary from 0.5 to 30% in fish muscle (Ackman 1980). Heme proteins have been reported to act as a catalyst of lipid oxidation (Baron and Andersen 2002; Richards and Hultin 2002; Richards and others 2005; Grunwald and Richards 2006). On the other hand, a product of lipid oxidation has been shown to bind Mb, altering tertiary structure (Alderton and others 2003), which increases the accessibility of Mb oxidation. Due to the strong correlation between lipid oxidation and heme proteins, the presence of lipids may cause some configuration change of heme proteins, resulting in their CO binding capability.

In this study, we are interested in investigating the effect of various factors, such as muscle system conditions and varying CO treatments on the extent of penetration and the total absorption of the CO into the muscle. We are furthermore interested in researching the fate of CO once incorporated into the muscle system.

Materials and Methods

Chemicals and Supplies

1-octanol (98%) was purchased from Acros Organics (Pittsburgh, PA). Sulfuric acid was diluted from a 50% (v/v) solution (RICCA Chemical company, Arlington, TX). Carbon monoxide gas, 99.5%, was purchased from Airgas (Airgas, Gainesville, FL). Carbon monoxide, 10 and 100 ppm, was purchased from Agilent Technology (Wilmington, DE). Gastight vials with septum-fitted caps (25 ml), were purchased from Fisher Scientific (Fair Lawn, NJ). Standard gas sample loops for GC injection was purchased from Agilent Technology (Wilmington, DE).

Preparation of OxyHb and MetHb

Tilapia was obtained locally from a tilapia aquaculture farm. It was placed on ice (pectoral side facing up) for 1 min and blood was drawn though the caudal vein. OxyHb was isolated and purified from the red blood cells according to Richards and Hultin (2000). Purity of oxyHb was verified by SDS-PAGE, and the results showed that the samples were more than 99% Hb.

Samples were stored in 500 µl aliquots at -80°C and thawed under 20°C running water before they were used. The ferric MetHb was made by adding 200ul Hb stock solution to 1.8 ml buffer (pH 6.5) with 2 mM sodium phosphate (dibasic) and 1mM sodium citrate, and then adding 0.43 mg potassium ferricyanide. The mixture was inverted a few times, followed by centrifugal filtration to remove the potassium ferricyanide. Then more buffer (pH 6.5) was used to rinse if necessary until all the potassium ferricyanide was washed out. Hb was kept on ice during all experiments. Hb levels were determined using the Bradford method (Chang 1998). The Coomassie plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL) was used with bovine Hb as the standard. To ensure the stability of Hb, all preparations were performed on ice.

Preparation of Ground Tilapia Muscle (GTM)

Fresh tilapia fillets were obtained from a local store. All dark muscle and blood spots were removed and the remaining white muscle was ground. Then the ground muscle was vacuum packed, stored at -80°C, and thawed overnight at 4 °C until needed. The final pH and moisture content of the samples was pH 6.4 and ~78% respectively.

Preparation of Ground Tilapia Muscle and Hb (GTM-Hb) Model System

The model system was prepared in the 100 ml plastic beaker by using ground tilapia muscle and oxyHb stock solution (around 25.15 mM Hb). An appropriate volume of the oxyHb stock solution was added to 50 g ground muscle in a 100 ml plastic beaker so that the final concentration was 5, 50 and 100 µmol Hb/kg muscle respectively. Samples were stirred with a glass stir bar for 3 min to distribute Hb in the muscle. Then they were flattened out in the bottom of the 100 ml plastic beaker (bottom diameter around 5.4 cm) giving rise to a sample thickness of around 2 cm. The pH value was measured using a pH meter (Model 220, Denver Instrument, Denver, CO) equipped with an Electrode (Accumet, Vernon Hills, IL) by manually stirring 1 part of sample with 9 parts of deionized water. Adjusting pH to 5.5 or 7.2 was carried out by

dropwise addition of 1 M HCl or 1 M NaOH into GTM followed by severe manual stirring with a stainless steel spatula. In order to ensure the acid or base had been evenly distributed into the GTM, the pH was measured in several samples taken at different locations of the GTM. Lipid content (10% w/w) was adjusted by adding fish oil (Nature Made, Mission Hills, CA) into GTM followed by stirring for around 2 min by using a stainless steel spatula. Ionic strength was adjusted by adding appropriate amount of KCl to GTM followed by stirring for 2 min to a final ionic strength of 150, 300 and 450 mM (water base). Hb oxidation status was adjusted by adding the corresponding amount of metHb into GTM instead of oxyHb. In controls, Hb was replaced by deionized water.

CO Treatment

The model systems were placed in a gastight bag, which was vacuumed, flushed with CO (100% or 20%) and stored at 4°C for up to 24 hr. After 15 min, 3 hr, 6 hr, 9 hr, 12 hr, and 24 hr of CO treatment, respectively, samples were taken for both spectrophotometric and GC analysis.

CO Standard Curve by GC and CO Mass Calculation

A CO calibration curve was established by injecting CO with various standard gas sample loops, 250, 500, and 1000 ml, and high pressured gas mixture with 10 and 100 ppm CO. CO was introduced directly from its cylinder into the gas sample loop, then into the column with the function of the gas sample valve. The actual CO mass was estimated by the ideal gas law equation: $PV=nRT$, where P (atm) is the barometric pressure, V (L) is the volume of CO gas, n (mol) is the number of moles of CO, R (0.082 $L \cdot atm \cdot K^{-1} \cdot mol^{-1}$) is the universal gas constant, and T is the temperature in Kelvin.

Sample Analysis by GC

After CO treatment, the center part of GTM-Hb was cut by using a meat cutter (1.8 cm diameter). Then for the cut center part, a sharp knife was used to cut 5 layers from the surface,

and each layer was 2 mm thick (0.5 g). Each layer was placed in a 25 ml vial (Fisher Scientific, Fair Lawn, NJ). After addition of 3 ml 20 mM sodium phosphate buffer at pH 8.5 (4°C), 2 drops of 1-octanol, and 5 ml 5% H_2SO_4, the vial was quickly sealed with a fluorosilicon septum and heavily shaken for 1 min, and then left to stand at room temperature for 10 min. Then 200 µl of headspace gas was taken for GC injection. All these experiments were duplicated and performed on ice.

GC Conditions for CO Analysis

An Agilent 6890N GC (Agilent Technologies, Palo Alto, CA) was used equipped with a Porapak Q column, 80/100 mesh, 6 FT x 1/8 IN (Supelco, Bellefonte, PA), a nickel catalyst (Agilent Technologies, Palo Alto, CA). A flame ionization detector (FID) (Agilent Technologies, Palo Alto, CA) was used for analysis. The samples were manually injected using a gastight syringe. The oven temperature was set at 35°C for 2 min. Nitrogen was used as a carrier gas and held at a constant flow of 30 ml/min. The injector temperature, nickel catalyst temperature and FID temperature were held at 100°C, 375°C and 200°C respectively.

Sample Analysis by Spectrophotometric Method

After CO treatment, the center part of the sample was cut by using a meat cutter (1.8 cm diameter). Then for the cut center part, a sharp knife was used to cut 5 layers from the surface, and each layer is 2 mm thickness (0.5 g). Each layer was added to 2 ml of sodium phosphate buffer at pH 8.5 (4°C), homogenized for 10 seconds using an homogenizer (Biospec Products Inc., Bartlesville, OK) and centrifuged at 4,000 rpm for 10 min with a centrifuge 5702 (Eppendorf North America Inc., New York, NY). The supernatant was scanned between 350-700 nm with an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA) and then sodium dithionite was added to give a final concentration of ~2 mg/ml. After standing

at room temperature for 5 min, the absorbance of the supernatant was measured at 419 nm and 430 nm by a spectrophotometer as described above. All experiments were duplicated.

Effect of Storage on the Fate of CO in Fish Muscle

After 24 hrs CO treatment, samples were stored in bags of high oxygen permeability at 4°C for up to 7 days. A sample was taken every day for CO detection by GC. Samples with both Hb concentrations, 5 μmol Hb/kg muscle and 50 μmol Hb/kg muscle, were investigated in this part.

Results and Discussion

The use of GTM as a model system provides a matrix which has the structure of muscle such as intact myofibrillar proteins and membranes. Muscle pH values, ionic strength, lipid content, Hb oxidation status and Hb concentration were adjusted in order to simulate the physiological conditions of different fish species. Generally speaking, CO could get into the first layer (0-2 mm from surface) after only 15 min treatment, and as CO treatment time increased, CO amount in each layer of the muscle increased (Figures 6-1, 6-4, 6-7, 6-10, 6-13, 6-16). The CO residue in the lower layers usually increased much more quickly and was higher than that in the deeper layers. CO concentration in the gas mixture has an effect on CO penetration rate and CO residue in the muscle. CO penetrating into the muscle was much slower for the 20% CO treatment compared to the 100% CO treatment. For the 100% CO treatment, CO could penetrate into 10 mm, the center of GTM-Hb system, within 6 hrs, while for the 20% CO treatment CO could only penetrate into 4 mm within 6 hours, and it took 24 hrs to get into 10 mm (Figure 6-3). Also, CO residue in the muscle after the 20% CO treatment was much lower than that after 100% CO treatment. After 24 hours of 20% CO treatment, CO levels in the first layer (0-2 from surface) and the fifth layer (8-10 mm from surface) were 875 and 356 μg/kg muscle respectively,

which were significantly lower ($p<0.05$) than those after 100% CO treatment, which were 3898 and 3637μg/kg muscle respectively (Figure 6-2). After 24 hr treatment, the CO penetration depth in beef steaks was 3.9 mm in 5% CO MAP and 1.8 mm in 0.5% CO MAP (Jayasingh and others 2001). It was reported (Krause and others 2003) that in a 0.5 % CO environment, CO reached 10 mm, 15 mm, and 25 mm after 8 days, 27 days and 36 days treatment respectively.

Hb concentration may have a big effect on CO penetration into the muscle and CO residue in the muscle. Mb concentration is highly dependent on fish species and the muscle type. Mb content was 2.3% in dark muscle and 0.06% in ordinary muscle in yellowfin tuna (Kanoh and others 1986). The total heme proteins concentration in muscles from trout to mackerel dark muscle ranged from 6.1 to 504.6 μg/kg muscle (Richards and Hultin 2002). Also, Mb concentration is affected by age, sex, stress, nutrition and environment (Livingston and Brown 1981). CO amount was high in red-flesh fish and low in white-flesh fish (Ishiwata and others 1996). It was also observed that a higher CO level was found in fish flesh with higher Mb concentrations (Chow and others 1997). Four levels of Hb concentrations, 0, 5, 50 and 100 μmol/kg muscle, which simulated the heme protein concentration of different fish species, were investigated in this study. During 24 hrs of CO treatment, the CO amount in each layer of GTM with 100 μmol Hb/kg muscle was much higher than that in GTM with 5 and 50 μmol Hb/kg muscle (Figure 6-4). After 24 hrs CO treatment, there was a significant difference ($p<0.05$) of CO amount between the GTM with various Hb concentrations (Figure 6-5). The CO amount got up to 11948 μg/kg muscle in GTM with 100 μmol Hb/kg muscle (Figure 6-4d), which is higher than 3898 and 9419 μg /kg muscle in the GTM with 5 and 50 μmol Hb/kg muscle respectively (Figures 6-4b, 6-4c). CO penetrated into the GTM with 50 μmol Hb/kg muscle much more quickly than into the GTM with 5 μmol Hb/kg muscle. CO needed only three hours to reach 10

mm in GTM with 50 μmol Hb/kg muscle whereas it needed 6 hours to get into that depth in GTM with 5 μmol Hb/kg muscle (Figure 6-6). It was interestingly found that in the GTM with 100 μmol Hb/kg muscle, CO could easily penetrate into the first and second layer in a short time, but it was difficult for it to get into deeper layers. It took CO 9 hours and 12 hours to get into the fourth and the fifth layer of the GTM with 100 μmol Hb/kg muscle, respectively (Figure 6-6). This is possibly because this high Hb concentration (100 μmol Hb/kg muscle) requires lots of CO to bind and dissolve in the upper layers of the muscle, which may decrease the CO penetration rate through the muscle. In the muscle system, Hb may act as a driving force for CO penetration. However, CO can penetrate and dissolve into the GTM without Hb. That result further demonstrated that after CO treatment, not all the CO in the muscle is bound to Hb/Mb and there is a significant amount of CO dissolved in the muscle tissue.

MetHb/Mb is an oxidation status of oxyHb/Mb. During refrigerated storage and specifically frozen storage, the reduced oxyHb/Mb and deoxyHb/Mb in fish muscle will be oxidized to metHb/Mb, which gives muscle an undesirable brown color. Several factors may promote the formation of this brown color, such as reduced pH value, increased temperature as well as freezing and thawing. CO can bind to reduced Hb/Mb and form the stable COHb/Mb, which can prevent Hb/Mb from oxidation. However, CO can not bind to the oxidized metHb/Mb (Kristinsson and others 2006b). A high concentration of CO was observed in tilapia samples in which the blood-colored parts of the fillet were bright red while the CO concentration was low in the sample in which the blood-colored parts were dark brown (Ishiwata and others 1996). However, it was reported that the use of high CO concentration, such as 100% CO, can either impart red color to frozen/thawed tuna or restore the red color of lower grade brown tuna to higher grade (Balaban and others 2006). This kind of color “enhancement” also applied to mahi

mahi, and a week-old mahimahi could be made to look like or even better than fresh after 100% CO treatment (Balaban and others 2006). CO binding to Hb/Mb in the muscle system may be different from its normal binding to the Hb/Mb molecule. An active reducing system may exist in the muscle and it can reduce the oxidized metHb/Mb to reduced forms, which then are able to bind CO (Lanier and others 1978). In order to simulate CO penetration into aged fish muscle, an appropriate amount of metHb (versus oxyHb) was added into the GTM with a final concentration of 5 μmol Hb/kg muscle and followed by 100% CO treatment. Compared to GTM with oxyHb, CO penetrated much slower in GTM with metHb and it reached 4 and 10 mm after 3 and 9 hours treatment respectively (Figure 6-9). After 24 hrs CO treatment, there was no significant difference ($p>0.05$) between the CO amount in the first layer of GTM-metHb and GTM-oxyHb, whereas there was a significant higher ($p<0.05$) of CO amount in the deeper layers of GTM-oxyHb compared to GTM-metHb (Figure 6-8). High concentration of CO may promote the conformational change of the area around the heme. During CO treatment, metHb in the first layer of GTM were exposed to 100% CO and may be converted to the reduced form by this CO induced metHb reduction activity, which would be able to bind CO. However, metHb in the deeper layers of GTM could not be reduced by CO due to the lack of CO there. MetHb reduction under a pure CO environment was very rapid, and this reduction activity appeared to peak over the pH range 6.2 -6.6 (Lanier and others 1978).

The post-mortem pH of fish species may range from 5.5 for some red muscle fish to 7.0 or even higher for white muscle fish (Daniels and others 1986; Shimizu and others 1992). Lowering pH from 7.2 to 6.0 reduced the oxygenation of Hb and enhanced the autoxidation rate of Hb (Richards and Hultin 2000; Undeland and others 2002; Undeland and others 2004). Lowering pH decreased CO penetration rate into the muscle, and it took CO 12 hours to penetrate into 10 mm

depth in the muscle with pH 5.5, which is much longer compared to 6 hours and 3 hours in the muscle with pH 6.4 and 7.2 respectively (Figure 6-12). After 24 hrs CO treatment, CO amount in the muscle with pH 5.5 is significantly lower ($p<0.05$) than that with pH 6.4 and 7.2 (Figure 6-11). CO levels can only reach 2763 μg/kg for pH 5.5 muscle while they were 4216 and 4334 μg/kg for pH 6.4 and 7.2, respectively (Figure 6-10). This may be explained by the formation of COHb that occurred more readily at higher pH. Raising the pH suggested an increase in CO penetration rate (Figure 6-12), which may because that muscle proteins would become increasingly ionized and separated from each other when pH increased, and that may help CO to get into the muscle system. Also, lowing pH promotes Hb to be oxidized to metHb, which may influence CO binding and penetration in the muscle.

We investigated the effect of lipid content on the CO penetration rate and CO residue in the muscle, and found that there was a significantly ($p<0.05$) higher amount of CO in the first and second layer of GTM with lipid, while in the other layers of GTM with lipid, CO amount was significantly ($p<0.05$) lower (Figure 6-14). These results indicated that CO dissolves easier in the GTM with lipid compared to GTM without lipid. However CO does not penetrate easily in the GTM with lipid, which results in a relatively low amount of CO in the deep layers of GTM with lipid. Ionic strength is another factor which may influence CO penetration into muscle. The presence of salt has a certain effect on CO uptake into the muscle. After 24 hrs CO treatment, CO amount can get up to 6092 and 6804 μg/kg in the muscle with 150 and 300 mM KCl, respectively, which was significantly ($p<0.05$) higher than 4216 and 4352μg/kg in the muscle with 0 and 450 mM KCL, respectively (Figures 6-16, 6-17). CO may be more easily dissolved or the complex COHb may be more easily formed in muscle with ionic strength of 150 and 300 mM compared to 0 and 450 mM. In this study, our aim was to investigate the effect of various

ionic strengths on the Hb conformation, and further on Hb binding with CO. Hb concentration used here was relatively small, which may be one reason that the differences in CO amount and penetration rate between GTM at various ionic strengths are relatively small in this study (Figures 6-17, 6-18)..

After 24 hrs CO treatment, samples were subjected to 2 or 7 days refrigerated storage in the oxygen permeable bags. CO amount decreased rapidly at the first day of storage, and then it decreased slowly or remained constant (Figure 6-19). In the first layer of GTM with 5 μmol Hb/kg muscle, CO amount decreased from 3898 to 43 μg/kg after one day storage, and it was undetectable after two days storage (Figure 6-19a). In the first layer of GM with 50 μmol Hb/kg muscle, CO amount reduced from 9419 to 849 μg/kg during the first day of refrigerated storage, and after that CO remained almost constant in the muscle. 980 μg/kg of CO could still be detected even after 7 days storage (Figure 6-19b). Even though CO has a strong affinity for heme proteins, it is being released from heme proteins during storage (Chow and others 1998). Oxygen, at relatively high concentrations, will accelerate dissociation of CO from heme proteins. The CO amount in the CO treated tuna steaks decreased drastically during the first day of iced storage and became undectectable in both the middle layer (2-4 mm from surface) and inner layer (4-6 mm from surface) after 7 days of storage (Chow and others 1998). The fate of CO in ground beef was investigated by using ^{14}C-labeled CO, and it was observed that ^{14}CO escaped from beef to the environment during storage (Watts and others 1978). CO amount in the lower layer was reduced more quickly than that in the deeper layer (Figure 6-19). CO release from the muscle surface to the environment is quicker than CO moving from deeper layer to lower layer in the muscle. Therefore, CO content was usually higher in the deeper layer than in the lower layer during the storage. The CO amount in the homogenate from tilapia treated for one-hour

decreased to less than 50 μg/kg muscle during 3 days of refrigerator storage (Ishiwata and others 1996). CO amount in a homogenate from tuna treated for one-hour decreased to less than 300 μg/kg muscle during 7 days of refrigerator storage (Ishiwata and others 1996). Chow and others (1998) also monitored the CO amount of CO treated tuna steaks during six-months of frozen storage. It was found that the CO amount in the surface layer (0-2 mm from surface) and middle layer (2-4 mm from surface) decreased during the early stage of six-month frozen storage followed by no apparent change; there was no apparent variation in CO amount in the inner layer (4-6 mm from surface) of tuna steaks during the frozen storage (Chow and others 1998), which indicated that there is a certain amount of CO still staying in the center of the muscle. That possibly can explain the reason that CO can protect muscle from browning during frozen storage. Even though CO residue decreased dramatically, the samples can still show desirable red color after 7 days of refrigerator storage, which was also supported by Chow and others (1998). However, from the sixth day of storage, the muscle smelled as spoiled. All of the frozen, commercially CO treated tuna samples were found to contain near or above 1000 μg/kg CO (Anderson and Wu 2005).

CO penetrating into the muscle and binding to heme proteins is a complex process. The binding status of CO with heme proteins in a muscle is reversible and may not be as strong as the binding of CO and heme proteins in an aqueous solution (Chow and others 1998; Chow and others 1997). In a muscle system, CO binding is reversible and there is an equilibrium existing between CO dissolution, binding and release. During refrigerated storage, CO release from the muscle may skew the balance between CO dissolution, binding and release, which is followed by CO being released from Hb/Mb and dissolving in the muscle.

One-wavelength and two-wavelength spectrophotometric methods have been developed recently to detect COHb (Chapters 3, 4). In this study, these spectrophotometric methods were also used to investigate CO penetration into the muscle. Any heme protein extraction which exhibits a maximum peak wavelength at 415 nm or above can be determined as containing CO (Chapter 3). For GTM with 5 μmol Hb/kg muscle, based on the results of one-wavelength spectrophotometric method (Table 6-1), CO penetrated into first layer after 15 min of CO treatment, into the fourth layer after 3 hours and fifth layer after 6 hours, which matched previous gas chromatography results. For GTM with 50 μmol Hb/kg muscle, CO can penetrate into the fifth layer after 9 hours of treatment (Table 6-2), which is longer than 3 hours according to previous gas chromatography results. The ratio of absorbance at 419 to 430 nm can be used to calculate COHb% (Chapter 4). Figure 6-20 shows the COHb%, in GTM with 5 and 50 μmol Hb/kg muscle respectively, as a function of CO treatment time. It was found that COHb% increased as treatment time increased, and COHb% in the lower layers rose more rapidly than that in the deeper layers. Higher Hb concentration may result in higher Hb saturation with CO after CO treatment. COHb% can get up to around 50% for 5 μmol Hb/kg muscle while around 80% for 50 μmol Hb/kg muscle (Figure 6-20). Hb is difficult to be fully saturated with CO even after 24 hours treatment with 100% CO. It is worth to point out that a strong characteristic absorption of COHb at 418-419 nm (Tables 6-1, 6-2) does not indicate that Hb is fully bound to CO, as there is still certain percentage of Hb not binding to CO (Figure 6-20).

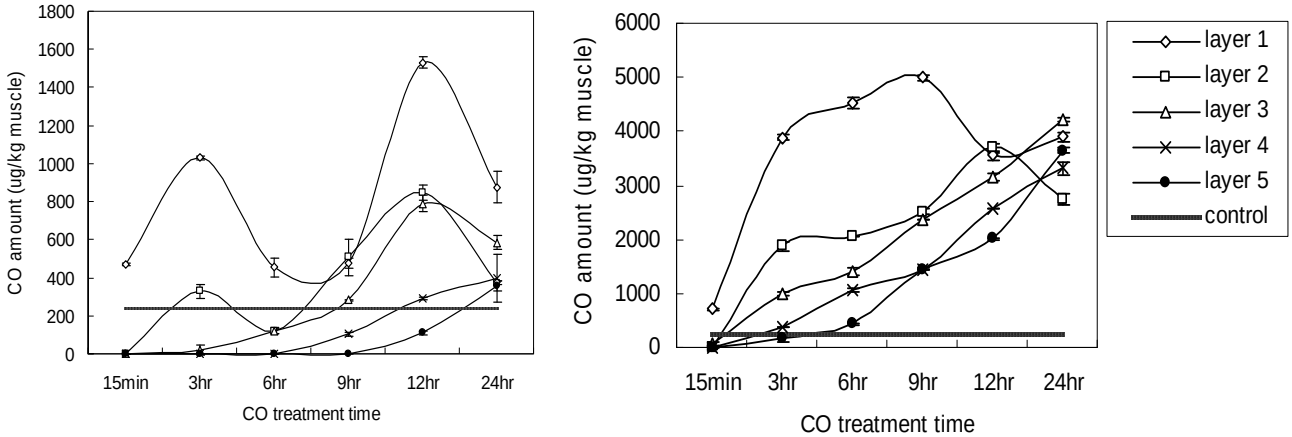
Chow and others (1997) also investigated the COMb in Mb extract after yellowfin tuna steak was subjected to 30 min CO treatment, based on the specific absorption spectra of COMb in the visible range. Their results show no characteristic absorption peak of COMb. After 30 min CO treatment, CO could only reach the surface of the steak and only Mb in the surface layer of

the steak could bind to CO to form COMb. According to our two-wavelength spectrophotometric results, only part of heme proteins were binding to CO. Using whole tuna steak to extract Mb, following homogenation and centrifugation may dilute the COMb and also cause CO release from Mb, which is possibly the reason why no COMb was detected. This emphasizes the importance of CO penetrating into the muscle gradually and why CO amount varies in different parts of the muscle.

Conclusion

Both internal factors and external factors will influence the CO penetration rate and CO amount in a muscle system. CO could get into the first layer (0-2 mm from surface) after only 15 min treatment, and as CO treatment time went on, CO amount in each layer of the muscle increased. CO residue in the lower layers normally increased much more quickly and was higher than that in the deeper layers. CO penetration into the muscle was much slower under 20% CO treatment compared to that under 100% CO treatment. CO residue in the muscle after 20% CO treatment was significantly ($p<0.05$) lower than that after 100% CO treatment. After 24hrs CO treatment, CO amount in the GTM with 50 and 100 μmol Hb/kg muscle was significantly ($p<0.05$) higher than that in the GTM with 5 μmol Hb/kg muscle or without Hb. CO penetrated into the GTM with 50 μmol Hb/kg muscle much faster than into the GTM with 5 μmol Hb/kg muscle. Compared to GTM with oxyHb, CO penetration rate was far lower in GTM with metHb, and after 24 hours treatment, there was no significant difference ($p>0.05$) between the CO amount in the first layer of GTM-metHb and GTM-oxyHb, whereas there was a significant higher ($p<0.05$) of CO amount in the other layers of GTM-oxyHb compared to GTM-metHb. Lowering pH decreased CO penetrating rate into the muscle, and it took CO 12 hours to get into 10 mm depth in the muscle with pH 5.5, which is much longer compared to 6 hours and 3 hours in the muscle with pH 6.4 and 7.2 respectively. After 24hrs CO treatment, CO amount in the

muscle at pH 5.5 was significantly ($p<0.05$) lower than muscle at pH 6.4 and 7.2. Regarding the effect of lipid content on CO amount in the muscle, there was a significantly ($p<0.05$) higher amount of CO in the first and second layer of GTM with lipid, while in the other layers of GTM with lipid, CO amount was significantly ($p<0.05$) lower. Ionic strength is another variable in muscle and muscle products. After 24 hrs CO treatment, CO amount in the muscle with 150 and 300 mM KCL was significantly ($p<0.05$) higher than that in the muscle with 0 and 450 mM KCL. There was no significant difference in CO penetration rate between various ionic strengths. During refrigerated storage of CO treated samples, CO amount decreased dramatically during the first day of storage, and it decreased slowly or remained constant during subsequent storage. For muscle with 50 μmol Hb/kg muscle, CO amount was as high as 1000 μg/kg even after 7 days of refrigerated storage. In addition, both one-wavelength and two-wavelength spectrophotometric methods were used to investigate CO penetration into the muscle, and the results almost matched our GC results. These results could be of importance for designing and processing CO or FS treatment, such as CO treatment time and CO concentration, in order to develop and maintain the red color as well as not cause safety problems.



(a) (b)

Figure 6-1. Carbon monoxide amount of each layer in the muscle after various CO treatments, (a) 20% CO, (b) 100% CO, as a function of CO treatment time. GTM-Hb system was treated with 20% or 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount by GC.

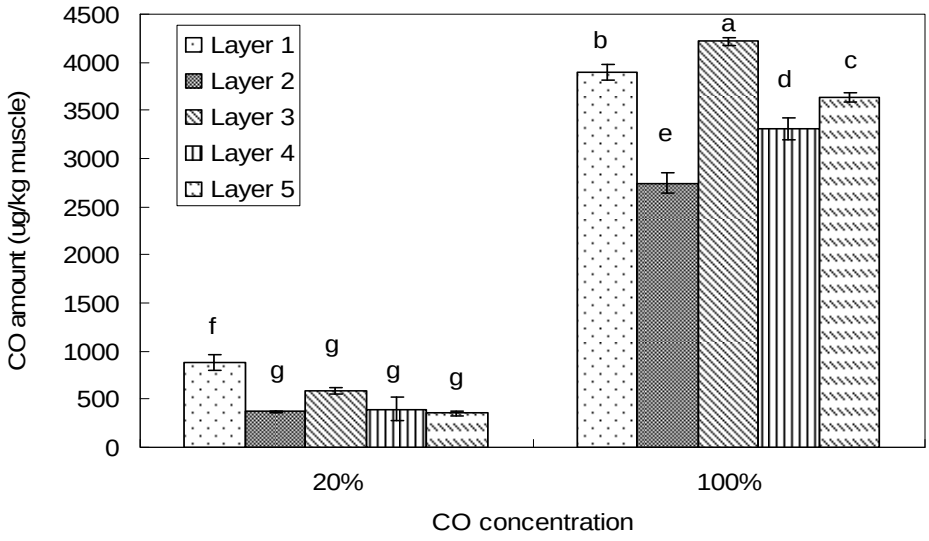


Figure 6-2. Carbon monoxide amount in the muscle after various CO treatments for 24 hrs. GTM-Hb system was treated with 20% or 100% CO for 24 hrs. Samples were taken and each layer was removed to measure CO amount by GC. Different small letters indicate a significant difference ($p<0.05$).

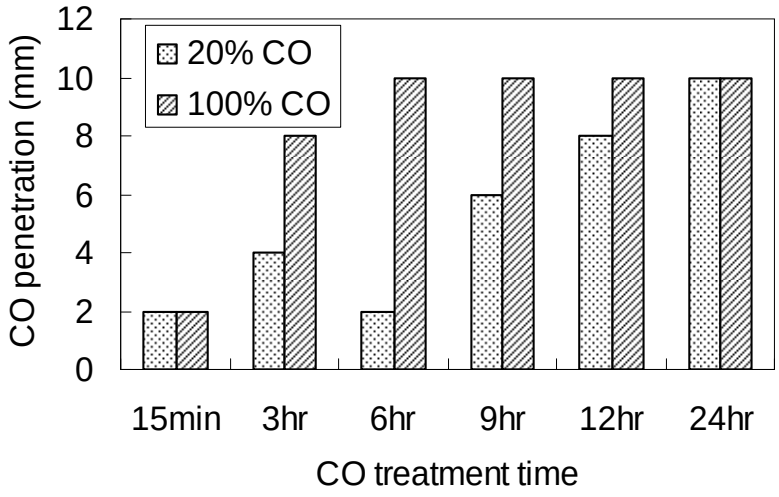


Figure 6-3. Effect of various CO concentrations on CO penetration rate into the muscle. GTM-Hb system was treated with 20% or 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. Layers where the CO amount was above the untreated controls, were considered to be penetrated by CO.

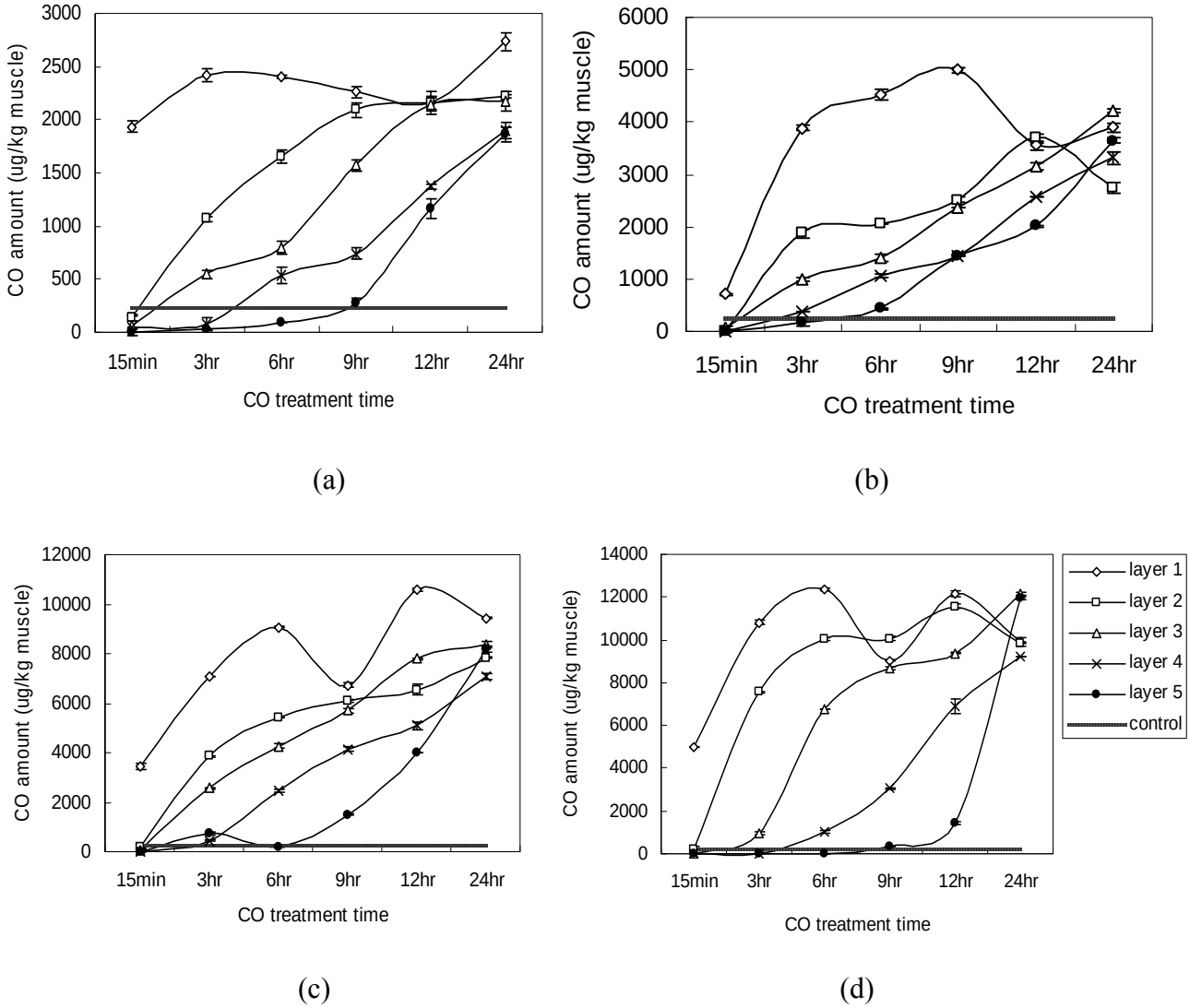


Figure 6-4. Carbon monoxide amount of each layer in the muscle with various Hb concentrations: (a) 0 μmol Hb/kg muscle, (b) 5 μmol Hb/kg muscle, (c) 50 μmol Hb/kg muscle (d) 100μmol Hb/kg muscle, as a function of CO treatment time. GTM-Hb system with various Hb concentrations was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount by GC.

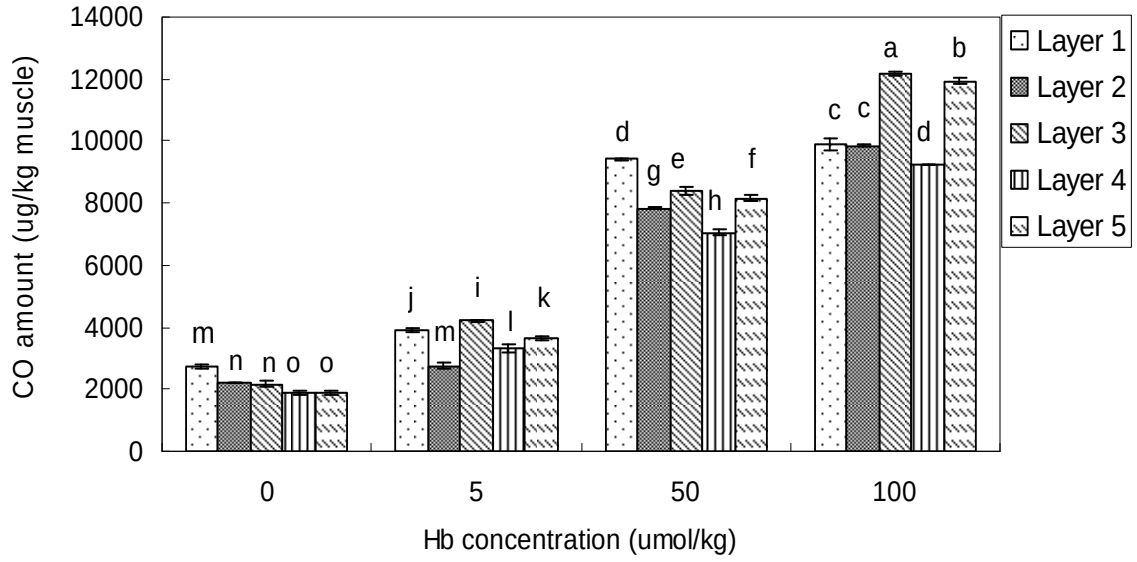


Figure 6-5. Carbon monoxide amount in the muscle with various Hb concentrations after 100% CO treatment for 24 hrs. GTM-Hb system with various Hb concentrations was treated with 100% CO for 24 hrs. Samples were taken and each layer was removed to measure CO amount by GC. Different small letter indicate a signicatant difference ($p<0.05$).

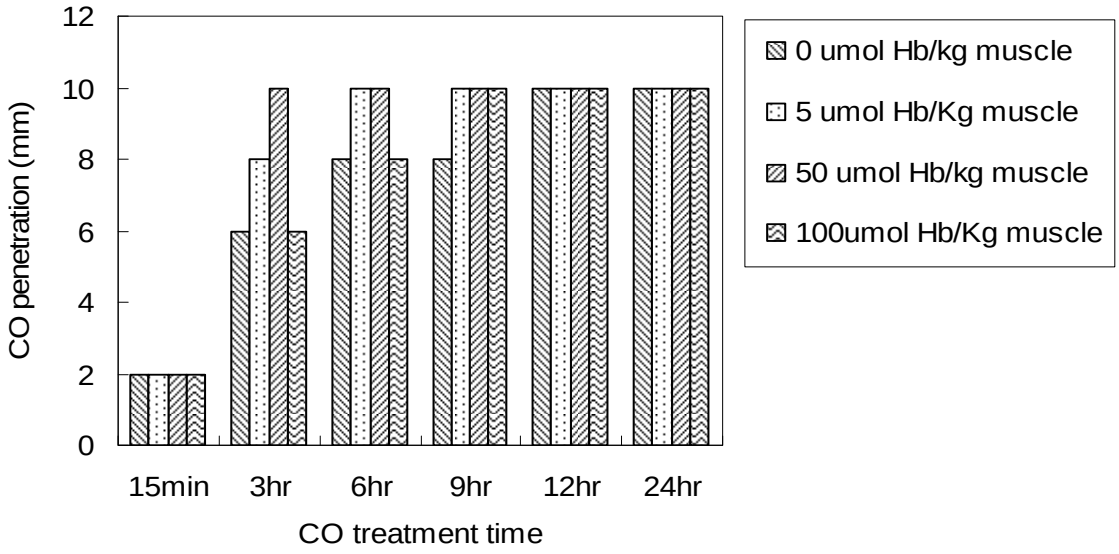


Figure 6-6. Effect of various Hb concentrations in the muscle on CO penetration rate. The muscle with various Hb concentrations was treated with 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. The layer, of which the CO amount was above the untreated controls, was considered to be penetrated by CO.

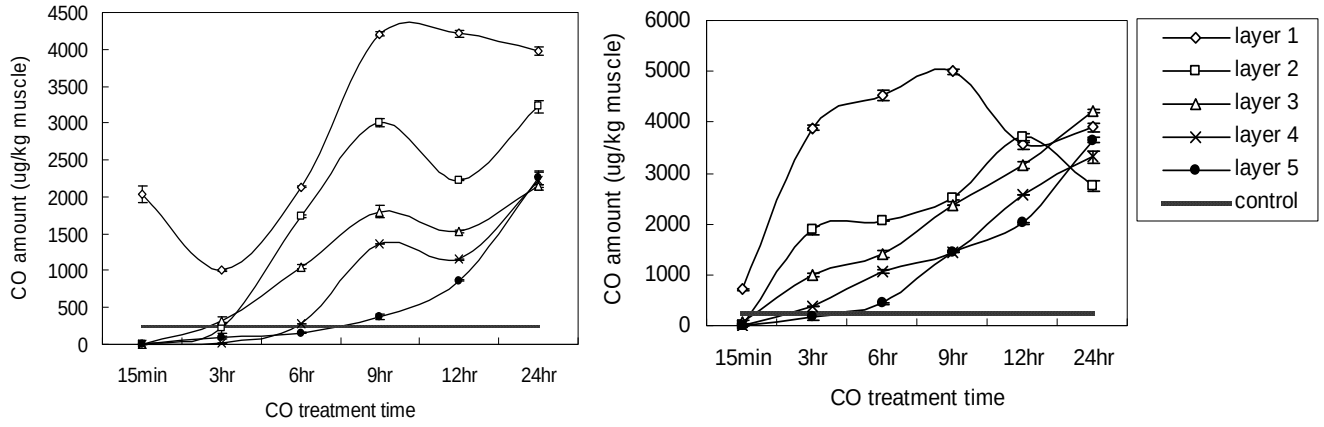


Figure 6-7. Carbon monoxide amount of each layer in the muscle with different Hb oxidation state, (a) metHb, (b) oxyHb, as a function of CO treatment time. The GTM-metHb or GTM-oxyHb system was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount by GC.

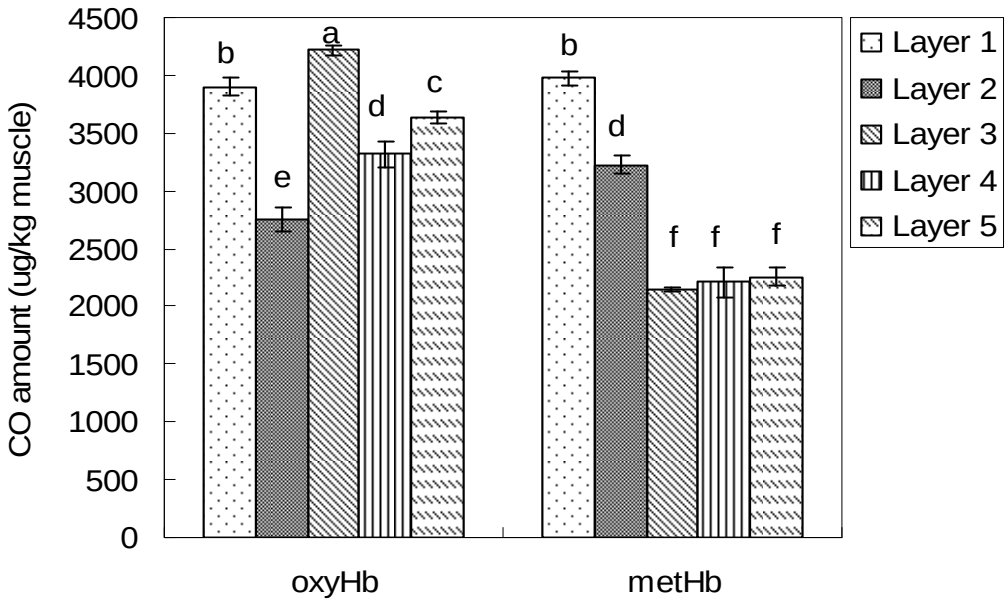


Figure 6-8. Carbon monoxide amount in the muscle with different Hb oxidation state after 100% CO treatment for 24 hrs. The GTM-metHb or GTM-oxyHb system was treated with 100% CO for 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO amount by GC. Different small letters indicate a significant difference ($p<0.05$).

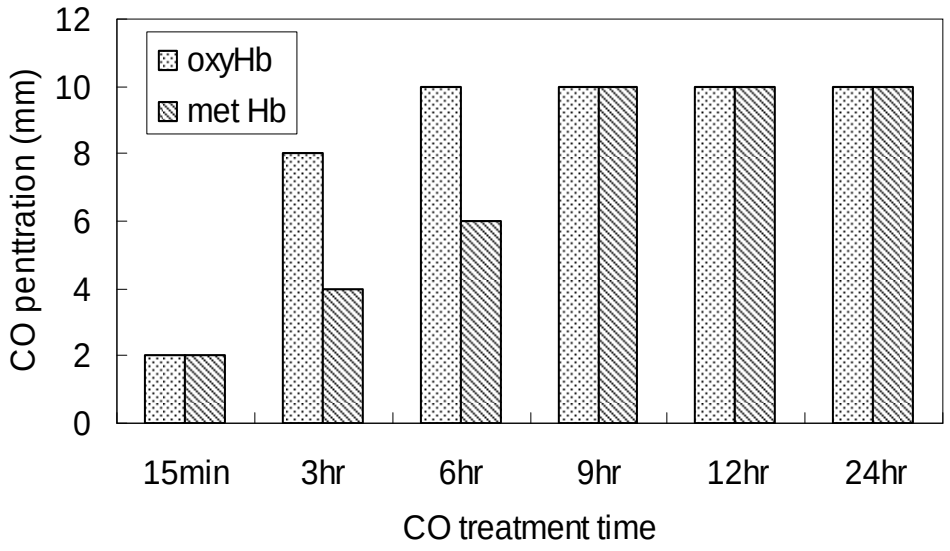


Figure 6-9. Effect of different Hb oxidation states in the muscle on CO penetration rate. GTM-metHb and GTM-oxyHb was treated with 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. Layers where the CO amount was above the untreated controls, were considered to be penetrated by CO.

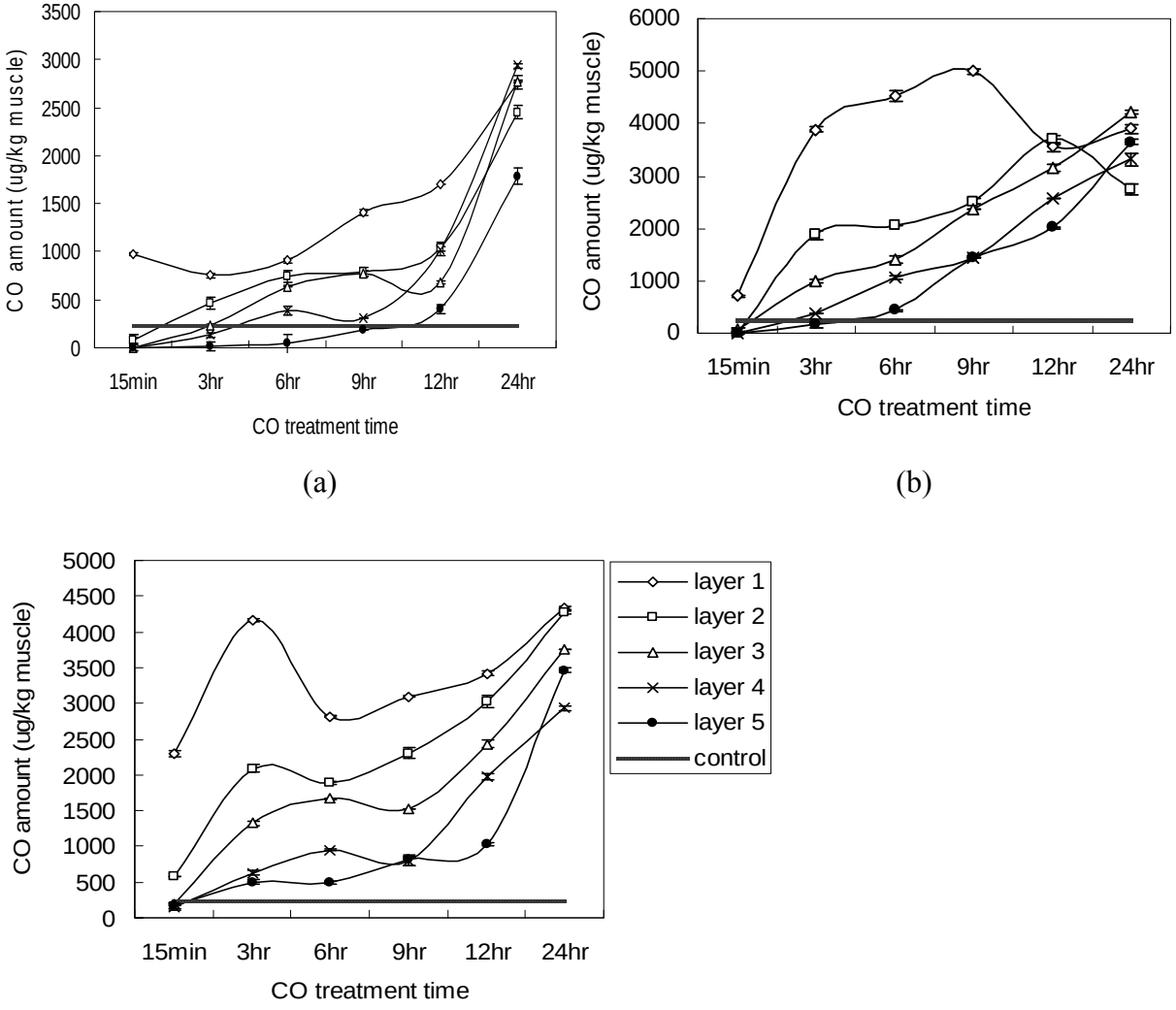


Figure 6-10. Carbon monoxide amount of each layer in the muscle with various pH values: (a) pH 5.5, (b) pH 6.4, (c) pH 7.2, as a function of CO treatment time. The GTM-Hb system with various pH values was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount by GC.

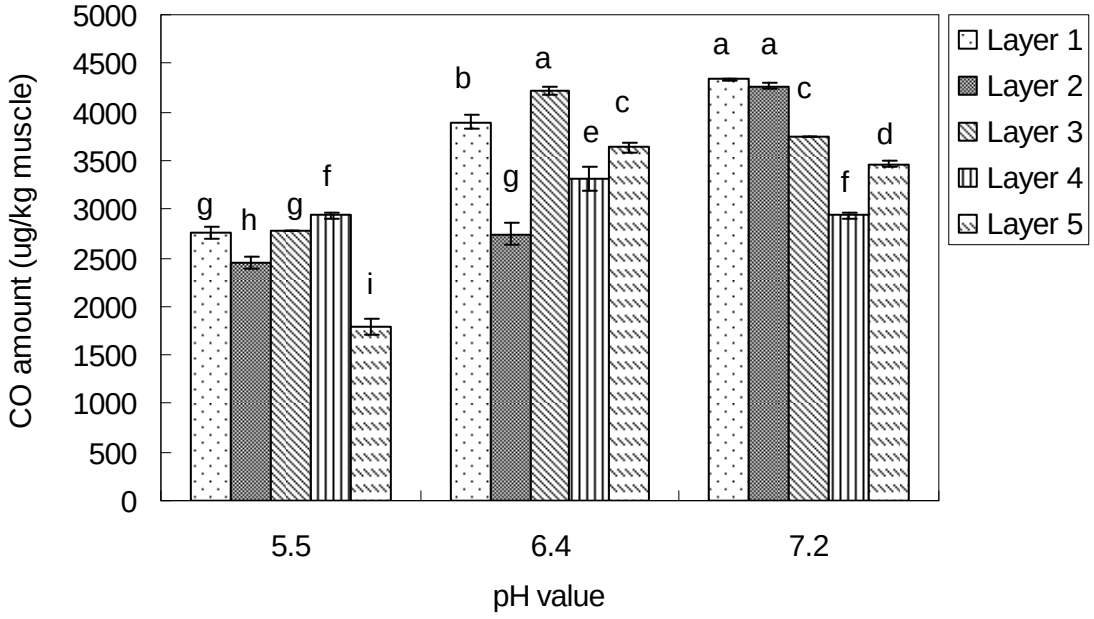


Figure 6-11. Carbon monoxide amount of in the muscle with various pH values after 100% CO treatment for 24 hrs. The GTM-Hb system with various pH values was treated with 100% CO for 24 hrs. Samples were taken and each layer was removed to measure CO amount by GC. Different small letters indicate a significant difference ($p<0.05$).

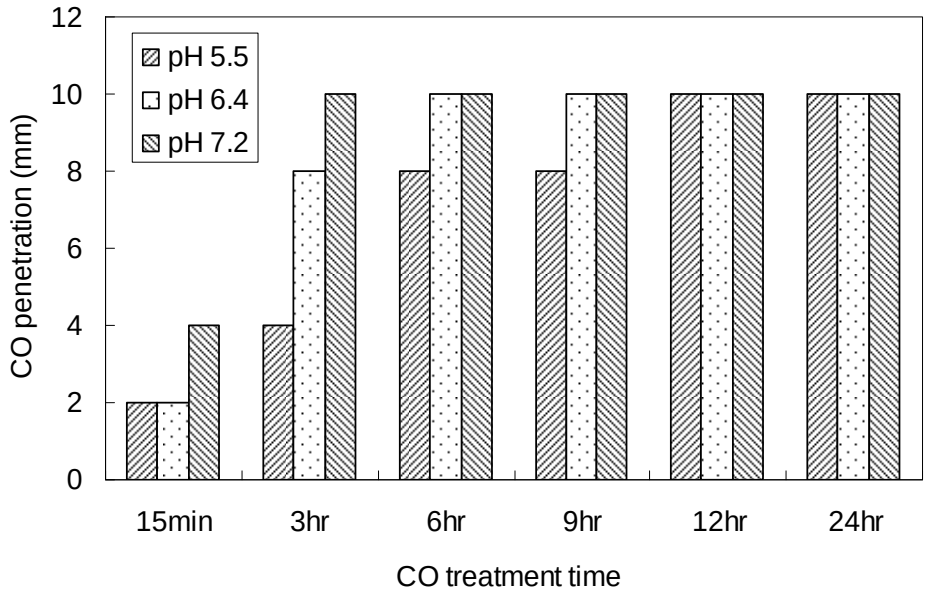
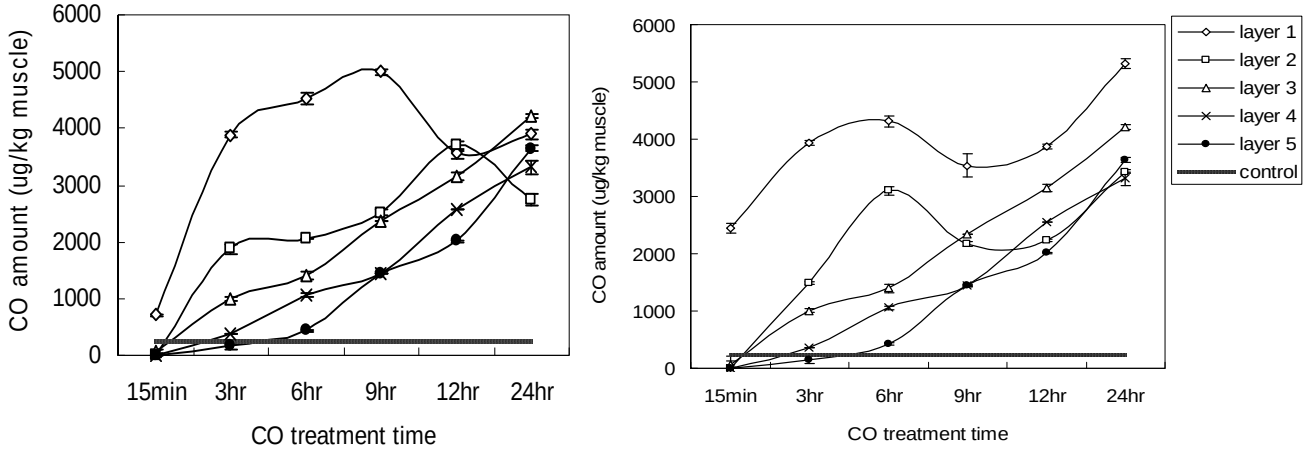


Figure 6-12. Effect of different pH values in the muscle on CO penetration rate. The GTM-Hb system at different pH values was treated with 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. Layers where the CO amount was above the untreated controls, were considered to be penetrated by CO.



(a) (b)

Figure 6-13. Carbon monoxide amount of each layer in the muscle with different lipid contents: (a) 0, (b) 10% lipid, as a function of CO treatment time. The GTM-Hb system with different lipid contents was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount by GC.

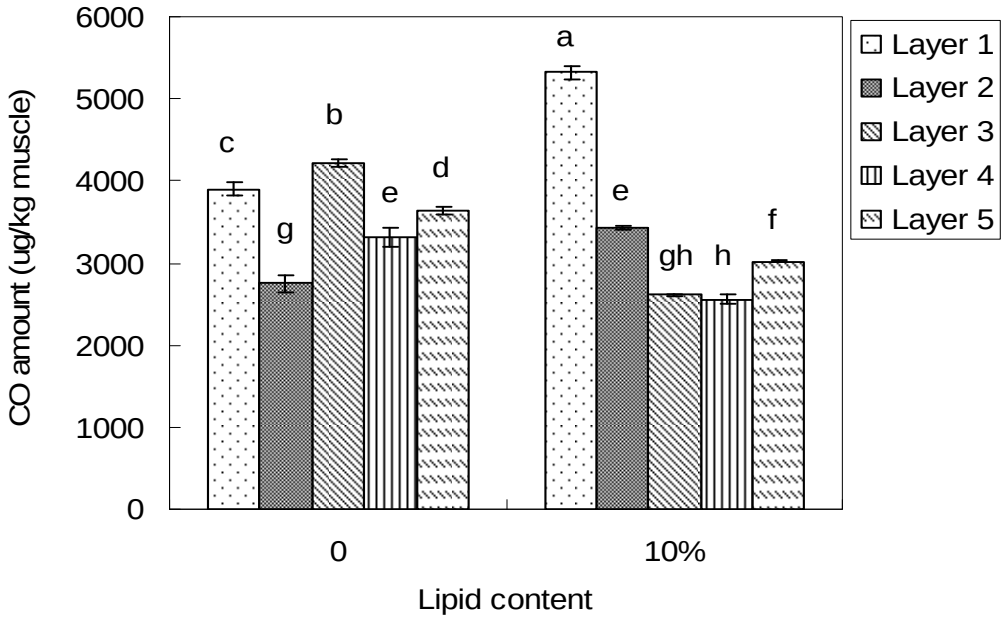


Figure 6-14. Carbon monoxide amount in the muscle with different lipid contents after 100% CO treatment for 24 hrs. The GTM-Hb system with different lipid contents was treated with 100% CO for 24 hrs. Samples were taken and each layer was removed to measure CO amount by GC. Different small letters indicate a significant difference ($p<0.05$).

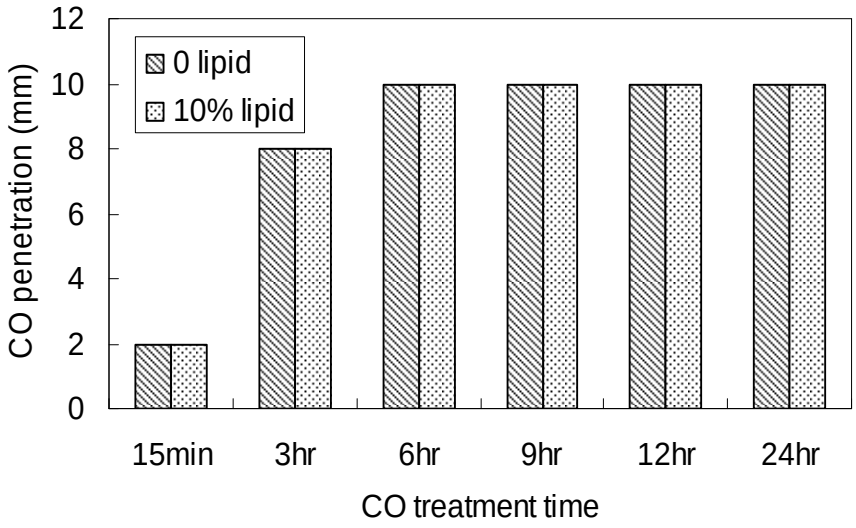


Figure 6-15. Effect of different lipid contents in the muscle on CO penetration rate. GTM-Hb system with different lipid contents was treated with 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. Layers where the CO amount was above the untreated controls, were considered to be penetrated by CO.

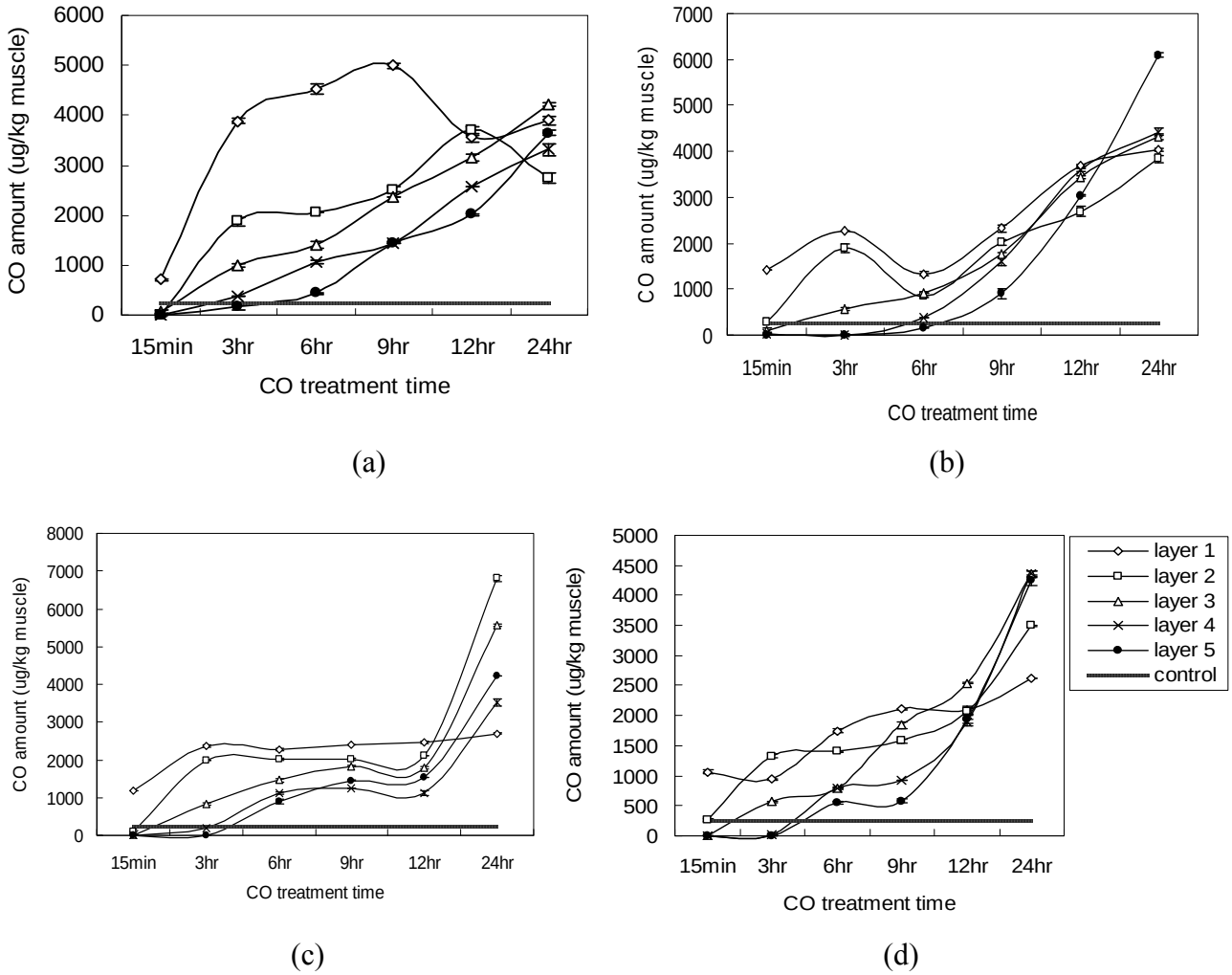


Figure 6-16. Carbon monoxide amount of each layer in the muscle with different ionic strength: (a) 0, (b) 150 mM, (c) 300 mM, (d) 450 mM, as a function of CO treatment time. The GTM-Hb system with various ionic strength was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO amount by GC.

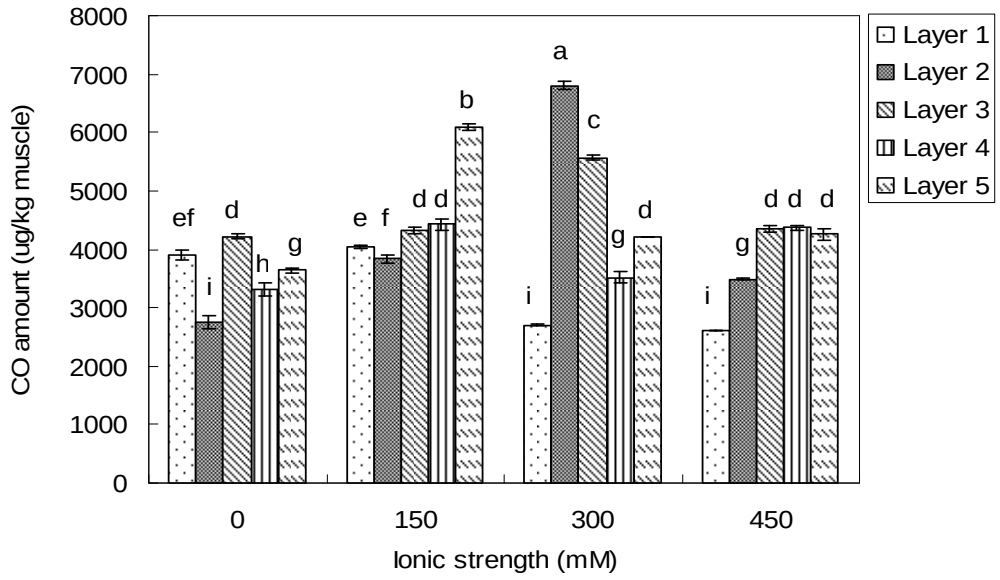


Figure 6-17. Carbon monoxide amount of each layer in the muscle with different ionic strength after 100% CO treatment for 24 hrs. The GTM-Hb system with various ionic strength was treated with 100% CO for 24 hrs. Samples were taken and each layer was removed to measure CO amount by GC. Different small letters indicate a significant difference ($p<0.05$).

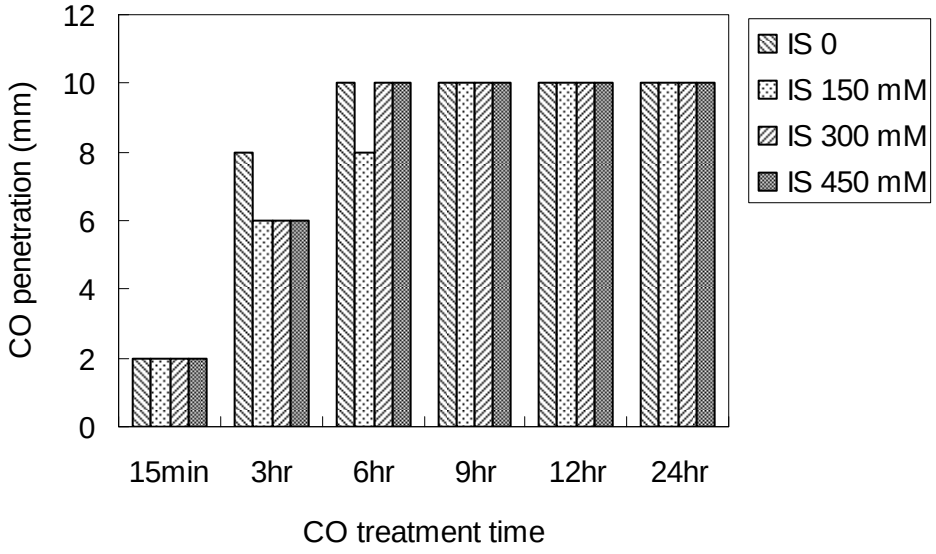
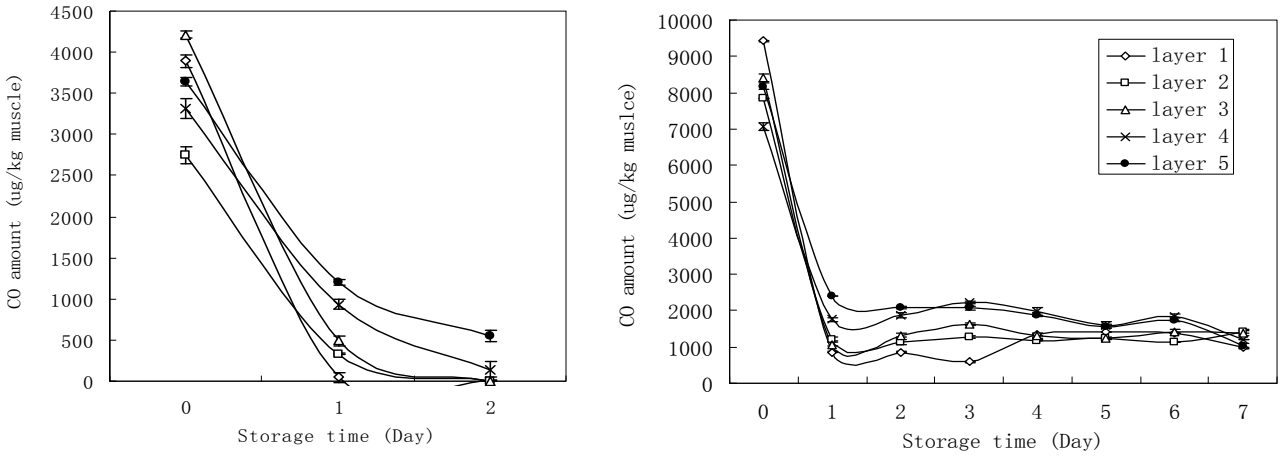
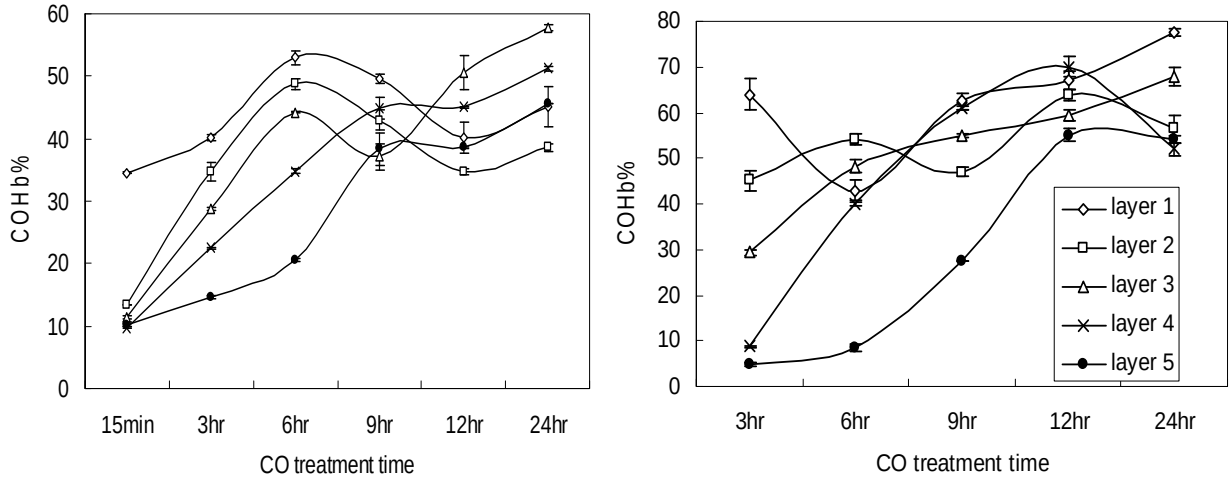


Figure 6-18. Effect of different ionic strength in the muscle on CO penetration rate. The GTM-Hb system with various ionic strength was treated with 100% CO for up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure CO amount. Layers where the CO amount was above the untreated controls, were considered to be penetrated by CO.



(a) (b)

Figure 6-19. Effect of storage at 4°C on CO amount in the muscle with (a) 5 µmol Hb/kg muscle and (b) 50 µmol Hb/kg muscle. The GTM-Hb system with different Hb concentrations was treated with 100% CO for 24 hrs and then placed in oxygen permeable bags for up to 7 days. Samples were taken each day during refrigerated storage and each layer was removed to measure CO amount by GC.



(a) (b)

Figure 6-20. Percentage of COHb in each layer in the muscle with different Hb concentrations: (a) 5 µmol Hb/kg muscle, (b) 50 µmol Hb/kg muscle, as a function of CO treatment time. The GTM-Hb system with with different Hb concentrations was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to measure COHb% by the spectrophotometer.

Table 6-1. Maximum absorption peak wavelength of Hb extracted from each layer in the muscle with 5 μmol Hb/kg muscle as a function of CO treatment time. The GTM-Hb system was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to extract Hb and measure CO by spectrophotometer.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	416.0±0.0	413.5±0.7	413.0±0.0	412.5±0.7	413.0±0.0
3 hr	417.5±0.7	417.5±0.7	417.0±0.0	416.0±0.0	413.5±0.7
6 hr	418.0±0.0	417.5±0.7	417.5±0.7	416.5±0.7	415.0±0.0
9 hr	417.5±0.7	418.0±0.0	417.0±0.0	417.5±0.7	418.0±0.0
12 hr	418.0±0.0	417.5±0.7	418.0±0.0	418.0±0.0	418.5±0.7
24 hr	418.5±0.7	418.5±0.7	417.5±0.7	418.0±0.0	417.5±0.7

Table 6-2. Maximum absorption peak wavelength of Hb extraction from each layer in the muscle with 50 μmol Hb/kg muscle as a function of CO treatment time. The GTM-Hb system was treated with 100% CO up to 24 hrs. Samples were taken at different times of CO treatment and each layer was removed to extract Hb and measure CO by spectrophotometer.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
3 hr	419.0±0.0	417.5±0.7	416.0±0.0	412.5±0.7	412.5±0.7
6 hr	418.0±0.0	418.0±0.0	419.0±0.0	418.0±0.0	412.5±0.7
9 hr	418.0±0.0	418.0±0.0	417.5±0.7	418.0±0.0	416.0±0.0
12 hr	418.5±0.7	419.0±0.0	418.0±0.0	418.0±0.0	418.0±0.0
24 hr	418.5±0.7	418.0±0.0	418.5±0.7	419.0±1.4	418.5±2.1

CHAPTER 7
CONCLUSION

The washed tilapia muscle and tilapia Hb were used as a model system in order to develop a spectrophotometic method to identify CO and further quantify CO in fish muscle. A simple one-wavelength spectrophotometric method was developed to determine the presence of CO in the muscle tissue. This method involves a simple extraction of heme proteins from fish muscle using a slightly alkaline, low ionic strength solution and centrifugation. Then the UV-visible spectrum of the supernatant was obtained by a spectrophotometer. Higher COHb recovery and stability were found when Hb was extracted at pH 8.0 compared to pH 6.0. This method can be used to determine various forms of Hb, which may reveal if the food products have been treated with CO based on COHb/Mb peak wavelength. It was concluded that any heme protein extract which exhibits a maximum peak wavelength at 415 nm or above can be determined as CO treated.

A two-wavelength spectrophotometric method was developed, which can give a good estimate of COHb/Mb% in the muscle tissue. This method is based on that sodium dithionite can deoxygenate oxyHb/Mb and metHb/Mb without influencing COHb/Mb. Therefore, a two-pigment mixture was produced and percentage of COHb/Mb was obtained by calculating directly from the ratio A_{419}/A_{430}. This two-wavelength spectrophotometric method was able to detect COHb/Mb% even as low as 3.33% in muscle tissues. There was an excellent correlation between expected COHb% and detected COHb% by the two-wavelength method. The high correlation of the two-wavelength method with the more sensitive GC method showed the results were accurate and reproducible. During the procedure, protecting solutions against exposure to air is not necessary. MetHb/Mb was reduced by sodium dithionite and it was not a source of interference, but the other Hb derivatives or pigments may possibly cause interference. Both the

one-wavelength and two-wavelength spectrophotometric methods can be used as simple, rapid and low-cost screening methods.

Our improved GC method can be used to determine CO in fish muscle effectively. There is a significant difference ($p<0.05$) between releasing CO from various sample sources. Liberating CO directly from the mixture of muscle and buffer was more efficient than liberating CO from muscle homogenate, which was more efficient than from Hb extract. There is also a significant difference ($p<0.05$) between various CO liberating method. As a CO liberating reagent, 5% sulfuric acid was more suitable than the others tested. Moreover, it was found that both the Soret range and the visible range have similar sensitivity in COHb/COMb% determination even though the absorbance in the Soret range was around 10 times higher than that in the visible range. For various pairs of wavelengths in both ranges, there was an excellent linearity ($r^2 \geq 0.992$) between expected and detected COHb/COMb%. Both spectrophotometric and GC method have their own specificity for determining CO in fish muscle, and only if we combine these two methods together, we can obtain the sound understanding of CO in the muscle system.

It was found that CO could diffuse into the first layer of the fish muscle after only 15 min of CO treatment.As CO treating time increased, CO amount in each layer of muscle increased. The CO residue in the lower layers generally increased much more quickly and was higher than that in the deeper layers. Both internal and external factors influenced CO penetration rate and CO amount in the muscle system. During refrigerated storage of CO treated samples, CO amount dropped dramatically during the first day of storage, and after that, it dropped slowly or remained constant. For the muscle with 50 μmol Hb/kg muscle, CO amount was as high as 1000 μg/kg even after 7 days refrigerated storage. In addition, both one-wavelength and two-wavelength spectrophotometric methods were used to investigate CO penetration into the muscle, and results

almost matched our GC results. These results could be of importance for designing and processing CO or FS treatment, such as CO treating time and CO concentration, in order to develop and maintain the red color as well as not cause other safety problems.

This study has direct implications for regulatory agencies interested in testing suspected seafood products and controlling CO treated products, and for seafood industries which process using CO or FS.

CHAPTER 8
FUTURE STUDIES

In the future, we will continue working on development and evaluation of both qualitative and quantitative methods for CO determination in the muscle. For qualitative methods, we will aim at looking for the simple methods which may be used to identify if the products have been treated with CO. For quantitative methods, we will use GC to quantify CO in the muscle. However, we will use TCD as a GC detector instead of FID. In the current study CO was converted to CH_4 and detected by FID to determine CO in the muscle because of its low detection limit. However, this specific equipment can only be used to detect CO or CO_2 and it is costly, which may limit its application in some agencies and industries. Based on this study, the detection limit of TCD is enough for determining CO in the treated products, and TCD is a normal GC detector and can be used for other analysis. In addition, using SPME (solid phase microextraction) for CO sampling followed by GC analysis would be worth pursuing.

It is recommended that we study the CO penetration into the muscle with a higher amount of Hb concentration. Based on our results, Hb concentration has a big influence on CO penetration into the muscle. In this study, we investigated the effect of various muscle conditions on the CO penetration into the muscle with low amount of Hb concentration. If we use higher Hb concentration to do this study, the various muscle conditions may have a more obvious influence on CO penetration.

Finally, it is advisable to investigate the CO fate during frozen storage of CO treated products. Since many CO treated seafood products are subjected to certain time of frozen storage, it is important to know the CO fate during frozen storage.

APPENDIX
ORIGINAL DATA ABOUT CO PENETRATION IN THE MUSCLE

Table A-1. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	705.61±12.73	15.57±22.01	71.62±47.72	0.00±0.00	0.00±0.00
3 hr	3884.75±52.23	1872.93±79.80	1006.86±34.20	366.50±3.45	155.61±65.22
6 hr	4523.61±90.93	2053.64±1.86	1396.03±67.60	1059.73±35.26	429.11±15.11
9 hr	4989.26±54.35	2518.73±64.69	2349.64±0.53	1451.52±24.66	1437.83±13.79
12 hr	3561.38±82.71	3690.73±76.35	3156.47±60.97	2558.84±2.65	2013.71±8.48
24 hr	3897.68±77.94	2746.68±104.98	4216.37±40.30	3314.68±116.65	3637.11±51.96

Table A-2. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, no addition of lipid and KCl, after 20% CO treatment as a function of CO treatment time. The GTM model system was treated with 20% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	469.60±6.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	1029.36±7.16	329.01±36.85	19.88±28.11	0.00±0.00	0.00±0.00
6 hr	454.23±51.70	119.05±15.11	121.86±15.38	0.00±0.00	0.00±0.00
9 hr	474.66±25.98	507.66±95.44	281.95±1.06	102.74±12.19	0.00±0.00
12 hr	1529.50±30.49	847.52±41.09	790.16±45.33	291.70±5.83	109.68±7.16
24 hr	874.52±83.51	373.43±9.01	585.08±37.91	395.56±126.19	356.38±25.72

Table A-3. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with pH 6.4, no addition of Hb, lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	1931.6±53.02	132.36±31.28	60.56±85.64	39.94±8.75	0.00±0.00
3 hr	2422.75±59.91	1066.66±22.80	543.46±35.79	77.06±53.82	24.57±7.69
6 hr	2400.07±14.58	1651.35±62.30	793.91±61.24	529.03±76.88	91.12±5.83
9 hr	2260.41±57.79	2092.63±71.84	1571.68±52.49	740.67±50.64	277.46±44.54
12 hr	2151.87±68.66	2161.62±104.19	2149.06±56.20	1370.72±28.63	1162.64±92.79
24 hr	2736.37±81.92	2220.48±16.17	2174.55±85.90	1896.55±81.92	1860.18±64.42

Table A-4. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 50 μmol Hb/kg, pH 6.4, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	3465.96±131.23	164.23±39.77	85.50±9.54	5.82±8.23	0.00±0.00
3 hr	7095.38±12.19	3851.94±18.03	2557.34±16.44	460.04±19.09	738.79±38.97
6 hr	9062.02±35.79	5387.99±0.53	4274.48±67.34	2448.06±78.74	202.47±4.24
9 hr	6716.33±89.61	6086.84±73.17	5701.61±101.54	4141.94±76.62	1507.57±10.60
12 hr	10595.25±46.13	6552.12±204.13	7802.85±34.46	5097.05±158.00	4005.10±20.41
24 hr	9419.13±27.04	7845.59±10.60	8397.66±108.96	7063.70±115.32	8170.08±90.40

Table A-5. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 100 μmol Hb/kg, pH 6.4, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	4971.64±2.92	235.09±75.29	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	10817.77±114.26	7561.96±31.55	942.75±56.47	30.75±43.49	0.00±0.00
6 hr	12385.87±59.91	10023.31±50.11	6764.88±2.92	1047.92±72.64	0.00±0.00
9 hr	9031.65±24.12	10065.68±91.46	8669.48±96.23	3039.12±30.22	317.57±0.53
12 hr	12153.99±109.49	11559.55±93.32	9388.01±51.43	6905.29±365.85	1439.71±48.78
24 hr	9892.28±221.63	9861.72±45.86	12163.92±48.78	9239.54±6.89	11947.97±68.93

Table A-6. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol metHb/kg, pH 6.4, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	2028.89±114.26	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	1001.24±5.57	226.09±72.11	324.32±44.54	20.45±0.27	88.50±15.38
6 hr	2132.37±8.75	1734.58±11.93	1050.35±26.78	272.39±5.57	151.86±9.54
9 hr	4208.12±28.10	2998.81±54.35	1798.88±89.08	1361.16±1.33	374.56±39.77
12 hr	4216.37±46.66	2227.60±6.10	1534.19±22.27	1152.33±7.69	867.39±14.05
24 hr	3975.85±55.94	3225.26±79.27	2146.99±24.12	2213.54±129.90	2253.28±79.53

Table A-7. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 5.5, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	976.31±15.38	71.81±32.08	1.51±35.00	0.00±143.42	0.00±134.68
3 hr	758.29±23.06	468.10±61.24	235.65±9.28	138.92±23.59	15.76±39.77
6 hr	907.70±18.56	740.11±63.63	642.44±5.57	386.00±49.05	43.32±98.36
9 hr	1408.03±34.20	789.03±50.11	781.16±30.49	309.89±1.33	185.60±4.24
12 hr	1702.90±5.83	1031.80±21.21	683.87±15.38	1025.80±68.40	400.80±49.31
24 hr	2762.61±63.89	2451.80±65.48	2779.11±6.63	2935.08±25.72	1786.51±80.06

Table A-8. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 7.2, no addition of lipid and KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	2304.27±40.83	577.02±7.95	199.28±17.23	159.36±43.48	168.17±42.68
3 hr	4171.00±21.21	2084.01±55.41	1322.73±29.16	629.13±31.81	503.91±34.46
6 hr	2819.41±10.60	1889.61±12.19	1664.47±8.22	949.69±15.91	497.35±15.11
9 hr	3093.86±10.60	2302.59±72.90	1524.06±0.53	793.91±66.01	807.97±73.70
12 hr	3419.66±28.63	3030.49±88.02	2430.06±55.94	1974.34±53.55	1026.73±17.23
24 hr	4334.28±11.40	4268.10±24.92	3750.9±3.45	2936.95±34.20	3462.03±27.04

Table A-9. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, 10% lipid, no addition of KCl, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	2446.37±92.79	0.00±221.10	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	3931.80±47.72	1480.57±22.27	539.52±71.05	44.44±129.64	0.00±7.95
6 hr	4312.16±91.46	3099.67±68.66	1411.59±31.81	770.66±90.93	125.24±29.69
9 hr	3537.01±204.66	2172.11±48.51	1656.41±42.95	983.24±37.38	597.64±44.01
12 hr	3871.81±38.71	2240.54±18.03	2771.99±11.40	1942.48±44.01	970.12±84.04
24 hr	5317.13±82.71	3432.41±28.63	2609.08±7.95	2553.6±54.61	3018.5±11.13

Table A-10. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, 150mM KCl, no addition of lipid, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	1431.27±0.27	284.95±2.12	107.80±35.79	27.01±4.77	0.00±0.00
3 hr	2266.22±5.04	1893.55±91.99	564.08±21.47	0.00±28.37	0.00±0.00
6 hr	1333.61±44.01	866.83±52.49	927.38±11.40	380.75±7.69	161.04±8.22
9 hr	2324.52±74.76	2012.96±59.38	1757.64±41.36	1610.11±90.40	903.38±113.20
12 hr	3687.54±43.74	2695.13±101.54	3443.28±27.04	3597.56±23.06	3042.68±22.53
24 hr	4034.53±30.22	3832.63±67.60	4314.59±55.67	4430.07±94.38	6092.47±45.07

Table A-11. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, 300mM KCl, no addition of lipid, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	1197.51±1.06	94.31±46.92	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	2378.88±29.16	1977.91±1.86	835.15±26.25	197.22±11.66	0.00±0.00
6 hr	2272.59±32.08	2018.40±43.21	1457.33±47.19	1120.84±18.29	895.32±57.26
9 hr	2406.81±0.27	2021.96±25.05	1837.12±23.33	1240.25±3.71	1446.46±15.38
12 hr	2466.80±23.06	2120.38±2.39	1780.32±34.20	1115.96±70.25	1531.75±2.39
24 hr	2701.50±17.76	6803.88±74.50	5567.76±39.50	3521.26±99.15	4219.74±4.24

Table A-12. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg, pH 6.4, 450mM KCl, no addition of lipid, after 100% CO treatment as a function of CO treatment time. The GTM model system was treated with 100% CO up to 24 hrs. Samples were taken after a certain time of CO treatment and each layer was removed to measure CO by GC.

CO treatment time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
15 min	1067.97±37.38	260.58±23.86	0.00±0.00	0.00±0.00	0.00±0.00
3 hr	939.56±10.60	1330.42±27.31	563.52±13.79	32.07±7.16	0.00±0.00
6 hr	1746.02±36.05	1400.34±8.48	789.97±13.26	802.91±14.58	542.34±23.06
9 hr	2108.57±9.54	1580.12±29.43	1839.94±51.70	918.38±0.80	581.14±41.36
12 hr	2098.82±62.04	2077.45±48.78	2535.41±10.87	1886.43±59.65	1942.85±68.93
24 hr	2618.64±0.27	3494.08±9.28	4351.52±58.59	4365.40±50.11	4254.79±93.58

Table A-13. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 5 μmol Hb/kg during storage at 4°C. The GTM-Hb system was treated with 100% CO for 24 hrs then placed in oxygen permeable bags for up to 2 days. Samples were taken each day during storage and each layer was removed to measure CO by GC.

Storage time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
0 day	3897.68±77.94	2746.68±104.98	4216.37±40.30	3314.68±116.65	3637.11±51.96
1 day	43.12±60.99	334.26±15.11	490.41±66.81	933.57±54.08	1201.63±34.46
2 day	0.00±0.00	0.00±0.00	5.44±7.70	143.42±88.81	549.46±60.71

Table A-14. Carbon monoxide amount (μg/kg muscle) of each layer in the GTM with 50 μmol Hb/kg during storage at 4°C. The GTM-Hb system was treated with 100% CO for 24 hrs then placed in oxygen permeable bags for up to 7 days. Samples were taken each day during storage and each layer was removed to measure CO by GC.

Storage time	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
0 day	9419.13±27.04	7845.59±10.60	8397.66±108.96	7063.70±115.32	8170.08±90.40
1 day	849.40±17.76	1213.44±46.92	1064.04±50.37	1749.39±51.43	2403.06±15.64
2 day	865.52±0.27	1140.33±65.48	1323.30±68.66	1874.99±85.90	2086.44±33.40
3 day	588.64±16.44	1273.81±26.25	1635.04±31.28	2227.79±46.13	2079.13±51.70
4 day	1336.23±40.30	1175.01±10.07	1313.17±70.78	1973.22±95.44	1889.24±55.14
5 day	1418.52±0.80	1224.88±30.75	1231.06±8.22	1601.30±81.12	1558.37±55.94
6 day	1372.22±11.13	1132.09±15.64	1426.21±45.60	1845.93±47.45	1748.83±45.86
7 day	980.06±20.15	1423.59±46.66	1379.53±55.94	1209.32±8.22	1009.86±70.25

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BIOGRAPHICAL SKETCH

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