EFFECTS OF CARBON MONOXIDE ON MUSCLE QUALITY OF SPANISH MACKEREL

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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The treatment of fish muscle flesh with carbon monoxide (CO) and filtered smoke (FS) containing CO is widely employed with the purpose of influencing the color of red muscle. More research is needed on the effect these processes have on various quality attributes of fish muscle.

The objective of this experiment was to determine if CO or FS treatment will have any effect on the following safety and quality parameters of Spanish mackerel muscle: 1) enhancement of color and color stability of the red muscle in fish flesh, 2) heme protein oxidation state, 3) the level of lipid oxidation, 4) growth of aerobic microorganisms, and 5) the level of histamine production in Spanish mackerel. Results from this study could help alleviate consumer fears that CO or FS treatment is masking inferior quality; and could expand the use of CO and FS treatment of seafood.

Fresh Spanish mackerel fillets were either 1) treated with different CO gas blends (18% CO, FS (containing 18% CO) and 100% CO) for 24 h, then exposed to air at 4°C

for 8 days, or 2) kept in the gas environments for 8 days at 4°C. Control samples were kept aerobically for 8 days at 4°C. Color changes (L*, a*, b* values) on storage were analyzed for white and dark muscle using a color machine vision system and Minolta color meter. Secondary oxidation products (thiobarbituric reactive substances) were analyzed for white and red muscle. Heme proteins were extracted from muscle; and CO binding was determined. The effect on aerobic microbial growth was determined for all treatments.

Results showed that all treatments significantly ($P<0.001$) influenced redness (a* value) on treatment and red color stability on storage. This stability was directly correlated with the binding of CO to heme proteins in muscle, with 100% CO treatment giving most binding. Development of yellowness was minimized for the samples with the most red color stability. Lipid oxidation development was retarded on treatment with CO, 100% CO treatment being the slowest, while it was significantly ($P<0.0001$) suppressed for all samples when kept in the gases for 8 days. Treatment with CO led to a significant ($P<0.01$) delay in microbial growth on storage. Histamine formation was low in all samples; and CO did not inhibit the production of histamine.

This study demonstrated that CO and FS can have multiple positive effects on the quality of fatty species rich in red muscle

CHAPTER 1
INTRODUCTION

Seafood is a major economic contributor in many countries, including the US. Since fish is a highly perishable commodity, proper processing and storage are very important factors to maintain or extend its shelf life. Fish muscle quality declines soon after harvest and continues once the fish has been processed. Primary contributors to fish muscle-quality decline are microorganisms, oxygen, lipids, heme proteins, and enzymes. Heme proteins in fish muscle are primarily responsible for lipid oxidation and color deterioration. Oxidation of the heme proteins hemoglobin and myoglobin occurs rapidly after processing and during storage, giving the red muscle of fish an undesirable brown appearance. The oxidized heme proteins are also known to promote lipid oxidation, leading to rancidity development in fish muscle. A recent method to prevent the undesirable brown color formation of fish muscle is the use of carbon monoxide (CO) or extensively filtered smoke (FS) that contains carbon monoxide.

The 2003 regulation outlined in 21CFR section 173.50 (c) addresses the use of carbon monoxide, a combustion product gas, that may be used up to 4.5% by volume to displace or remove oxygen in the processing, storage, or packaging of beverage products and other food, except fresh meats (CFR). The U.S. Department of Agriculture (USDA) has limited the use of CO to smoked products including beef, lamb, poultry, and other similar mammalian meats. However, the Food Drug and Administration has included tuna and other fish species as long as they comply with food code regulation 9CFR318.7

that states the use of a substance is not permitted if it renders the product adultered or misbranded.

Carbon monoxide causes red muscle to obtain a highly stable cherry red color. Experiments have shown that carbon monoxide binds strongly with myoglobin and hemoglobin, forming carboxymyoglobin (CO-Mb) and carboxyhemoglobin (CO-Hb) which greatly stabilizes the proteins to oxidation (Chow 1998, Ross 2000; Kristinsson et al. 2003a). Although no studies have been done on Spanish mackerel several studies have been done on mahi-mahi and yellowfin tuna. These studies showed that the a-value (redness) of tuna treated with either 4% or close to 100% CO for 4 to 48 h significantly increased compared to untreated muscle (Chow 1998, Ross 2000; Kristinsson et al.. 2003). Studies done with tuna using 4% (48 h treatment) and 100% CO (8 h treatment) indicated that there was no significant effect on microbial growth (Ross 2000; (Kristinsson et al. 2003a)). Limited knowledge is available on the effect CO or FS treatment has on other quality properties (lipid oxidation) and safety issues (e.g. histamine formation) of fish muscle. Most research so far has focused on tuna, but more species of fish are being introduced to this process without any knowledge of its effect (Kristinsson et al 2003, Ross 2000).

Therefore, the purpose of this study was to determine if CO or FS treatment will have any effect on the following safety and quality parameters: 1) enhancement of color and color stability of the red muscle in fish flesh, 2) heme protein oxidation state, 3) the level of lipid oxidation, 4) growth of aerobic microorganisms, and 5) the level of histamine production in Spanish mackerel. Mackerel are highly susceptible to oxidation, contain unsaturated fatty acids, and are rich in dark muscle which makes them highly

prone to oxidation (Ackman and Eaton 1971). Mackerel are also prone to histamine development. For this reason, Spanish mackerel is a good model species to investigate with respect to this process.

CHAPTER 2
REVIEW OF LITERATURE

Scomberomorous maculatus

Spanish mackerel (*Scomberomorous maculatus*) is a popular gamefish of the mackerel family, Scombridae, which has a sizable commercial fishery in the US. Spanish mackerel are fast swimming and inhabit the coastal ocean waters of the western Atlantic Ocean from the Gulf of Mexico to the Yucatan Peninsula. Spanish mackerel has an elongated, compressed body and a pointy snout, has a dusky blue dorsal area with a silver underside, averages 1 to 3 feet long and weighs 2 to 3 pounds. Spanish mackerel have a significant commercial interest as a human food fish along with using whole small fish as bait for big game fishing. During the 1850s along the coasts of Long Island and New Jersey the Spanish mackerel fishery was born. In 1880, the Chesapeake Bay area produced 86% of the total coastal catch, 1.9 million pounds; however by 1887 the number decreased to 64%, (Collete and Nouen 1983). In 1985 Florida accounted for more than 92% of the Spanish mackerel commercial landings. In July 1995, Florida instituted a statewide net ban, which affected the commercial harvest of Spanish mackerel and changed the characteristics of the fishing industry (Collete and Nouen 1983). Atlantic coast commercial landings have generally fluctuated between 2 and 8 million pounds since 1950, peaking at 10 and 11 million pounds from the mid-70s to the mid-80s (Collete and Nouen, 1983). Since 1986, ex-vessel value of commercial landings has ranged between $1.3 and 2.0 million (Collete and Nouen 1983). Spanish mackerel support extensive recreational fisheries in many states, with the primary fishing grounds

in the South Atlantic and Mid-Atlantic areas. Spanish mackerel are a pelagic fish high in dark muscle and pro-oxidants and are very susceptible to lipid oxidation (Everse and Hsia 1997). As time progresses, the heme protein in the dark muscle oxidizes creating an undesirable brown appearance of its fillets. In addition, being part of the Scombridae, family it can develop high levels of histamine on storage, which can lead to serious allergic reactions if consumed (Eitenmiller et al. 1982). For these and more reasons, Spanish mackerel is a good model species to work with when investigating the effect of new processing methods to extend quality and assure safety of seafoods.

Quality and Shelf Life of Seafood

The quality of seafood is perceived through the integration of sensory or organoleptic characteristics. These characteristics are usually grouped under the following headings: appearance, odor, flavor, and texture. Ideally, freshly caught fish are at their highest quality level. From the moment they are slaughtered their quality begins to deteriorate (Gorga and Ronsivalli 1988). Factors that influence the rate of spoilage in fresh fish include temperature, muscle pH, level of microorganisms, activity of endogenous enzymes, amount and type of heme proteins, fat content and fatty acid profile, and the availability of oxygen. Immediate concerns for fresh fish are the development of off flavors, odors, and color changes (Gorga and Ronsivalli 1988). Refrigeration, frozen storage, and atmosphere modification are effective methods used to extend the shelf life of seafood products. Spoilage of seafood products can be characterized into two categories: autolytic quality changes and microbial quality changes.

Autolytic Processes and Quality

Properties of heme proteins**.** There are many autolytic processes that impact the quality of seafood products; however, the function of the heme proteins will be emphasized here. Heme proteins play a very important role in the quality of muscle of many fish species. A heme molecule consists of an organic part and an iron atom, illustrated in Figure 2-1. Hemoglobin (Hb) and Myoglobin (Mb) dictate the color of red muscle. Hb is viewed as a tetramer of myoglobin-like monomers with similar properties (Haard 1992). In some fish, Hb may constitute as much as 30% of the heme protein in red muscle and most of the hemoprotein of white muscle (Haard 1992). Mb is a globular heme protein found in red muscle. The Mb content of muscle tissue is related to a myriad of factors: makeup of fiber types, muscular activity, oxygen availability, blood circulation, and the age of the animal (Gorga and Ronsivalli 1988). It is a general belief that Hb is lost easily during the bleeding of fish and contributes less to the appearance, unlike Mb which is retained by the intracellular structure (Richards et al. 1998). Recent evidence however point to a significant retention of Hb even after bleeding (Richards et al., 1998; Richards and Hultin, 2002). The iron containing heme group found in Hb and Mb is capable of binding to different gas molecules (O_2, NO and CO) and can also be found in various oxidation states (Fe 2+, Fe3+, and Fe4+). Upon storage the heme proteins in the fish begin to autoxidize, which refers to the conversion of the ferrous (+2) heme protein to ferric met (+3) heme protein. This yields a brown discoloration of the fish which is undesirable to the consumer. Met Mb/Hb is also considered one of the principal pro-oxidant in fish muscle leading to lipid oxidation and accompanying off-odors and off-flavors (Hultin, 1988).

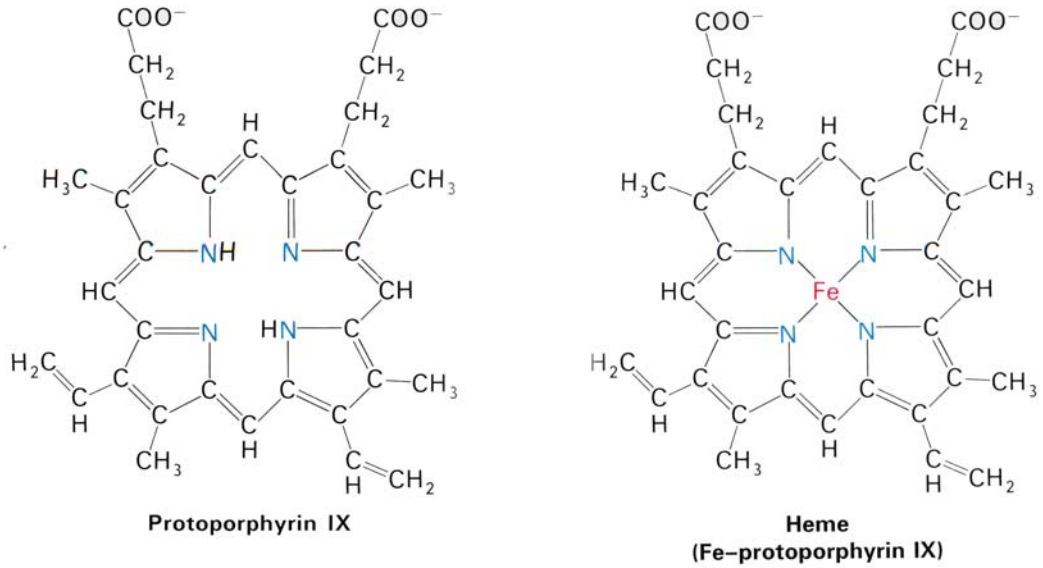

Figure 2-1. Organic portion and iron atom of a heme molecule (Stryer 1999)

When oxygen interacts with the ferrous (+2) binding site of Hb it is called oxyhemoglobin. This complex is loosely bound and dissociates easily when the concentration of oxygen is low or pH is reduced (Stryer 1999). When the iron binding site is vacant this is called deoxyhemoglobin. The interaction of carbon monoxide with Hb forms carboxyhemoglobin. Carbon monoxide has a much higher binding affinity to the heme protein than oxygen. An isolated heme in solution binds CO with 25,000 times more affinity as O_2 while binding affinity of heme as a part of Mb and Hb for CO is about 200 times greater than O_2 (Stryer 1999). It can be determined to which gas molecule Hb/Mb are bound to by their visible spectra. Figure 2-2 illustrates the visible absorption spectrum of hemoglobin, from 450-650 nm.

Due to its tetrameric structure Hb binds oxygen and other gas compounds differently (cooperatively) compared to myoglobin (Stryer 1999). The binding of gas compounds to Hb is also greatly influenced by pH to a different extent compared to Mb.

Hemoglobin looses its oxygen readily as pH is reduced and also oxidizes more easily at lower pH's (Richards and Hultin 2000).

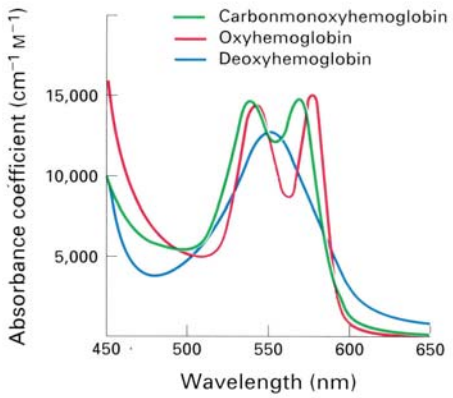

Figure 2-2. Visible absorption spectrum of Hb in different ligand binding states (Stryer 1999)

Figure 2-3 illustrates the major difference between Hb and Mb in terms of their oxygen binding properties. Hb has a sigmodial curve, when oxygen binds to the first subunit of deoxyhemoglobin it increases the affinity of the remaining subunits for oxygen. As additional oxygen is bound to the second and third subunits oxygen binding is strengthened further. However, with Mb the curve is hyperbolic; this means that O_2 molecules bind independently of each other since it is a monomeric protein. Besides the heme pigments interaction with O_2, they can also catalyze lipid oxidation in meat (Rhee 1988) which will be elaborated on in more detail below.

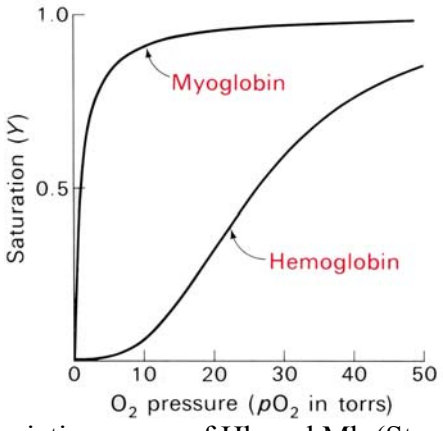

Figure 2-3. Oxygen disassociation curve of Hb and Mb (Stryer 1999)

The Role of Heme Proteins in Lipid Oxidation

Lipid oxidation is a major cause of spoilage in fish muscle, due to the highly unsaturated nature of the fatty acids in fish tissue and abundance of pro-oxidants (Hultin 1994). Fatty fish like mackerel can undergo lipid oxidation rapidly at 4°C (Shahidi and Spurvey 1996). The primary quality deterioration observed during lipid oxidation is the production of off-flavor and off-odor compounds. Lipid oxidation can also lower the nutritional quality and modify texture and color. Transition metals including iron (Fe) and copper to a lesser extend are known to catalyze lipid oxidation (Hultin 1994). The transition metals are often found as a part of other molecules, e.g. iron can be found in the iron storage protein ferritin and also as a part of heme proteins (Decker and Welch 1990; Hultin 1994).

Heme proteins including Hb and Mb are primary catalysts of lipid oxidation in muscle foods. The presence of blood in fish muscle has been reported to lead to color and lipid oxidation problems (Connell 1975; Wheaton and Lawson 1985, Botta et al. 1986; Richards et al. 1998) with hemoglobin being identified as the primary catalyst (Richards and Hultin 2002). A comparative analysis of hemoglobin and myoglobin with respect of their relative role in oxidation has not been conducted. The pro-oxidative power of Hb and Mb is due to the iron containing heme. It is believed that activated heme proteins are the primary form which catalyzes oxidation (Everse and Hsia, 1997). Autoxidation occurs when iron in the ferrous (Fe^{+2}) form is converted to the ferric met form (Fe^{+3}). Autoxidation appears to be a critical step in the ability of heme proteins to catalyze lipid oxidation since the oxidized met form can break down preformed lipid hydroperoxides to stimulate formation of compounds capable of initiating and propagating lipid oxidation

(Everse and Hsia 1997). The met form is further oxidized during this reaction to form a hypervalent ferryl form (Fe^{+4}) with a free radical nature, which is believed to have the capacity to initiate oxidation (Reeder and Wilson 1998). Thus, being able to stabilize hemoglobin and myoglobin in the reduced ferrous form is expected to lead to significant retardation of lipid oxidation in fish muscle.

Limited research has been conducted on the role of heme proteins with respect to the quality of fish muscle. It was demonstrated by Richards and Hultin (2002) that heme proteins appeared to play a major role in the oxidation of both bled and unbled trout and mackerel. The same researchers found that hemoglobin constituted the majority of the heme proteins extracted from the muscle of bled and unbled fish (Richards, 2002) which is interesting considering that bleeding is expected to reduce the amount of hemoglobin. Using model systems Richards and Hultin (2000) found that hemoglobin was a very potent catalyst of membrane lipid oxidation, more as pH was reduced within the post-mortem pH range of a fish muscle (from pH 7.6 to 6.2). They accounted this to increased deoxygenation and autoxidation of Hb as pH was reduced. Kristinsson (2003a, 2003b) reported that the conformation of hemoglobin as influenced by pH has a strong impact on the proteins pro-oxidative activity. At lower pH's the protein is partly unfolded thus enhancing autoxidation and giving the heme more access to participate in oxidation. It has been reported that fish heme proteins are more unstable (autoxidize and denature more easily) than their mammalian counterparts, and that cold water fish have more unstable heme proteins than warm water fish (Cashon et al. 1997; Undeland et al. 2003). The difference between mammalian and fish species may be due to the difference in amino acid composition; this in turn may affect the relative oxidative color stability of

postmortem muscle. (Hultin 1994). A study performed by Richards and Detmann (2003) compared hemoglobins from different species in terms of their rate of autoxidation after reacting with peroxide and analyzed there ability to catalyze lipid oxidation. This study illustrated that trout Hb autoxidized faster than chicken or beef Hb this was because of the proton dependent equation: oxy (Fe^{+2}) Hb + H^{+} met (Fe^{+3}) Hb + $HOO^{\cdot}$. This is a key reaction in heme protein autoxidation (Kanner 1994). It was also shown that autoxidation is dependent on deoxyHb content; such that fully oxygenated heme proteins should be more resistant to autoxidation than heme proteins containing substantial amounts of deoxyHb (Richards et al. 2002). Richards and Hultin (2002) illustrated that there was a good correlation between hemoglobin content and lipid oxidation (Richards and Hultin 2002). This was hypothesized to be the case because the mackerel was minced which aided in the release of Hb from the erythrocytes which then distributed Hb throughout the muscle. Thus, mincing of the muscle will rupture blood vessels causing extracellular and intracellular components (including Hb) to mix. This would cause a dilution of hemoglobin formerly housed in the erythrocytes .These studies demonstrated that the rates of lipid oxidation in various muscles foods may depend on the relative ability of hemoglobins from different animal species (mackerel, beef, trout, and chicken), Hb concentration and oxidation states of Hb to promote lipid oxidation.

Shahidi and Spurvey (1996) illustrated that over time there was generally an increase in thiobarbituric acid reactive substances (TBARS) value of mackerel. This is attributed to the fact that during storage oxidation continues, lipid hydroperoxides breakdown and produce secondary oxidation products, as supported by Kim and Park (1984). Chapman et al. (1993) demonstrated that extra-cold storage (-30°C and -40°C) of

hake and mackerel showed quality deterioration. As time increased the quality of mackerel decreased according to sensory and chemical indices, unlike fillets stored at 18°C tended to retain the fresh fish and sweet taste. Extra-cold storage temperatures slowed deterioration in mackerel as measured by rate of hardening of mince and slower free fatty acid (FFA) formation (Chapman et al. 1993).

Microorganisms and Quality

Microbial spoilage is another important factor compromising the safety and acceptability of seafood products. The presence of spoilage means there are microorganisms present and the enumeration and identification of these microorganisms are imperative. Common spoilage organisms associated with seafood are gram-negative psychrotrophic bacteria such as *Pseudomonas, Alteromonas,* and *Shewanella* (Lopez-Galvez et al. 1995). Microorganisms are found on all the outer surfaces (skin and gills) and in the intestines of live (Huss, 1995). The range of the total number of organisms vary from 10^2- 10^7 cfu (colony forming units/cm^2) (Liston 1980) depending on the location of the fish, species, fishing ground and season. The bacterial loads in fresh fish are usually relatively high; however, many of these bacteria are unimportant during spoilage (Huss 1988). The bacteria that contribute to off-flavors and odors are the organisms of main interest and importance. The compound trimethylamine (TMA) is responsible for off odors at late stages of spoilage for many marine species.

The flesh of most marine fish and shellfish contains trimethylamine oxide (TMAO) which is thought to have an osmoregulatory function in the live animal (Parry 1993). Upon death TMAO can serve as a terminal electron acceptor for some of the spoilage bacteria, enabling them to grow when oxygen levels are depleted (Parry 1993), as a

consequence TMA is released. With some species of seafood such as herring, mackerel, sardine, tuna and some species of crab, the TMAO content is unevenly distributed throughout the body, which could lead to uneven quality problems in the fillets (Hebard et al. 1982). TMA production used as an indicator of freshness is dependent on the presence of TMA-producing bacteria such as *Pseudomonas putrefaciens* in the spoilage flora (Laycock and Regier 1971). TMA does not increase during the early stages of storage it is not considered, thus it is not suitable for fish to be stored on ice for less than six days (Howgate 1982).

Spoilage is not the only microbial issue to worry about; certain bacteria can cause food borne illnesses. A number of health hazards, including pathogenic organisms and biotoxins are associated with seafood products. The expanded practice of consuming raw fish may make consumers more vulnerable to serious foodborne illnesses, especially if appearance is stabilized and masks inferior food quality. From 1993 to 1997 approximately 3% of all reported foodborne illnesses came from seafoods, a slight rise from the 1973 to 1987 survey (Olsen et al. 2000). Illness resulting from pathogens and toxins in seafoods can lead to great economic losses for consumers, industry and government, estimated to be in the hundreds of millions each year (WHOI 1998; Anonymous 1999). Scombroid (histamine) poisoning is a primary concern with scombroid fish species that have large amounts of free-histidine in their muscle such as Spanish mackerel (Huss 1994)). Histamine, a biogenic amine, is formed *post mortem* by bacterial decarboxylation of the amino acid histidine (Eitenmiller 1982) as a result of time/temperature abuse. Free histidine can be converted to histamine through the enzyme action of histidine decarboxylase, which cleaves the carboxy group of the amino acid

histidine to form histamine (Budavari 1989). Between 1973 and 1987, 116 outbreaks (757 cases) of scombroid fish poisoning were reported to Center for Disease Control and Prevention (CDC), 37% of the total reported seafood associated poisonings. Between 1988 and 1992, scombrotoxin accounted for 2.1 to 3.7% of foodborne outbreaks with one case resulting in death (Morbidity and Mortality Weekly Report (MMWR) 1996). Scombroid poisoning occurs after the ingestion of foods that contain high levels of histamine. Scombroid fish poisoning usual manifests as a mild discomfort for a few hours, but is rarely fatal. Common symptoms are facial flushing, urticaria, edema, but also the gastrointestinal tract may be affected (nausea, vomiting, diarrhea) as well as neurological involvement (headache, tingling, burning sensation in the mouth) (Huss 1994)).

Fish with high histidine content are from the *Scrombridae* family. However, non-scombroid fish such as *Clupeidae* (herring) and mahi-mahi , may be involved in histamine poisoning. Histamine producing bacteria are *Enterobacteriaceae,* some *Vibrio* sp. a few *Clostridium* sp. and *Lactobacillus* sp. However, the most potent histamine producers are *Morganella morganii, Klebsiella pneumoniae* and *Hafnia alvei* (Taylor et al. 1978).*M. morgananii* forms large amounts of histamine at low temperatures (0-5°C) following storage for up to 24 hours at high temperatures (10-25 °C) (Taylor et al. 1978). *Morganella morganii* is capable of producing a significant amount of histamine, >1000 ppm when cultured in a broth. According to Taylor et al (1978) *M. morganii* formed high levels (40 umols) of histamine in a culture broth when isolated from spoiled fish and stored above 15°C. Histamine formation is more rapid at high-abuse temperatures (21.1°C) than at moderate-abuse temperatures (7.2°C) (US Food and Drug

Administration 2001). Histamine formation is more a result of high temperature abuse than anything else.

Once histamine has been produced in a fish, the risk of provoking disease is extremely high. Histamine is resistant to heat, thus even if the fish is cooked, canned or in any way heat treated before consumption, the histamine is not destroyed. In order to limit histamine formation fish should be stored at or near 0°C or very near to 0°C. The US Food and Drug Administration has stated that histamine levels >50 ppm is prohibited for human consumption. Other indicators of decomposition are the development of putrescine and cadverine, which are also biogenic amines. Putrescine and cadaverine are not particularly toxic but may potentiate the toxicity of histamine. These putrefactive amines are also formed in fish as a result of bacterial spoilage from the decarboxylation of ornithine and lysine (Taylor and Sumner 1986). Putrescine and cadverine may potentiate the toxicity of histamine by inhibiting its detoxification by diamine oxidase and histamine N-methyltransferase *in vivo* (Taylor and Sumner 1986).

Extension of Shelf Life by Atmospheric Modification

Vacuum and MAP Packaging

Packaging of food makes it more convenient and inhibits the food from microorganisms, biological and chemical changes to extend its shelf life. There has been a significant growth in the food packaging industry over the past ten years; the leaders in these new innovations are modified atmospheric packaging (MAP) and vacuum packaging. In MAP an initial atmosphere is generated by either permitting air to be enclosed or by injecting a desired initial gas mixture (Skandamis and Nychas 2002). This blend then changes as a result of multiple variables including: (i) permeation of oxygen, carbon dioxide, and water vapor through the package material; (ii) transmission of

oxygen, carbon dioxide, and water vapor through the seal and defective structural areas; (iii) temperature of the package material which may lead to small changes in permeation; (iv) surface area of the package material; and (v) thickness of the package material (Skandamis and Nychas 2002). In vacuum packaging a product is placed in a film of reduced oxygen permeability, and removing air from the packaging, followed by employing a hermetic seal (Smith et al. 1990). Clingman and Hopper (1986) demonstrated that fresh fish products stored under vacuum packaging (32°C) were microbially acceptable($<10^7$) on the ninth day, unlike the products stored aerobically which were unacceptable (10^7) on third day. Overall there was a shelf life extension of seven days when using vacuum packaging. Studies conducted by Shewan and Hobbs (1960), Pelroy and Seman (1969), and Huss (1972) illustrated that vacuum packaging extended the shelf life as pertaining to development of rancidity since Oxygen was excluded. Even though the MAP packaging was slowing down the aerobic respirations of the product may deteriorate due to growth of anaerobic microorganisms. A drawback to using vacuum packaging and MAP is that there is an increased survival of *Clostridium botulinum*, which could grow and produce toxin in the absence of oxygen, especially if temperature is above 3.3°C (Shewan and Hobbs (1960), Pelroy and Seman (1969), and Huss (1972). For this reason the FDA has specified a minimum oxygen transmission rate (OTR) for seafood packaging films of 10,000 cc O_2/m^2/day (Gnanaraj 2003).

The Use of Carbon Monoxide and Filtered Smoke Treatment to Extend Shelf Life Regulations

The 2003 regulation outlined in 21CFR section 173.50 (c) addresses the use of CO, a combustion product gas, may be used up to 4.5% by volume to displace or remove

oxygen in the processing, storage, or packaging of beverage products and other food, except fresh meats (CFR). The U.S. Department of Agriculture (USDA) has limited the use of CO to smoked products including beef, lamb, poultry, and other similar mammalian meats. However, the Food Drug and Administration has included tuna as long as they comply with the food code regulation 9CFR318.7. 9CFR318.7 states that the use of a substance is not permitted if it renders the product adultered or misbranded. According to CFR301.2 adulteration is not approved as Generally Recognized as Safe (GRAS) or of a substance that is not permitted based on the Federal Food, Drug and Cosmetic Act is considered adulterated. Smoked products traditionally have been place under GRAS Status.

Since 2000, Hawaii International's cold filtered smoking process called "tasteless smoke" is considered GRAS for tuna (Hawaii International Seafood Inc 1999; US Food and Drug Administration 2000). This smoking process introduces CO into a tissue at levels of 7-30% and is applied to raw tuna before freezing with the goal to preserve the taste, aroma, texture, and color of the frozen product (US Food and Drug Administration 2000; Hawaii International Seafood Inc 1999). Carbon monoxide is accepted as a component of smoke but not as a color preservative by itself. Carbon monoxide can be classified in the same general category as "tasteless smoke". Based on the information provided by Hawaii International, as well as other information available from the FDA, the agency has no question at this time regarding the conclusion of Hawaii International that tasteless smoke is GRAS for use on raw tuna, before it is frozen, to preserve its taste, aroma, texture, and color. The use of this process must be included on the label (as outlined in the Food Drug and Cosmetic Act) pertaining to adulterated or misbranded

products (US Food and Drug Administration, 2000). Further, the product may not be referred to as "fresh frozen" on the packaging in accordance with 21 CFR 101.95. Currently a variety of different forms of filtered smoke and carbon monoxide gas blends are being used widely in the seafood industry without proper control or understanding how this type of processing may affect the quality and safety of seafood products (Kristinsson et al. 2003).

Effect on Color

Consumers buying decisions are based on the appearance of the product. Color is very important to the quality of food but can be difficult to measure. Thus, color measurements must be done as carefully as if one is measuring chemical and physical traits (AMSA 1991). Cooking, smoking, curing and other processing not only present opportunities to enhance product appearance and color stability but often create colors that vary from the exterior and the interior (AMSA 1991). After CO is introduced as a color preservation or enhancement method, measuring the degree of color and color change can be useful in monitoring the effectiveness of the method.

Appearance, particularly color, is a primary factor involved in consumer acceptance of red meat (Brewer et al. 1993). Consumers frequently interpret the color of muscle on retail display as an indicator of wholesomeness (Kropf 1980). Consumers prefer their red meat to have a bright red color, the muscle heme proteins in the oxy-state, and not a purple-red color, the heme proteins in the deoxy-state. Upon making decisions on safety and acceptability of what type of red meat to buy consumers rely on sensory characteristics such as odor and color (Brewer et al. 1993). Currently new methods are needed to extend the shelf-life of red meat products. The use of CO on red meat was introduced over 100 years ago for meat products but was soon banned due to the fear that

this method may increase the practice of adulteration (Church 1994)). Recently there has been a renewed interest in using CO to stabilize red color of red meat and in the US it has been petitioned to use up to 0.5% CO in MAP packing of red meat. For example, in Norway up to 0.5% CO is legally used in about 50-60% of all MAP packed retail meat and up to 85% of ground beef (Sorheim et al. 1997). The European Commission's Scientific Committee on Food (Dec 13th 2001) concluded that there is no health concern associated with the levels used in Norway (0.3-0.5%) in MAP provided that temperature during packaging, distribution, retailing and storage does not exceed 4°C (European Commission Scientific Committee on Food, 2001). The use of FS has recently been allowed in several European countries.

An attractive red color is also an important quality trait of fish with dark muscle such as tuna, mahi mahi, tilapia, snapper, grouper and Spanish mackerel. The challenge facing commercial fish processors is to stabilize and maintain the red color attribute of dark muscle species during processing, transport, storage and display for as long as possible (Ross, 2000). The usage of carbon monoxide and filtered smoke containing CO to maintain and enhance red color characteristics of fish muscle has been increasing over the years (Kristinsson et al. 2003a). Dark muscle is a mixture of oxygenated and reduced myoglobin which gives the dark muscle its red color. However, upon being cut the dark muscle very rapidly turns from red to brown as hemoglobin is oxidized to met-hemoglobin. By using carbon monoxide the red color is it stabilized even after the muscle is cut. A study done by Sorheim et al. (1997) demonstrated that it is enough to use CO in concentration of 0.5%-2.0% to enhance and stabilize a bright red color (Sorheim et al. 1997). As discussed previously, when CO binds to Hb/Mb it forms a cherry red color,

carboxyMb/Hb. These pigments are spectrally similar to the bright red oxyMb/Hb which normally develops at the surface of fresh meat in air (Sorheim et al. 1997). Since carboxymyoglobin has a stronger binding effect to the iron-porphyring ring then oxymyoglobin it is not easily oxidized to brown metmyoglobin (Kristinsson et al. 2003). The heme's preference for carbon monoxide is largely responsible for the asphyxiation that results from carbon monoxide poisoning (Sorheim et al. 1997).

There have only been a few studies conducted on the effect of CO treatment on fish muscle. Studies indicate that upon treating fish muscle with CO there will be an increase in the a*value and little effects on the L and b values. An increase in the a* value is attributed to the binding of CO to the heme proteins. According to Kristinsson et al. (2003a) this binding shifts the visible spectra of the heme peak in hemoglobin and myoglobin have a bathochromic shift. A study conducted by Ross (2000) on treated tuna with 4% and 100% CO, found 100% CO more effectively increased and maintained the red color on frozen storage. Kristinsson and coworkers (2003) reported a significant increase in redness and color stability of tuna stored at 3°C after being treated for 48 hr with 4% CO.

More studies have been conducted on the effects of CO on beef and poultry. Brewer et al. (1993) showed that vacuum-packaged beef steaks stored in the refrigerator had an increase in lightness, redness, and yellowness. The end product was more red-orange colored, comparable to the color of beef when allowed to "bloom" as deoxymyoglobin is oxygenated. The CO treatment increased both the color stability and the microbiological shelf-life of vacuum-packaged refrigerated beef steaks. A study done by Nam and Ahn (2002) found that irradiated chicken breasts had a definite change from

the usual brown/purple color to a more vibrant pink/red color. The pink/red color was postulated to be from a ferrous myoglobin derivative such as CO-Hb/Mb or nitric oxide-Hb/Mb. This study showed that CO-Hb/Mb was a major heme pigment responsible for the red or pink color in irradiated turkey breast. To effectively extend the red color stability of fresh meats there is a need for not only new processing but new packaging too. Most fresh beef is displayed in Styrofoam trays wrapped with oxygen permeable polyvinyl chloride (PVC) films. However, this method allows rapid surface pigment oxygenation and red color development (bloom), but within 1-7 days depending on the muscle cut there is brown discoloration (Madhavi and Carpenter, 1993). Manu-Tawiah et al. (1991) concluded that the red surface color of beef cuts packaged in modified atmospheres containing oxygen for bloom, CO_2 as an antimicrobial, and nitrogen as a filler, can be prolonged for 3-5 days. After exposing steaks to 100% CO for 1 hr before vacuum packaging Brewer and coworkers (1993) and Jayasingh et al. (2001) were able to maintain a stable red color for 6 weeks. Steaks and ground beef in 0.5% CO in modified atmosphere packages maintained a desirable red color for 8 weeks (Jayasingh et al. 2001). Beef steaks stored in modified atmosphere packaging with 5% CO for 24 hrs had an extension of redness for 21 days (Jayasingh et al. 2001).The major disadvantage of modified atmosphere packaging is that it is bulky (gas and steak) and there is an increased chance of puncturing the package during distribution as compared to vacuum packaging. The main disadvantages of using CO are that there is a possibility that the product could be spoiled but appear to be fresh and/or that it could mislead consumers if not properly labeled.

Effect on Microbial Growth

There is little information about the effect of CO on the growth of microorganisms. In the late 1970's there was a renewed interest in modified atmosphere storage of fresh meats and fish (Gee and Brown 1978). However, the preservative action was CO_2 and this caused a surface discoloration on meats subjected to atmospheres containing high levels of CO_2 (Gee and Brown 1978) . In order to counteract the discoloration by CO_2, and to extend the color shelf-life of fresh meats, the use of CO was considered (Gee and Brown 1978).CO used in low concentrations, <1%, reportedly have little affect on the microflora of red meat (Gee and Brown, 1978). However, there is concern that the use of CO will mask the microbiological spoilage because the cherry red coloring can last longer than the microbiological shelf life of red meat (Kropf 1980)**.** However, more importantly a product treated with CO could hide underlying safety problems, such as elevated levels of pathogens or histamines in certain fish species, including Spanish mackerel. There is a lack of information on the effects of CO on the growth of microorganisms. In an experiment done on pure bacteria, exposure to pure CO atmosphere had little effect on *Staphylococcus aureus, Clostridium botulinum or Escherichia coli* on apple or potato plugs (Kaffegakis et al. 1969)**.** It was also reported that an atmosphere of 1% CO and 99% N_2 inhibited the growth of psychrophillic bacteria on the surface of beef at 5°C (Clark et al. 1976). According to Brewer et al. (1993) vacuum packed steaks treated with CO resulted in samples with lower microbial loads compared to untreated vacuum packaged steaks. Luno et al. (2000) demonstrated that the growth of spoilage psychrotrophic flora was significantly inhibited when beef steaks where packaged in modified atmospheres with 50% CO_2. Clark et al. (1976) reported that

a low level of CO does not have a major effect on meat microflora. However, Silliker et. al. (1977) found that sustaining an atmosphere of 10% CO, 90% nitrogen or CO_2 inhibited bacterial growth. None of these studies however investigated whether the effect of using CO was from the direct action of CO or due to the exclusion of oxygen, or the action of CO_2 which is an effective anti-microbial (Huss 1994)).

Studies about the microbial effects of CO on fish are far and few between. Ross (2000) reported that CO treatment of tuna did not affect the growth rate microorganisms when it was treated with 4% and 100% CO for 16 and 24 hours, followed by 1 month of freezing and then thawing and refrigerated storage. Stiet al (2003) found that treating tuna at 4% CO for 48h led to no significant reduction in microorganisms. Recent studies by Kristinsson et al. (2003a) however show that 18% CO, filtered smoke and 100% CO led to a reduction in aerobic microorganisms after a 48 h gas treatment, while a 4% CO mixture did not. There is limited information known about the effects CO will have on histamine. Ross (2000) and Kristinsson et al. (2003a) demonstrated that when tuna is frozen after treatment with CO gases histamine production is substantially retarded, which is also seen for frozen untreated tuna. Thermal abuse studies however indicate that high histamine levels can form in tuna treated with 100% CO while color is still acceptable, which is a concern (Kristinsson et al. 2003b). It is thus believed that the usage of CO could mask the formation of histamine since the red color of the product becomes highly stable to abusive temperature conditions (Kristinsson et al. 2003b) but further investigation is needed.

CHAPTER 3
MATERIALS AND METHODS

Gas Treatment and Storage Procedure

Fresh Spanish mackerel, sushi-grade, was obtained from Save-on-Seafood (St. Petersburg, FL). The fish were filleted and skinned immediately upon arrival to the laboratory and placed in FoodSaver™ vacuum bags (2-3 fillets per treatment). Air was expelled from the fillets by vacuum packing in a FoodSaver vacuum packing machine (Food Saver model 550 from Tilia in San Francisco, CA). Three experiments were then conducted:

Storage in Atmosphere

Bags were opened and the samples were transferred into Glad® Zipper Freezer bags (1 gallon) and stored at 4°C for 8 days.

Storage after 24 h Gas Treatment

Bags were opened and flushed and filled with different CO gas blends: (a) 18% CO which also contained 21% CO_2, 1.1% O_2, and balanced with N_2,(b) filtered smoke (containing 18% CO, 21% CO_2, 1.5% O_2 and balanced N_2, (c)100% CO, and (d)100% N_2. After gas treatment samples were thermally sealed. The final treatment (d) performed in 100% N_2 was done to determine if any possible effects of 100% CO was simply due to the exclusion of oxygen or a direct effect of CO. The 18% gas blend was used to see if filtered smoke had any additional effect over a blend of approximately the same chemical composition. After gassing the bags were placed at 4°C for 24 h. After the 24 h gas treatment was completed the gas was expelled and the samples were transferred

into Glad® Zipper Freezer bags (1 gallon) and stored at 4°C for 6 days (total storage time was 8 days).

Storage in Gas

After vacuum packing the bags were opened and flushed/filled with different CO gas blends (see above) and 100% N_2 and stored at 4°C for 8 days.

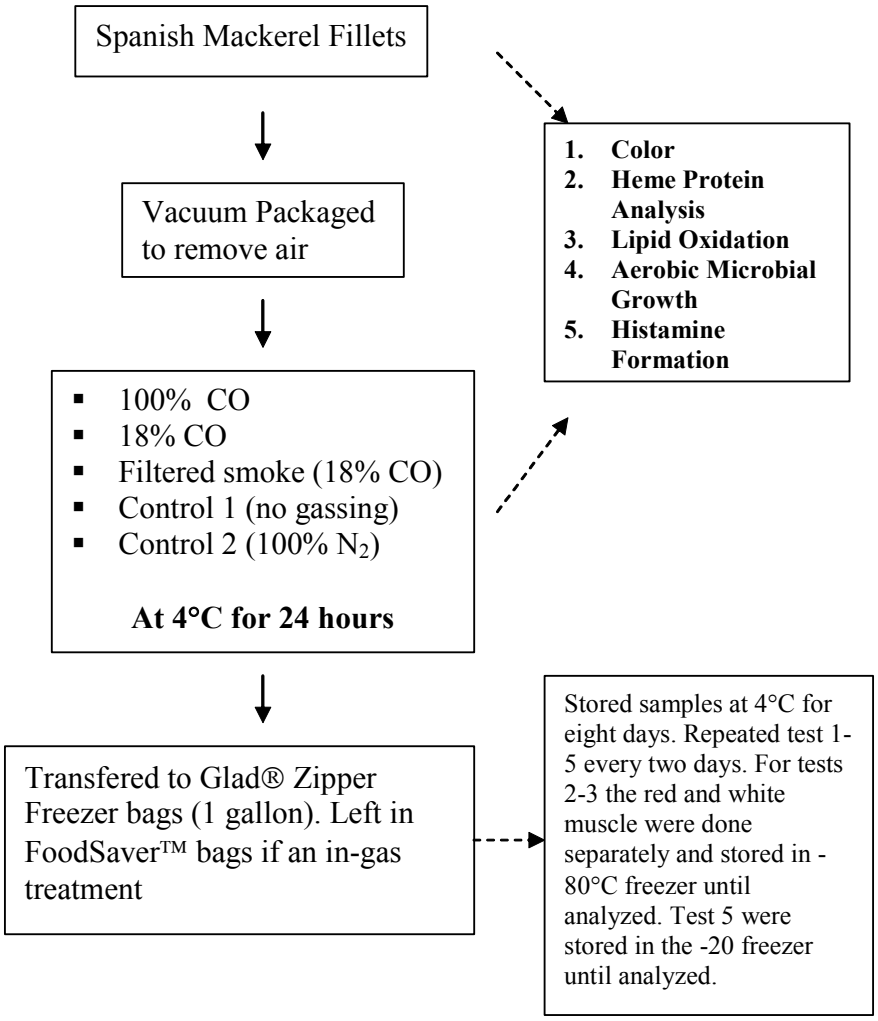

Figure 3-1. Experimental Procedure.

At day 0 (before gassing), 1, 2, 4, 6 and 8 fillets were removed and the red muscle and white muscle carefully excised with a surgical scalpel and individually vacuum packed in FoodSaver™ bags and placed in a -80°C freezer (Forma Scientific, Marietta,

Ohio) until samples were analyzed for lipid oxidation, heme protein oxidation/ligand binding state and histamine. Determination of color and growth of aerobic microorganisms was performed on fresh samples at the day of analysis. The experimental protocol is outlined in Figure 3-1. For each treatment 2-3 fillets were analyzed for each sampling day. Color analysis was performed on the same two fillets throughout the experiment, with red and white muscle analyzed separately. Heme protein analysis and lipid oxidation were analyzed from the same fillets (2 fillets, 3 samples from each), with red and white muscle analyzed separately. The same fillets were used for microbial analysis and histamine formation.

Color Analysis

Two color analysis methods were employed to follow color changes of Spanish mackerel fillets. A Color Machine Vision System was used for detailed color analysis analyzing RGA and L* a* b* values along with identifying important color blocks for each treatment (Luzuriaga et al., 1999). This system consisted of a light box, a ccd camera, a frame grabber and a computer to acquire the images and processing it with color image software. The second system was a hand held Minolta colorimeter (Minolta Camera Ltd, Ramsey, New Jersey) which was used for comparison by measuring L* a* b* values of the muscle.

Any color can be described in three dimension, among the many scales available the L* a* b* system is the most commonly used in food science research. L* expresses lightness of a product and the range is 0-100. Where 0 represents black and 100 represents white. The a* value represents greenness (-a) and redness (+a) and the b* value represents blueness (Hutchins 1999).

Oxidation and Ligand Binding State of Heme Proteins

In order to determine the oxidation and ligand binding state of Spanish mackerel muscle heme proteins, the heme proteins were extracted and their UV-vis absorption spectra analyzed. This analysis reveals if CO is taken up by the heme proteins and how stable the CO is bound to heme proteins on storage. A sample of 1 g of red muscle was mixed with 39 ml of cold (4°C) 20 mM Na_2HPO_4 (Sigma, St. Louis, MO) buffer (pH 8) and homogenized using a Tissue Tearor model 398 (Biospec Products, Bartlesville, OK) at speed five (lowest speed). This solution was then centrifuged at 3000 x g (Eppendorf, centrifuge 5702, Hamburg, Germany) in capped plastic tubes at 4°C for 10 min to separate the soluble heme proteins from the insoluble myofibrillar proteins. The supernatant was collected and its UV-visible absorbance spectrum was read from 350-700 nm in an Agilent diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). The spectrum reveals the oxidation and ligand binding state of extracted heme proteins. The wavelength of the heme peak is 419 nm for CO-Hb/Mb, 414 nm for oxyHb/Mb and 405 nm for metHb/Mb.

Lipid Oxidation

Development of lipid oxidation in Spanish mackerel fillets was assessed by analyzing secondary products of oxidation in the red muscle and the white muscle, according to the thiobarbituric acid reactive substances (TBARS) method by Lemon (1975). These products are responsible for most of the oxidative rancidity odor in fish. Samples were kept frozen at -80°C (Forma Scientific, Marietta, Ohio) prior to analysis. Upon analysis the samples were partially thawed to minimize further oxidation and then prepared to extract the secondary oxidations products followed by spetrophotometric

analysis for their quantification as described byLemon (1975). A standard curve was constructed with tetraethoxypropane. Moisture content of the samples was analyzed with a Cenco moisture balance (CSC Scientific Company, Inc., Fairfax, VA), to be able to report lipid oxidation values on muscle wet weight basis (μmol malondialdehyde/kg tissue).

Aerobic Microbial Growth

Aerobic microbial growth before and after treatment was determined using Petrifilm™ (3M Laboratories, St. Paul MN) according to the official AOAC method (AOAC, 2000). The analysis was done on 25g of aseptically ground muscle mixed with 225 ml (0.2g/ml) sterile phosphate buffer solution (58 mM Na_2HPO_4 (Sigma, St. Louis, MO), 10.0 mM mM NaH_2PO_4 (Sigma, St. Louis, MO), and 85.0 mM NaCl (Sigma, St. Louis, MO), and 1 L of deionized water). The solution was then blended in a stomacher for 1 minute followed by serially diluting the samples (10^{-1}-10^{-7}). For inoculation, the Petrifilm TM was placed on a sterile flat surface and 1.0 ml of the sample was placed at the center of the film and spread by a sterile plastic spreader to an area of ~20 cm^2. Duplicate inoculations were conducted for each dilution and no more than 20 plates were stacked at 25°C for an incubation time of 48 hours.

Histamine Analysis

Samples were kept frozen at -80°C prior to histamine analysis. For the analysis the AOAC Official Method (AOAC, 1987) was followed where histamine was determined by fluormetric determination after post-column derivation using a Sequioa-Turner model 450 fluorometer (Turner Designs, Sunnyvale, CA).

Statistical Analysis

Each analysis was conducted in a minimum of duplicate samples. Analysis of variance (ANOVA) was used to determine significant differences between treatments. The Statistical Analysis System Software Version (8.2)was used for statistical treatment of data.

CHAPTER 4
RESULTS AND DISCUSSION

Effect of Carbon Monoxide and Filtered Smoke on Color

Visual evaluations made during the study showed that there was very little change in the L*value between all treatments; however, there was a difference in the a* and b* values. The Minolta color meter reads only a small part of the sample, which requires multiple readings, while the Color Machine Vision System (CMVS) analyzes every pixel of the sample of interest and allows the user to select regions to analyze. Furthermore the CMVS was standardized with several different color tiles which the Minolta meter was standardized with one white color tile. The CMVS method is expected to give a more thorough analysis of the color and color changes of the Spanish mackerel. Results however indicated that the color reading from the CMVS were on average considerably higher than the values from the Minolta. This is in part due to the fact that samples are illuminated by a light source for the CMVS. To get a meaningful comparison between the two methods the change in L, a, and b value were plotted.

There was no significant trend observed for the L* value for both red and white muscle, indicating that there was not much of an overall change in the degree of lightness over time. There was considerable fluctuation in the data which made interpretation difficult. The most pronounced change was seen with samples kept in 100% N_2 and analyzed with the Minolta color meter, which showed a decrease in L* value. Keeping the muscle in this environment would lead to deoxygenation of heme proteins as well as promote heme oxidation, which is expected to lead to a darker color of red muscle. The

Minolta color meter indicated a slightly decreasing L*value over time for samples kept in gas (both red and white muscle).

The a*value, degree of redness, is the most important indicator of the quality of species rich in red muscle and heme pigments, such as Spanish mackerel, mahi-mahi, tuna, and swordfish. It is apparent from figures of the a*values that by treating the fish with CO or FS it led to a significant increase in redness compared to the control and also in the stability of the red color. This pattern coincides with previous work done by Hsieh et al. (1998) with fish flesh, and Brewer et al. (1993) with beef, Ross (2000) and Kristinsson et al. (2003a) with tuna.

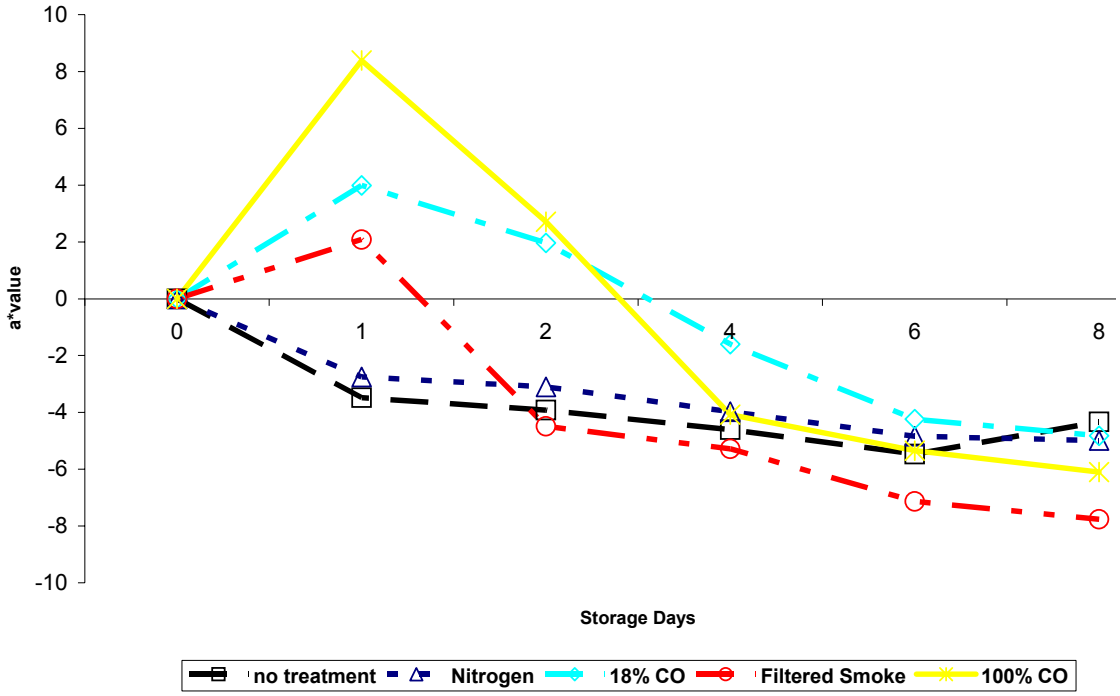

Figure 4-1. Changes in a*values over time for the red muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Minolta colorimeter.

This increase was attributed to the formation of carboxyhemoglobin and carboxymyoglobin in the tissue, as will be addressed in the heme section. The main goal of the CO/FS process is to either enhance or preserve red color. If the a*value increases

over its original value there is an enhancement in color, as seen in this work with Spanish mackerel. It is beneficial to determine how long the enhanced color can be stabilized. Enhancement of the red color is determined by the time the a*value falls below the initial a*value. From Figures 4-1 and 4-2 it is clear that the 100% CO gave a higher increase in redness of the dark muscle than the other CO/FS treatments.

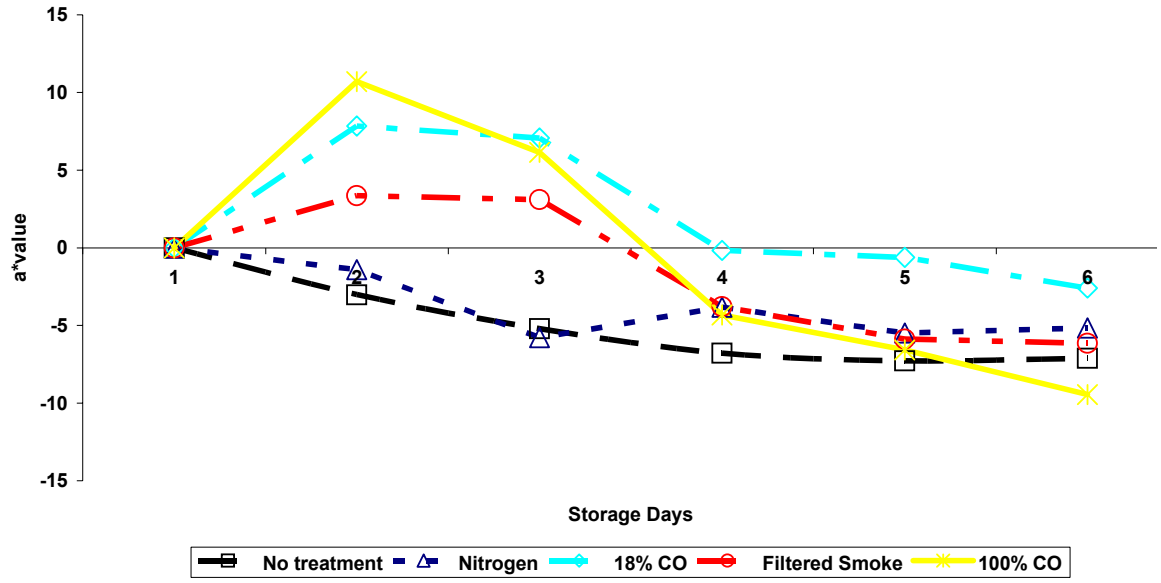

Figure 4-2. Change in a*values for the red muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Color Vision Machine.

The control and 100% N_2 treated samples showed a decrease in a*value at the same time, which could be attributed to oxidation of the heme proteins on storage. 100% N_2 would have yielded deoxygenatation of the heme proteins, thus leading to a darker purple color of the sample. When samples were kept in the gas environment for the duration of the storage experiment (Figure 4-3) it was evident from the Minolta color meter that it took more than 24 hours to reach a maximum a*value for the red muscle while the CMVS data indicated that maximum redness was achieved after 1 day.

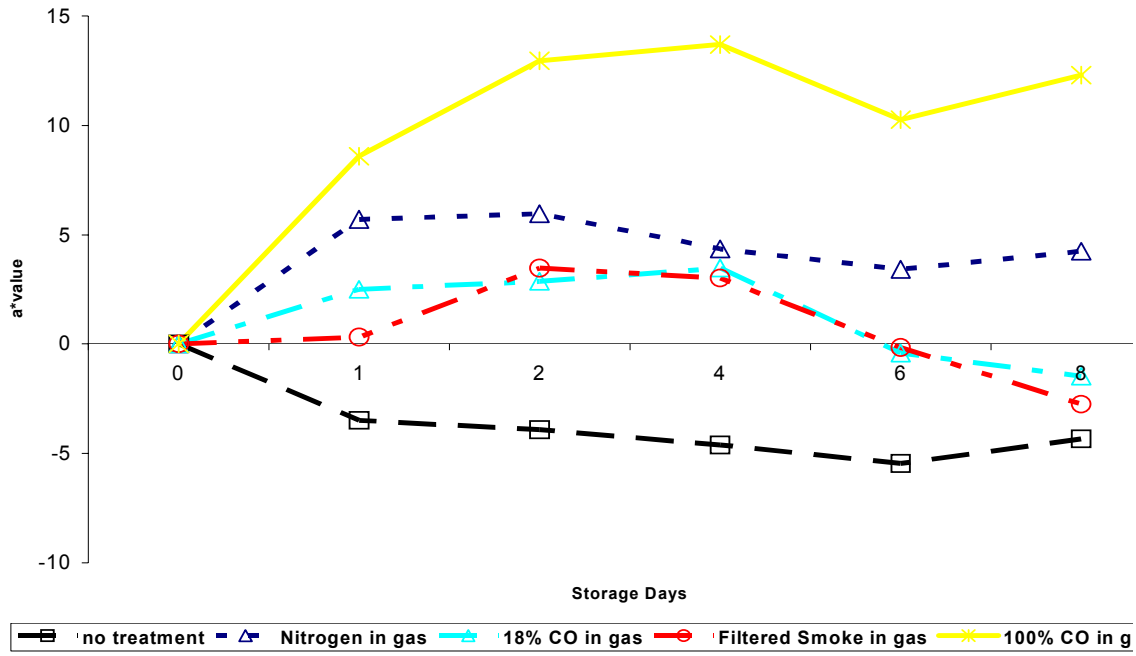

Figure 4-3. Changes in mean a*values for the red muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Minolta colorimeter.

According to both the Minolta color meter and the CMVS data the a*value for the red muscle stored in gas began to decline on day 6 for the 18% CO and filtered smoke but the 100% CO maintained a high a*value. This is attributed to the stable binding of the CO to the heme proteins, and an increased binding/stability would be expected in an environment saturated with CO. Samples kept in 100% N_2 showed a slight increase in a*value after 24 hours, which contradicted the results for the 24 hour treatments. This may have been due to the fillet variations. Little change was seen after day 1, suggesting that the red muscle was stabilized against heme oxidation. An increase in white muscle redness (more properly described as “pinkness”) on the 24 hour CO/FS treatments was also noted in a similar manner as the red muscle, however, the a*values were substantially lower since white muscle of mackerel has considerably lower heme protein content than the red muscle (Richards et al. 1998). Both the 100% and 18% CO and the

FS treatments for the samples kept in gas gave the same extension in red color for the white muscle. The white muscle data indicated that red color enhancement after treatment was higher than for the red muscle when CMVS is looked at, while the Minolta color meter data shows similar red color stability of the white vs. red muscle, where the filtered smoke and 18% CO treatments reverted back to the original a*value after about 3 days, while color was extended until day 6 for the 100% CO treatment.

The data collected with the Minolta color meter revealed an overall increase in yellowness for all treatments and the control regardless of muscle type. On the other hand the CMVS showed either little change in the b*value for the first 2 days or a slight drop, followed by an increase CMVS was found to be a highly sensitive method detecting subtle color changes in fish samples better than the Minolta color meter (Demir and Kristinsson 2003a), including changes in b*value. Increase in yellowness in fish muscle can be attributed in part to more browning of the muscle due to heme protein oxidation and thus reduction in a*value (Richards et al. 2002). If CMVS data is compared for both a* and b* values it is observed that an increase in the b*value (Figures 4-4, 4-5, 4-6, and 4-7) corresponded to a decrease in a*values (Figures 4-8, 4-9, 4-10, and 4-11) for samples treated for 24 hours. Samples kept in gas changed less in yellowness with storage than samples treated for 24 hours. These samples also changed little in a*value after the initial rapid increase in a*value when placed in the gas. Increase in yellowness has also been attributed to compounds developing from lipid oxidation (Demir et al. 2003).

The three variables, treatment, color, and day were compared against one another for L*a*b*. On every day there was a significant difference between the red muscle's L*a*b* values and the white muscle's L*a*b* values. For the L* values there was a

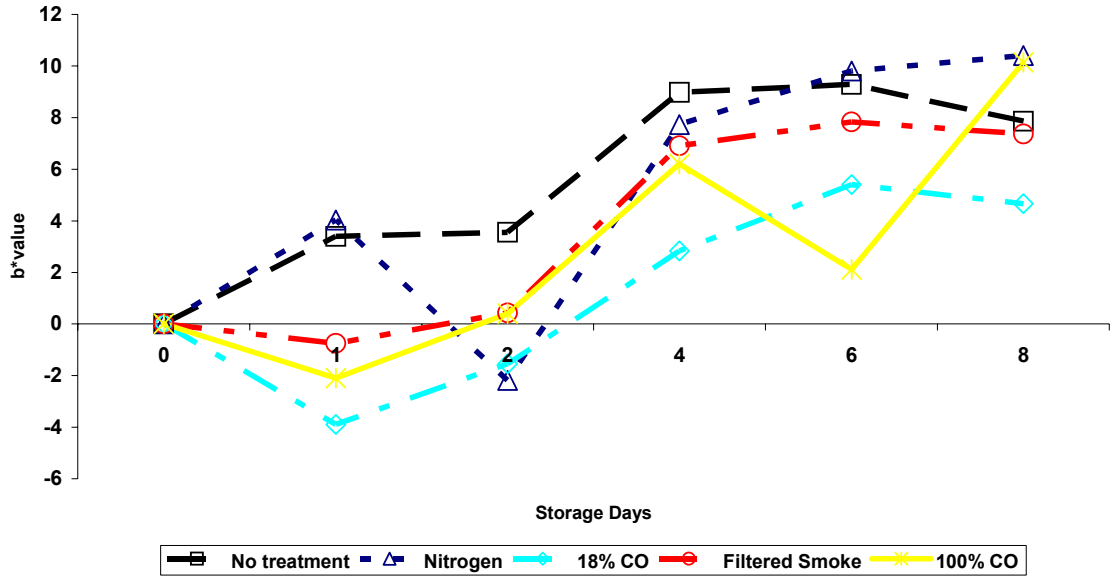

Figure 4-4. Change in b*values for the red muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Minolta colorimeter.

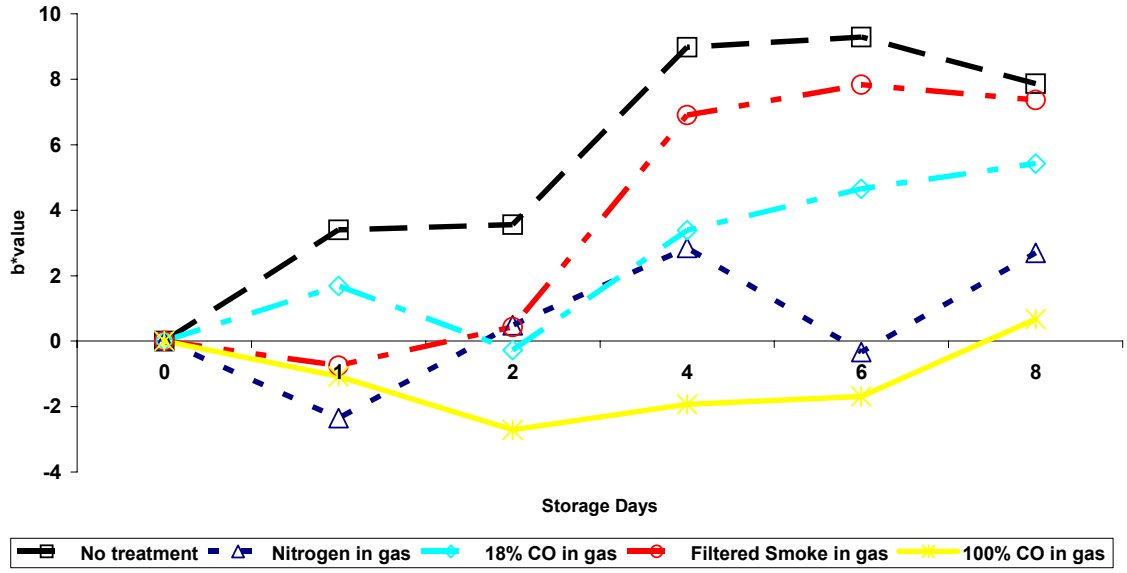

Figure 4-5. Change in b*values for the red muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Minolta colorimeter.

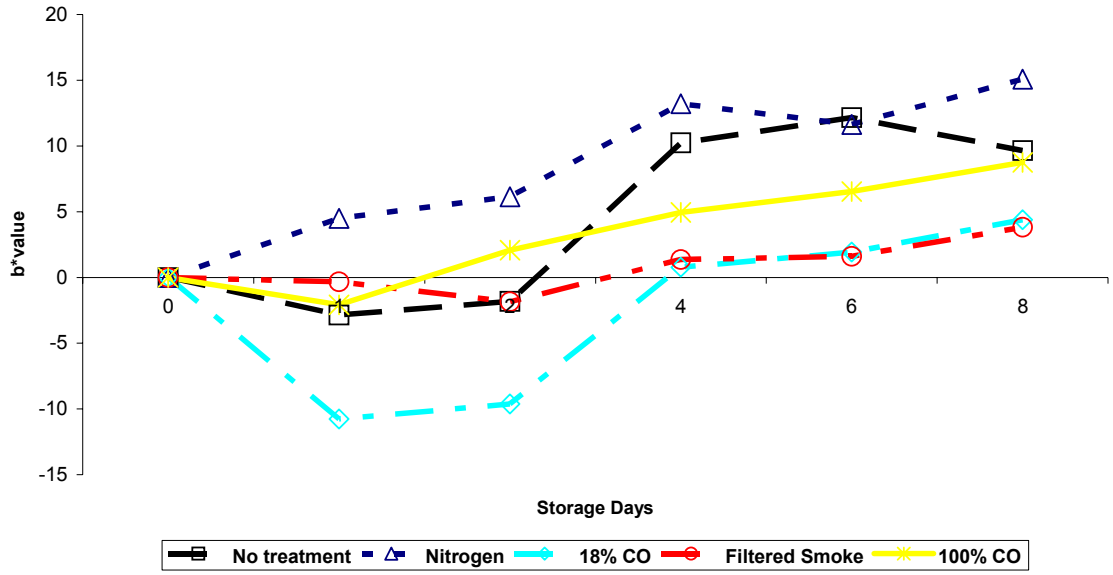

Figure 4-6. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Color Vision Machine.

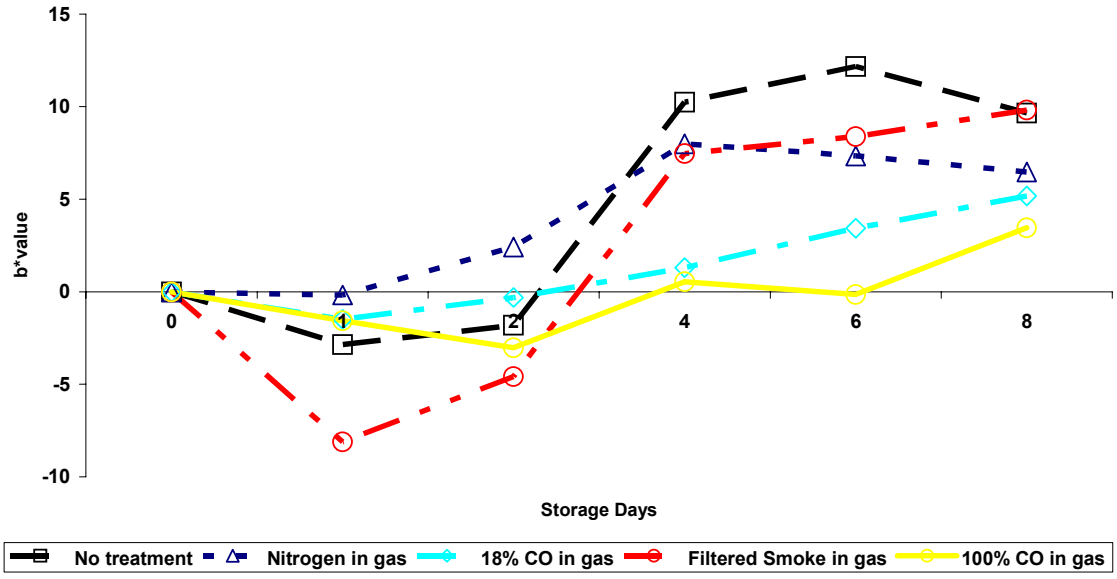

Figure 4-7. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Color Vision Machine.

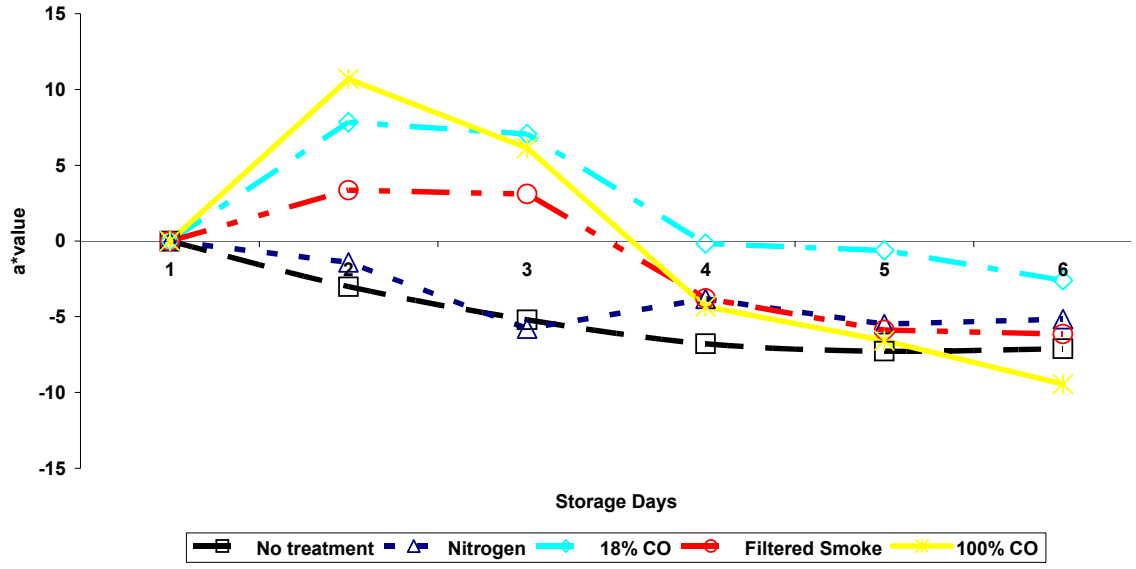

Figure 4-8. Change in a*values for the red muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Color Vision Machine.

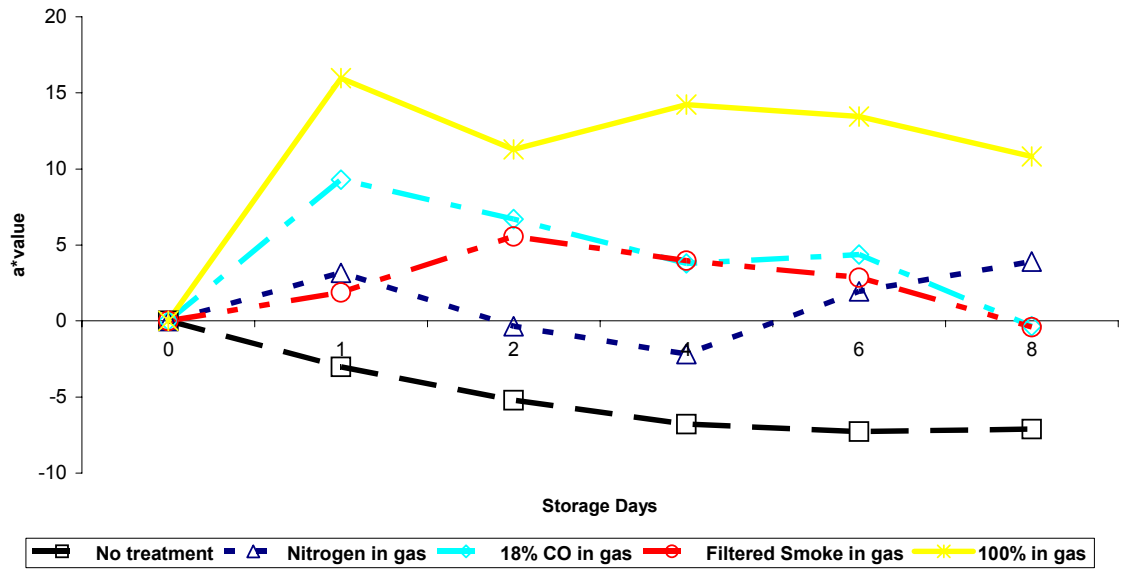

Figure 4-9. Change in a*values for the red muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Color Vision Machine.

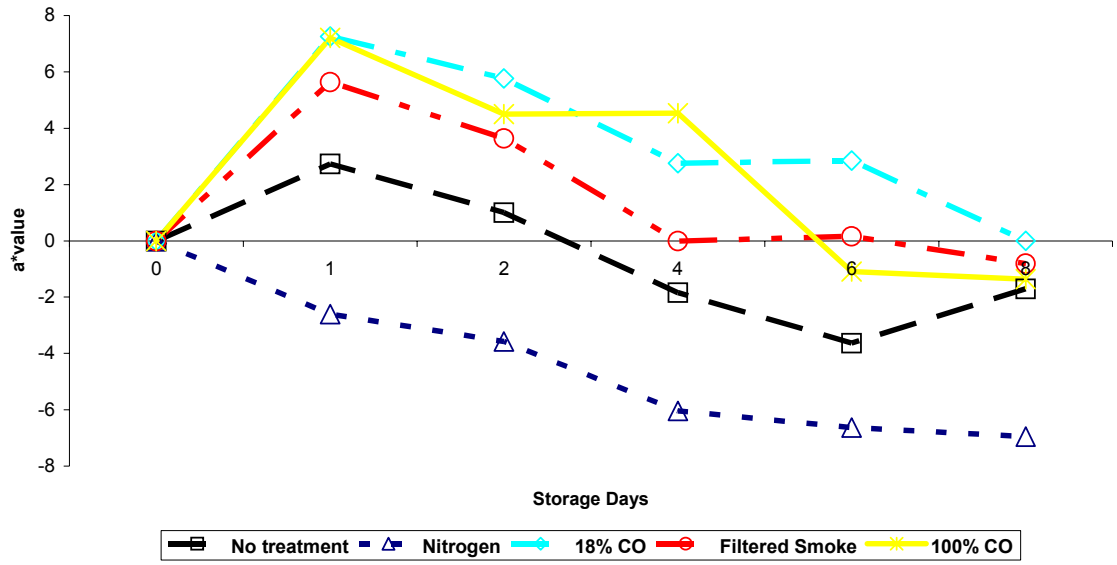

Figure 4-10. Change in a*values for the white muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Color Vision Machine.

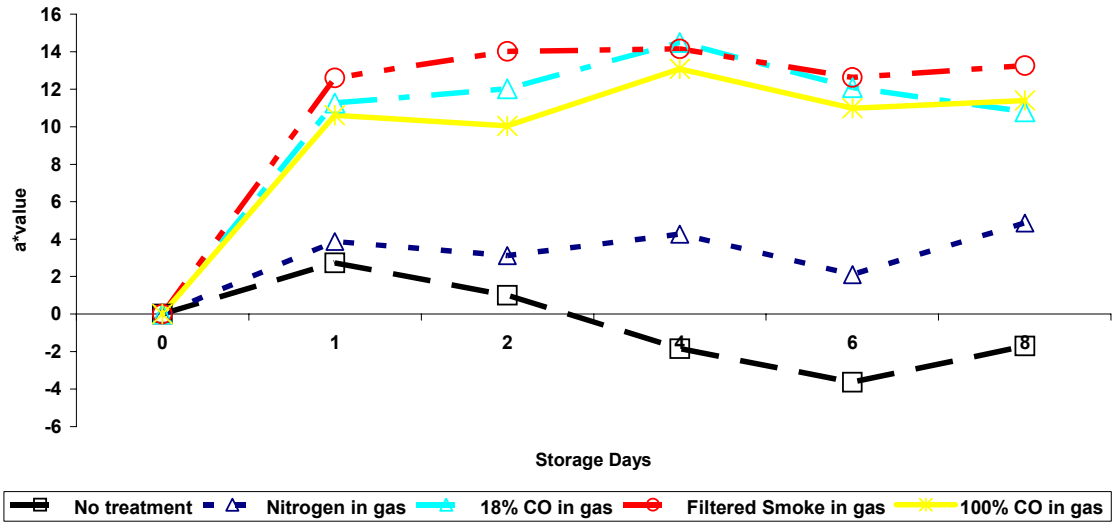

Figure 4-11. Change in a*values for the white muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Color Vision Machine.

significant difference between color and treatment and treatment and day ($P<0.0001$).For the "a" values there was a significant difference between color and treatment, treatment and day, and color, treatment, and day ($P<0.0001$). For the "b" value there was a significant difference between color and treatment and between treatment and day ($P<0.0001$).

Over time there was not a significant effects of the gas blends on the "L" and "b" values as time increased so did the "L" and "b" values; however, the gas blends had a significant effect on the "a" value. As time increased there was an enhancement in the "a" value. Over time there was an increase in the difference between the 24 hour treated Spanish mackerel and the in gas treated Spanish mackerel.

Heme Analysis

In the color analysis section it was demonstrated that CO/FS treatments lead to an increase in the redness and color stability when compared to the control. This increase was likely attributed to the formation of carboxyhemoglobin (CO-Hb) and carboxymyoglobin (CO-Mb) in the fish tissue. When complexed with CO hemoglobin was found to be substantially stabilized against oxidation compared to oxy-Hb (Kristinsson et al. 2003a). From Figures 4-12 and 4-13, it is apparent that the CO/FS treatments led to an initial increase in the heme peak wavelength.

This is attributed to the CO complexing with the heme proteins, unlike the control and 100% N_2 which exhibited a decrease in heme peak wavelength, signifying oxidation of the heme (Kristinsson et al. 2003a). The samples treated with CO/FS for 24 hour treatments started to lose CO immediately after treatment and exposure to air since the heme peak wavelength shifted down. The FS and 18% treatments had a similar

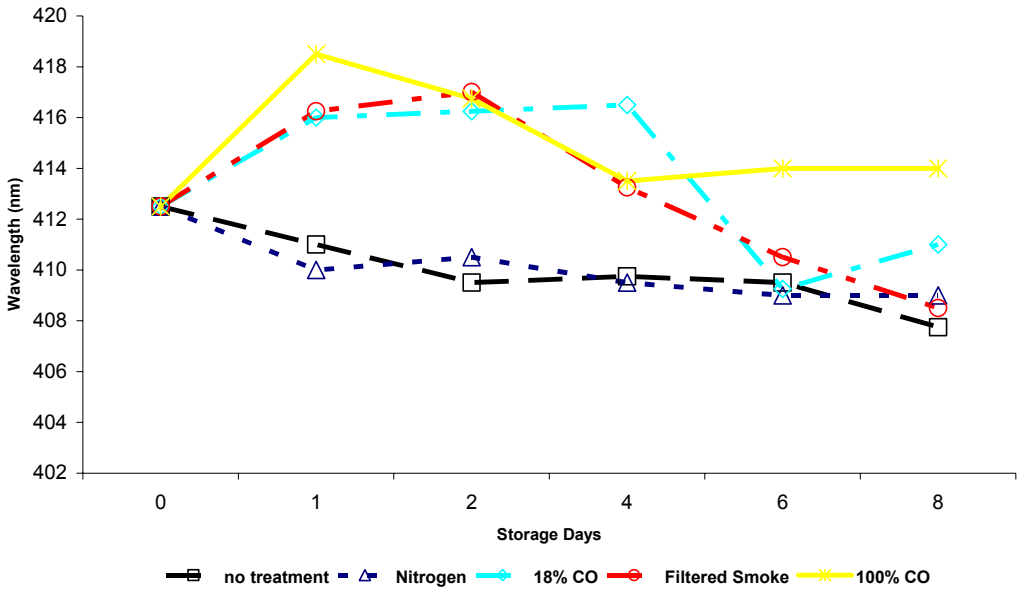

Figure 4-12. Mean heme values for Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h.

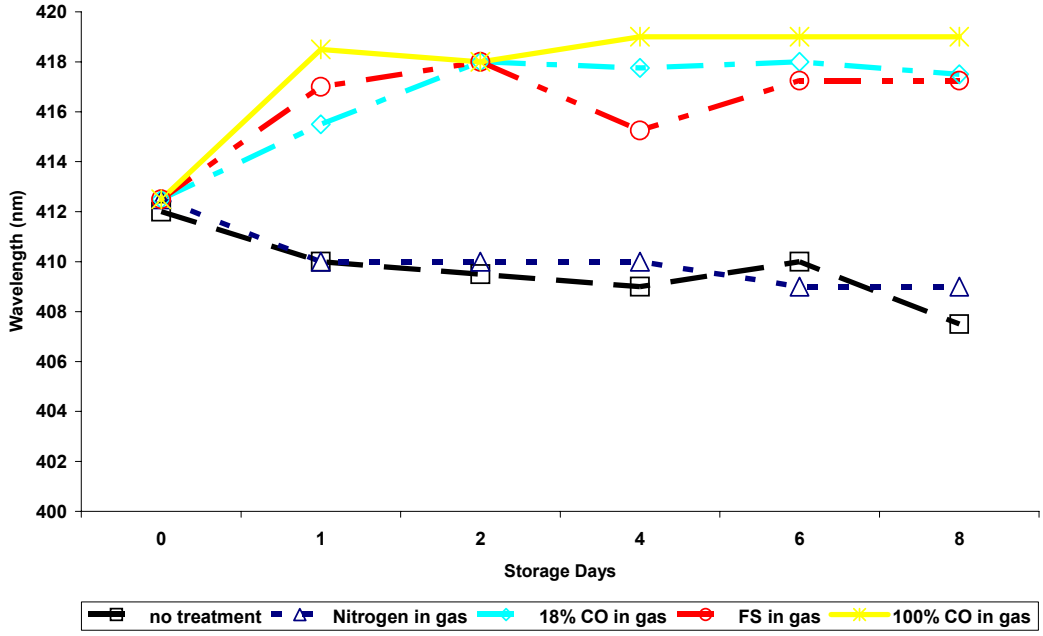

Figure 4-13. Mean heme values for Spanish mackerel stored at 4°C for 8 days while stored in the different gas blends.

stabilizing effect on the heme proteins and they reverted back to the oxy-Hb state after an estimated 5 days, while the 100% CO treatment remained above the original heme state throughout the study. The 100% CO treatment thus stabilized the heme proteins the most, which is in good agreement with the color stabilization results. This is likely since the 100% CO treatment also led to more binding after 24 hour, which is expected due to the higher concentration compared to the other gases.

Keeping the muscle in the CO/FS environment was expected to lead to an increase in the stability of muscle heme proteins since the presence of CO is expected to provide a continuous supply of CO and thus retard oxidation. Figure 4-13 shows that keeping the muscle in CO/FS resulted in most of the heme proteins in the muscle to bind to CO and remain bound to it throughout the study. Again the 100% treatment led to the most binding and stabilization compared to the other treatments, and the data suggests that all the heme proteins were bound to CO. This data is in good concord with a recent study by Kristinsson and coworkers (2003a) with tuna where red color enhancement and stability were found to be directly proportional to the percent CO used to treat the fish and subsequently the CO binding and stability of the heme proteins. Case in point Figure 4-14 illustrates the close relationship in red muscle a*value and heme peak wavelength of the extracted heme protein for the 100% CO in gas treatment. Thus the data clearly shows the CO binding to the heme proteins and the effect on their oxidation stabilization is the reason behind the color enhancement and stability for Spanish mackerel.

The two variables, treatment and day were compared against on another. There was a significant interaction between the two variables ($P<0.0001$). It was interpreted that as

time increased the in gas treated Spanish mackerel had an increase in color stability thus increasing heme stability.

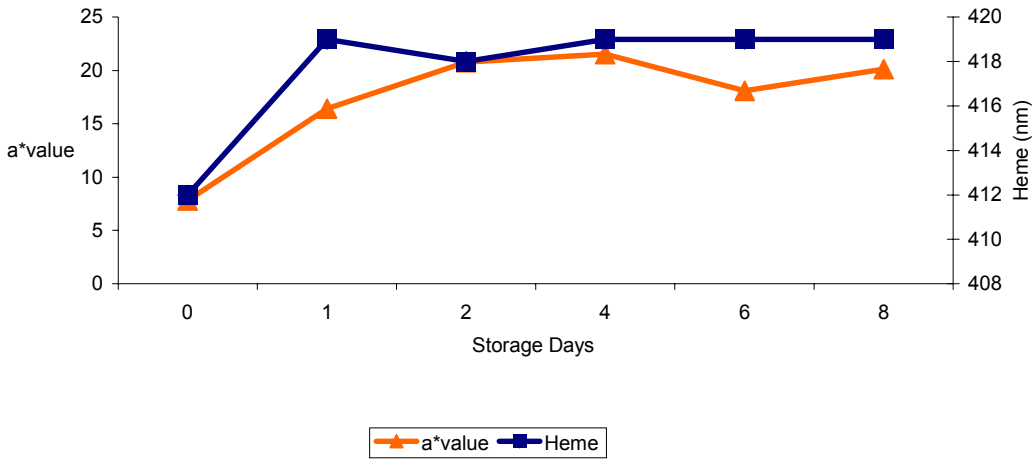

Figure 4-14. Change in a*value and heme over 8 days for 100% in gas treated Spanish mackerel.

Lipid Oxidation Development

Mackerel is a species that is rich in dark muscle relative to other species and high in unstable unsaturated lipids (Kelleher et al. 1992). Fat distribution is not uniform in mackerel since almost half of the fat is found below the skin in contact with the dark muscle, while about 30% is found in the white muscle (Ackman et al. 1991). It is well known that the dark muscle oxidized faster and more extensively than the white muscle since it has more pro-oxidants and more unstable lipids (Kelleher et al. 1994; Richards et al. 1998). Successful stabilization of mackerel muscle has been obtained by antioxidant treatments (Kelleher et al. 1992; 1994; Richards et al. 1998), which retard the activity of active pro-oxidants such as heme proteins, which are believed to be the main pro-

oxidants in fish muscle (Richards et al. 1998; Kristinsson 2002). Stabilization of heme proteins to oxidation is thus expected to reduce oxidation of lipids.

The results show that CO/FS treatment of the muscle had an effect on lipid oxidation formation. From Figure 4-15 it is apparent there is a steady increase in oxidation with all treatments. Samples treated with 100% N_2 for 24 h and the control showing the most rapid oxidation formation, maximizing at day 2, while all the CO treatments developed maximum TBARS values at day 4 (FS), 6 (100% CO) and 8 (18% CO).

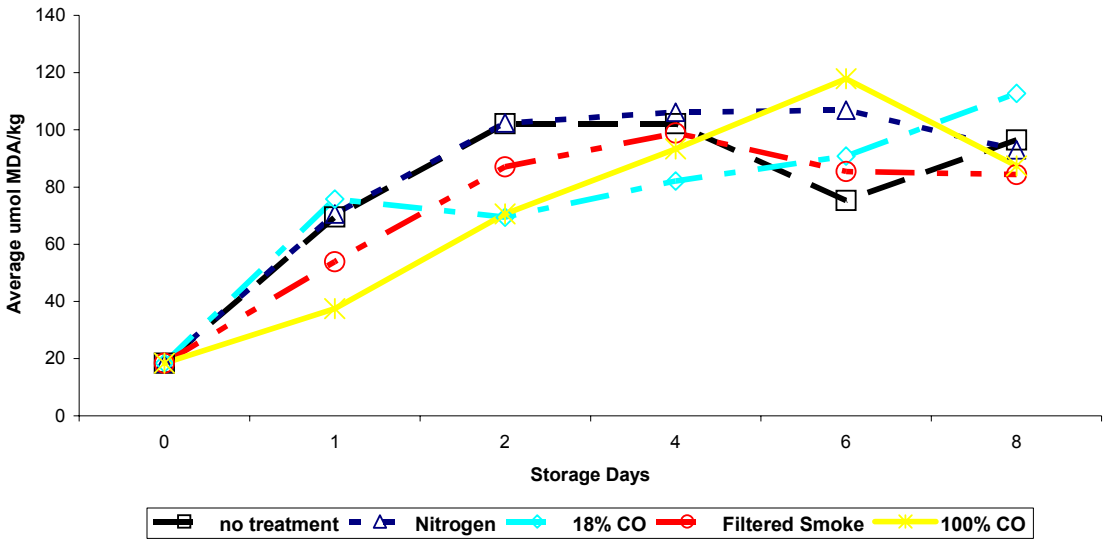

Figure 4-15. Mean malonaldehyde values of red muscle for Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h.

It was interesting to note the rapid development oxidation of the 18% samples at day 1 especially since it had a significantly lower TBARS value at day 1 for the in-gas study. Figure 4-16, shows the results for the fillets kept in gas environments for 8 days. It was unambiguous that keeping the fillets in the gas environments led to significantly lower oxidation formation and slower onset of oxidation than the 24h treatments.

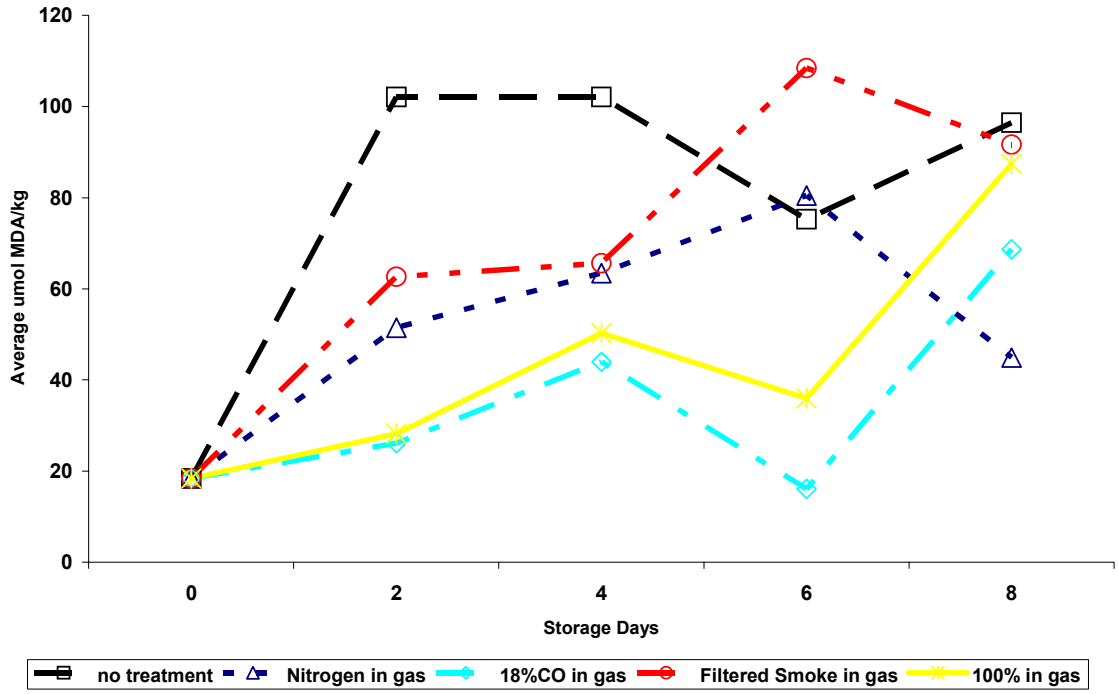

Figure 4-16. Mean malonaldehyde values of red muscle for Spanish mackerel stored at 4°C for 8 days while stored in the different gas blends.

Samples kept in 100% and 18% CO led to the most stabilization against lipid oxidation since they had the lowest levels of oxidation. Fillets kept in 100% N_2 and filtered smoke were not as effective in retarding oxidation. These results provide evidence that CO has a direct antioxidative effect since if oxygen is the reason behind the stabilization the 100% N_2 and 100% CO should have been equally effective. It is therefore highly probably that the stabilization of the heme proteins via CO binding plays an important role in this stabilization against oxidation. FS was less effective against lipid oxidation compared to the commercial gas of similar composition, contrary to expectations since FS contains phenolic smoke components. There is however a possibility that phenolic components could serve as pro-oxidants, which appears to be the case here.

Figures 4-17 and 4-18 corresponding to lipid oxidation in white muscle show a similar trend as Figures 4-15 and 4-16, except the oxidation level is significantly lower.

This is in agreements with studies performed by Kelleher and coworkers (1992; 1994) and Richards and coworkers (1998).

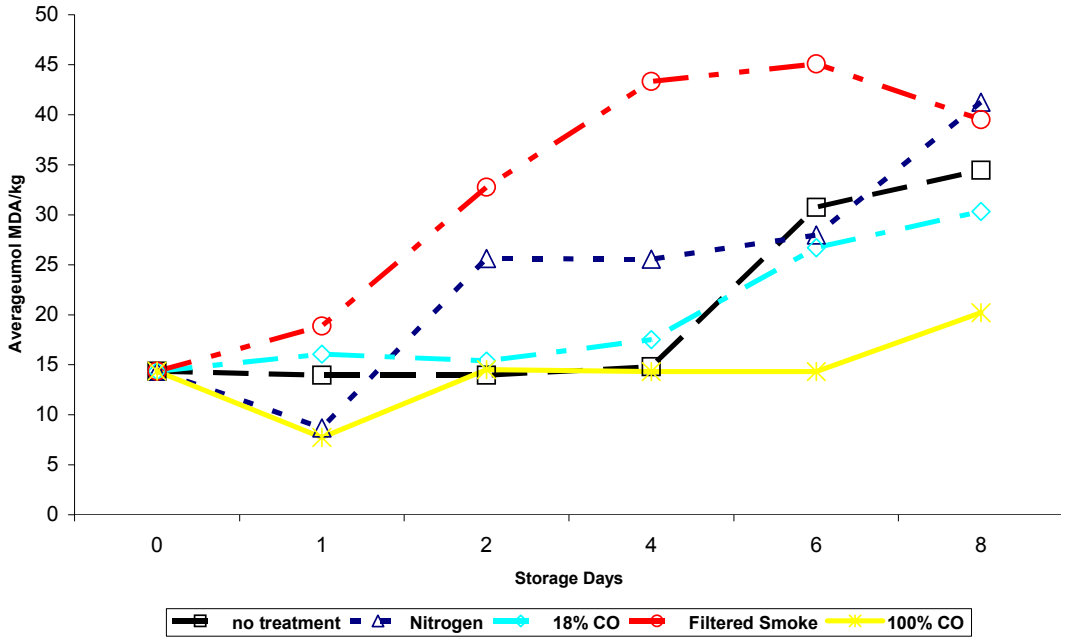

Figure 4-17. Mean malonaldehyde values of white muscle for Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h.

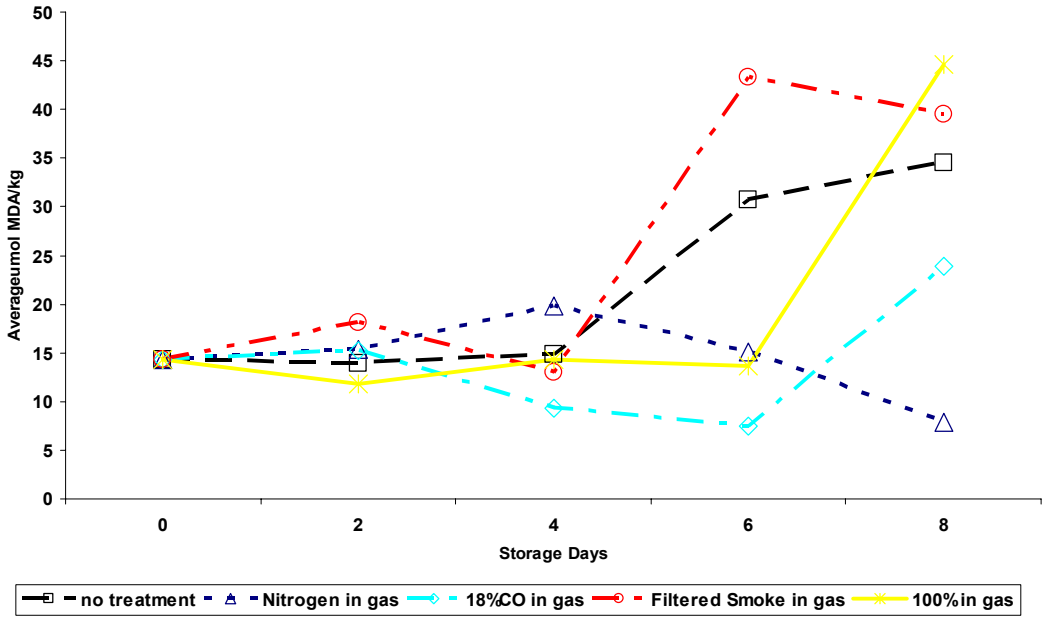

Figure 4-18. Mean malonaldehyde values of white muscle for Spanish mackerel stored at 4°C for 8 days while stored in the different gas blends.

The white muscle showed a steady increase in lipid oxidation but is significantly lower than the red muscle, presumably because there is considerably lower heme protein content and lipid content in white muscle (Richards et al. 1998). The pro-oxidative effect of FS was again noticed for white muscle, while the other CO treatments effectively retarded oxidation development. That the 24 h 100% N_2 treatment promoted oxidation could be due to fact that the deoxy-form of Hb and Mb was formed which has been reported to be more pro-oxidative than the reduced oxy-Hb form in the presence of air (Richards and Hultin 2000).

The variables, treatment, day, and color were compared against one another. There was a significant difference between treatment and day ($P<0.0001$). It was interpreted that as time increased there was an increase in lipid oxidation more so for the red muscle than the white muscle.

Microbial Analysis

Microbial evaluation for aerobic plate count was performed on each treatment using Petrifilm™ media. There was no identification of individual microbial strains, just an overall count was obtained. The plate counts ranged from 10^2- 10^8 log cfu/g. Beginning on day 3 there was at least a 1 log difference between the controls (no treatment, N_2 and N_2 in gas) and the treatments (filtered smoke, filtered smoke in gas, 18% CO, 18% CO in gas, 100% CO, and 100% CO in gas). The largest log difference was 4 log, seen on day 4 (Figures 4-19 and 4-20).

The Spanish mackerel treated for 24 hours and the Spanish mackerel in gas, both show an increase in microbial growth as time increases; however, there is a larger difference between the controls and the in gas treated Spanish mackerel (Figure 4-20),

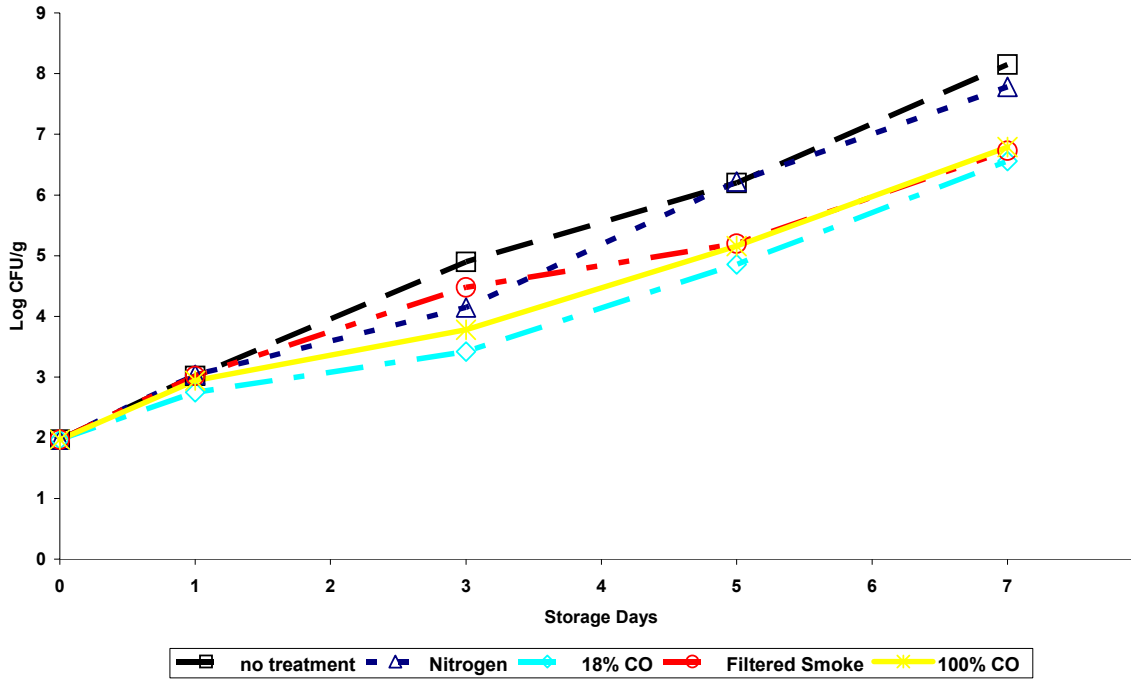

Figure 4-19. Mean log cfu/g values of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h.

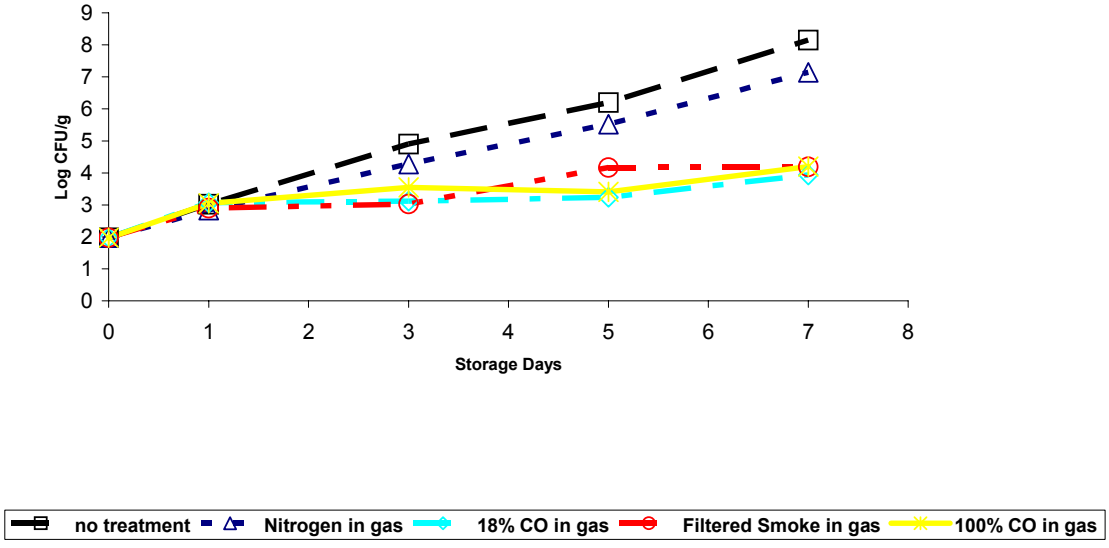

Figure 4-20. Mean log cfu/g values of Spanish mackerel stored at 4°C for 8 days while stored in the different gas blends.

showing that CO/FS treatments may be effectively used to extend the microbial shelf life.

This is in part due to the exclusion of oxygen, which is necessary for aerobic microbial

growth. However the data also indicated a direct effect of CO, since the 100% CO treatment more effectively retarded microbial growth compared to the 100% N_2 treatment. Claims have been made from companies producing and using FS that it has an antimicrobial effect due to the presence of wood phenolics (Hawaii International Seafood Inc. 1999). These studies with Spanish mackerel do not support these claims since the 18% CO gas mixture was found to be even better than the FS which also contained 18% CO. Studies published on FS and tuna report similar findings (Kristinsson et al. 2003). Low levels of CO have been reported to have little effect on the microflora of meat (Clark et al. 1976; Gee and Brown 1978; Luno et al. 2000) but studies have shown that CO may suppress aerobic microorganisms during prolonged storage in gaseous environments (Luno et al. 2000, Kristinsson et al. 2003), which is supported by the data with the Spanish mackerel fillets.

There was a significant difference between the variables treatment and day ($P<0.05$). As the days went on there was an increase in the difference between the 24 hour treated samples and the in gas treated samples. It was interpreted that, as the storage time increased the effectiveness of the gases increased.

Histamine Analysis

Histamine analysis was conducted on all samples. Histamine forming bacteria were present on all the samples (treated and untreated) at some point post-harvest. This is indicated by the presence of histamine. The control and 100% N_2 treated Spanish mackerel on day 5 had the highest level of histamine formation. Although this sample had the highest level of histamine formation, it was well below both the legal safety action level of 50 ppm and the 5 ppm level indicative of decomposition. Histamine

formation is normally an indicator of thermal abuse since the bacteria producing the enzyme that converts histidine to histamine only grows rapidly at temperatures higher than normal refrigerated storage (Huss 1994)). The Spanish mackerel were kept at 4°C throughout the study, which can explain the low histamine formation. Histamine formation is also highly variable from fish to fish, and depends on season and fishing ground (Huss 1994)). The results for Spanish mackerel concurred with the histamine levels reported by Middlebrooks et. al (1988) in fish decomposed at 0°C which remained low (<0.6 ppm) throughout a 380 hour incubation period. The slight differences seen between histamine levels in for the mackerel control, filtered smoke, filtered smoke in gas, 18% CO, 18% CO in gas, 100% CO, 100% CO in gas, N_2, and N_2 in gas could be attributed to the gas treatment, the storage in -80°C freezer (until analyzed), or a combination of both. Like the study conducted by Ross (2000), histamine formation did not appear to be directly inhibited by CO treatment, however this study cannot conclude this since levels were low. Thermal abuse studies are needed to examine the effect CO and FS has on histamine production.

The two variables, treatment and day were compared against on another. There was a significant interaction between the two variables ($P<0.0001$). It was interpreted that, CO gas blends effectiveness did not have much of an effect on histamine formation as time increased.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Over the past few years, the use of carbon monoxide has garnered a lot of attention. This study demonstrated that CO/FS treatments will significantly affect the red color development and intensity in Spanish mackerel muscle. Depending on which color analysis instrument you use, the Minolta color meter or the CMVS, the extent of the color development will vary. The Minolta color meter only looks at a small part of the sample of interest and is standardized by a white tile; whereas the CMVS analyzes every pixel of the sample of interest, allows the user to select the region of interest, and is standardized with several different tiles. The CO/FS treatments also significantly stabilize the heme proteins, especially when kept in the presence of gas containing CO, which in turn leads to a delayed oxidation formation. Although the microbial formation increases as the Storage days went by it appears that the 24 h treatment with CO/FS does lead to an extension in microbial shelf life while the in gas treatment may be more effectively used to extend the microbial shelf life. Histamine analysis showed that treatment with CO does not inhibit histamine formation, however since production was very low in this study little information can be derived from the data. This study also demonstrated that filtered smoke does not provide any benefit over a commercial gas mixture with similar composition, and in fact FS may be less effective for lipid oxidation stabilization and suppression of microbial growth. Of all the treatments tested the 100% CO treatment stored in gas appeared to lead to the best overall muscle stability and thus quality. 100% CO treatment stored in gas stabilized the heme proteins, leading to delayed oxidation

formation and effectively retarded microbial growth. These results justify the usage of CO/FS treatments as a means for preserving Spanish mackerel. Further experiments need to be done to address the following things: the effects of CO in inferior quality Spanish mackerel identify the microbial strains and determine if *C. botulinum* can grow in the modified atmosphere packaging, the level of CO residue found in the Spanish mackerel tissue, and the effects of these residues on human health.

APPENDIX
DATA

Table A-1. Mean and standard deviation for L* values of Spanish mackerel red muscle stored at 4°C for 8 days using the Minolta colorimeter.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	43.8±6.21 (a)	40.6±5.17 (a)	43.0±3.69 (a)	39.1±3.04 (b)	45.6±3.92 (a)	39.2±2.43 (b)	46.5±3.09 (a)	44.4±3.19 (a)	46.5±4.4 (a)
1	45.1±3.66 (a,b)	43.1±5.27 (a,b)	48.1±4.35 (a,b)	37.4±1.28 (c)	45.5±3.10 (a,b)	39.7±2.55 (c)	45.5±3.54 (a,b)	46.3± 2.43 (a,b)	42.8±3.57.(a,b)
2	44.9±3.56 (a)	43.7±5.35 (a)	46.1±2.98 (a)	39.3±3.19 (b)	45.5±5.06 (a)	38.0±2.08 (b)	44.1±3.70 (b)	44.9±2.93 (a)	42.3±3.02 (a)
4	46.3±5.46 (a,b)	40.9±2.70 (b)	42.9±2.18 (b)	38.4±2.41 (c)	44.7±3.42 (a,b)	40.5±3.71 (c)	42.4±1.42 (b)	46.5±2.55 (a)	42.2±2.74 (a,b)
6	44.3±3.09 (b)	42.9±3.86 (b)	43.9±1.45 (b)	41.9±1.37 (b)	42.8±1.96 (b)	40.8±3.21 (b)	44.8±1.50 (a)	46.5±4.99 (a)	42.5±3.14 (b)
8	45.1 ±3.84 (b,c)	41.8±4.22 (b,c)	45.9±2.35 (b)	41.0±1.80 (c)	46.2±4.26 (b)	43.5±4.55 (b)	44.1±1.34 (b)	45.1±3.84 (a)	41.0±1.18 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-2. Mean and standard deviation for L* values of Spanish mackerel red muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	59.6±10.7 (a)	58.4±10.9 (a)	62.7±11.4 (a)	58.9±10.8 (b)	63.2±10.0 (a)	57.2±10.8 (b)	64.90±15.1 (a)	74.5±12.2 (a)	56.9±8.23 (a)
1	62.5±10.1(a,b)	61.6±11.0 (a,b)	65.3±11.3 (a,b)	60.6±8.80 (c)	59.0±8.37 (a,b)	57.2±9.71 (c)	57.8±8.36 (a,b)	62.6±7.88 (a,b)	58.7±9.88 (a,b)
2	65.5±11.1 (a)	56.1±9.00 (a)	63.7±11.0 (a)	58.8±7.64 (b)	64.3±9.04 (a)	53.1±8.17 (b)	62.4±9.58 (b)	66.0±3.69 (a)	57.1±7.85 (a)
4	65.2±8.41 (a,b)	57.6±9.48 (b)	64.6±9.15 (b)	62.2±9.73 (c)	62.2±8.45 (a,b)	58.2±9.51(c)	60.0±8.12 (b)	71.6±7.14 (a)	54.4±8.00 (a,b)
6	67.2±9.75 (b)	63.8±11.1 (b)	66.7±9.54 (b)	62.2±8.43 (b)	65.1±8.27 (b)	60.2±8.70 (b)	61.5±6.27 (a)	69.7±5.44 (a)	58.3±6.99 (b)
8	65.3±8.76 (b,c)	58.2±8.26 (b,c)	64.8±8.76 (b)	59.3±7.11 (c)	66.0±9.61 (b)	62.6±7.99 (b)	65.6±8.24 (b)	69.8±5.59 (a)	57.4±5.95 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-3. Mean and standard deviation for L* values of Spanish mackerel white muscle stored at 4°C for 8 days using the Minolta.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	46.5±1.10 (a)	46.7±1.95 (a)	47.6±3.06 (a)	45.2±1.87 (b)	48.5±3.90 (a)	46.1±2.06 (b)	40.0±2.07 (a)	48.4±3.19 (a)	49.6±3.84 (a)
1	46.2±0.726 (a,b)	47.8±2.76 (a,b)	50.1±2.28 (a,b)	43.3±1.03 (c)	50.7±2.73 (a,b)	44.3±1.52 (c)	52.7±3.88 (a,b)	51.3±4.02 (a,b)	51.1±2.15 (a,b)
2	44.6±1.80 (a)	49.0±3.19 (a)	47.4±3.45 (a)	43.1±3.19 (b)	43.3±3.16 (a)	44.7±3.01 (b)	51.0±2.20 (b)	50.3±2.45 (a)	49.9±1.99 (a)
4	48.8±1.56 (a,b)	51.6±1.34 (b)	48.3±5.00 (b)	45.4±2.19 (c)	51.2±4.89 (a,b)	45.5±1.84 (c)	49.7±3.42 (b)	53.5±2.67 (a)	50.1±2.55 (a,b)
6	45.9±2.14 (b)	40.6±3.23 (b)	48.1±3.15 (b)	45.3±1.64 (b)	49.1±4.29 (b)	46.7±3.58 (b)	48.9±2.90 (a)	54.7±1.78 (a)	48.7±2.52 (b)
8	44.4±1.83 (b,c)	46.9±2.10 (b,c)	47.2±2.71 (b)	43.4±1.79 (c)	49.6±4.78 (b)	48.8±3.28 (b)	48.8±2.33 (b)	57.6±2.32 (a)	46.7±2.94 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-4. Mean and standard deviation for L* values of Spanish mackerel white muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	76.9±3.49 (a)	79.9±2.73 (a)	74.9±4.67 (a)	79.3±2.20 (b)	80.4±5.5 (a)	79.7±3.34 (b)	80.1±3.57 (a)	87.6±2.96 (a)	80.2±4.57 (a)
1	85.2±3.10 (a,b)	87.2±2.70 (a,b)	75.7±5.07 (a,b)	85.5±3.66 (c)	78.9±4.93 (a,b)	76.8±3.08 (c)	80.3±3.98 (a,b)	87.8±3.13 (a,b)	82.1±4.41 (a,b)
2	86.0±2.93 (a)	85.8±4.52 (a)	77.4±5.62 (a)	83.3±3.43 (b)	79.9±6.17 (a)	79.24±2.81 (b)	80.2±4.33 (b)	87.5±3.69 (a)	82.9±4.70 (a)
4	80.3±2.48 (a,b)	83.7±4.68 (b)	75.0±5.24 (b)	78.8±3.27 (c)	77.01±5.47 (a,b)	84.73±3.16 (c)	79.0±5.80 (b)	92.2±2.76 (a)	84.2±19.5 (a,b)
6	88.3±2.86 (b)	90.1±5.69 (b)	76.2±5.47 (b)	85.7±5.31 (b)	78.6±7.75 (b)	81.0±3.63 (b)	79.8±5.1 (a)	89.2±3.79 (a)	80.3±5.23 (b)
8	81.1±4.83 (b,c)	83.6±5.70 (b,c)	83.6±5.70 (b)	78.5±361(c)	76.8±5.97 (b)	81.6±3.78 (b)	79.8±3.39 (b)	89.9±4.66 (a)	78.4±6.89 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-5. Mean and standard deviation for a* values of Spanish mackerel red muscle stored at 4°C for 8 days using the Minolta.

Storage Day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	8.18±3.35 (a)	11.8±450 (a)	11.7±1.56 (a)	7.26±0.774 (a)	10.8±1.92 (a)	11.2±2.81 (a)	7.83±2.20 (a)	9.08±0.150 (a)	8.34±3.65 (a)
1	4.69±3.20 (a)	13.9±3.64 (c)	12.0±2.36 (c)	11.3±0.771 (b,c)	13.3±5.32 (c)	19.6±4.67 (c)	16.4±3.94 (c)	6.33±1.16 (a,b)	14.0±1.99 (c)
2	4.23±1.47 (a)	7.33±5.58 (a,b)	15.2±3.27 (e)	9.23±2.66 (a,b,c)	13.6±4.21 (b,c,d)	13.9±4.43 (b,c,e)	20.8±4.19 (b,d)	5.98±1.87 (a)	14.3±2.06 (b,c,d)
4	3.56±2.15 (a)	6.54±2.68 (a,b)	14.7±2.38 (c)	5.67±2.40 (a,b)	14.2±3.59 (c)	7.13±2.73 (b)	21.5±1.61 (b)	5.10±0.960 (d)	12.7±1.11 (c)
6	2.71±1.97 (a)	4.69±1.57 (a)	11.5±1.61 (c)	3.03±1.37 (a)	10.3±1.55 (b)	5.85±2.29 (a)	18.1±2.39 (b)	4.24±1.82 (a)	11.8±2.14 (b)
8	3.34±1.55 (a)	4.06±0.943 (a)	8.94±0.852 (c)	2.44±0.988 (a)	9.28±1.24 (b)	5.10±1.59 (b)	20.1±2.06 (c)	4.09±1.02 (a)	12.6±1.25 (b)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-6. Mean and standard deviation for a* values of Spanish mackerel red muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	13.5±4.88 (a)	14.4±5.67 (a)	17.5±5.14 (a)	10.3±4.50 (a)	20.0±6.45 (a)	17.4±6.48 (b)	19.1±6.16 (a)	12.8±5.17 (a)	21.7±4.36 (a)
1	10.4±2.99 (a)	17.8±4.80 (c)	19.3±5.15 (c)	18.2±4.40 (b,c)	29.3±6.24 (c)	28.1±8.72 (c)	35.1±6.03 (c)	11.4±4.05 (a,b)	24.8±7.14 (c)
2	8.25±2.79 (a)	17.5±4.37 (a,b)	23.0±5.40 (e)	17.4±3.31 (a,b,c)	26.7±6.34 (b,c,d)	23.6±6.31 (b,c,e)	30.4±8.17 (b,d)	7.06±2.82 (a)	21.3±4.28 (b,c,d)
4	6.68±2.80 (a)	10.6±3.66 (a,b)	21.4±3.96 (c)	10.2±3.53 (a)	23.8±7.03 (c)	13.1±4.01 (b)	33.4±6.54 (b)	9.02±2.13 (d)	19.5±4.04 (c)
6	6.20±2.84 (a)	8.51±2.92 (a)	20.31±2.07 (c)	9.73±2.84 (a)	24.1±6.68 (b)	10.8±2.99 (a)	32.6±4.45 (b)	7.35±2.43 (a)	23.6±4.04 (b)
8	6.36±3.21 (a)	8.25±2.40 (a)	17.0±4.05 (c)	7.75±2.98 (a)	19.7±7.41 (b)	7.97±3.06 (b)	30.0±5.4 (c)	7.68±2.02 (a)	25.6±3.35 (b)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-7. Mean and standard deviation for a* values of Spanish mackerel white muscle stored at 4°C for 8 days using the Minolta.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	0.463±0.579 (a)	0.1±0.687 (a)	3.75±0.604 (a)	0.788±0.703 (a)	2.93±0.900 (b)	-0.425±0.333 (c)	3.14±0.834 (d)	0.488±0.150 (a)	2.45±0.560 (b)
1	0.05±0.726 (a)	0.813±1.03 (a)	6.54±1.31 (c)	1.99±0.679 (a)	5.18±1.51 (b)	0.975±1.18 (a)	4.80±0.690 (c)	-0.625±0.390 (b)	2.88±1.06 (c)
2	0.788±.513 (a)	0.963±0.764 (a)	6.83±2.83 (d)	1.45±0.761 (a)	5.03±1.57 (c)	0.638±0.446 (a)	5.38±1.36 (d)	-1.13±0.657 (b)	2.60±0.549 (c)
4	-0.863±0.636 (a)	-0.813±0.458 (d)	6.81±1.15 (c,d)	0.15±0.303 (b)	5.66±1.87 (c)	0.275±0.709 (a)	4.91±3.59 (c,d)	-1.75±0.634 (a)	2.53±1.13 (c)
6	1.78±0.757 (a)	3.03±0.486 (a,b)	6.63±2.14 (e)	0.213±0.505 (c)	3.65±0.997 (d,e)	-0.338±0.836 (a,b)	5.04±1.39 (e)	-2.09±0.503 (a,b)	1.78±1.11 (c,d)
8	4.06±.360 (a)	-1.63±0.326 (a)	5.46±1.86 (c)	-0.35±0.619 (b)	3.63±1.00 (b,c)	1.04±0.420 (b)	4.15±1.46 (c)	2.23±0.507 (c)	1.49±0.486 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-8. Mean and standard deviation for a* values of Spanish mackerel white muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	7.09±2.64 (a)	6.84±2.67 (a)	19.9±2.68 (a)	8.00±2.90 (a)	13.2±3.50 (b)	7.74±2.45 (c)	12.7±2.54 (d)	10.6±2.48 (a)	13.6±2.49 (b)
1	9.82±2.71 (a)	12.48±2.99 (a)	32.5±3.60 (c)	15.3±3.37 (a)	24.5±4.32 (b)	15.0±2.90 (a)	23.3±3.32 (c)	8.02±3.38 (b)	17.4±2.50 (c)
2	8.09±3.37 (a)	10.5±4.31 (a)	33.9±4.65 (d)	13.8±3.84 (a)	25.3±4.74 (c)	12.2±3.30 (a)	22.8±3.48 (d)	7.06±2.82 (b)	16.7±3.12 (c)
4	5.24±2.67 (a)	6.84±4.36 (d)	34.0±5.48 (d)	10.8±3.63 (b)	27.7±4.84 (c)	12.3±3.13 (a)	25.8±2.13 (c,d)	4.59±2.60 (a)	17.8±3.68 (c)0
6	3.45±2.54 (a)	7.01±3.79 (a,b)	32.5±5.05 (e)	10.9±3.62 (c)	25.3±5.74 (c)	6.65±3.47 (a)	23.7±5.16 (c,d)	4.00±2.76 (a)	15.7±3.57 (c)
8	5.38±2.99 (a)	6.03±3.81 (a)	33.2±3.49 (c)	8.01±3.29 (b)	24.0±5.31 (b,c)	6.38±3.29 (b)	24.1±4.89 (c)	3.67±2.66 (c)	6.03±3.81 (c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-9. Mean and standard deviation for b* values of Spanish mackerel red muscle stored at 4°C for 8 days using the Minolta.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	5.00±2.23 (b)	4.43±1.74 (b)	6.75±0.780 (a,b)	3.76±1.92 (b)	6.30±1.72 (b)	6.35±0.895 (a,b)	4.68±1.35 (b)	8.23±0.598 (a)	4.91±1.59 (b)
1	5.11±2.94 (c)	5.58±1.42 (b)	6.15±1.84 (b)	5.34±1.16 (c)	7.52±1.76 (a,b)	6.98±0.833 (a,b)	6.58±1.70 (a,b)	7.94±1.39 (a)	5.94±0.900 (b)
2	6.60±2.31 (b,c)	3.90±1.43 (c)	7.95±1.22 (a,b)	6.26±1.04 (b,c)	7.58±2.04 (b)	7.04±0.891 (b,c)	8.69±1.05 (a,b)	9.49±1.11 (a)	7.03±1.14 (b)
4	8.14±2.59 (a,b)	6.30±0.745 (c)	8.78±1.00 (a,b)	7.11±1.31 (b,c)	7.60±1.38 (b,c)	7.94±1.16 (b,c)	8.54±0.909 (a,b)	9.85±2.22 (a)	7.48±0.826 (b,c)
6	9.65±2.35 (b,c)	7.93±1.61 (b,c)	7.63±0.800 (b,c,d)	6.50±4.45 (c,d)	5.90±0.818 (c,d)	9.14±2.40 (a,b)	8.00±1.29 (b)	11.6±1.37 (a)	5.65±1.07 (d)
8	9.29±3.50 (b,c)	8.05±2.51 (b,c)	8.25±2.58 (b,c)	7.21±2.60 (c)	8.93±1.05 (b,c)	10.3±1.67 (b)	8.79±0.514 (b,c)	12.5±3.50 (a)	4.80±0.562 (e)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-10. Mean and standard deviation for b* values of Spanish mackerel red muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	8.56±3.09 (b)	6.24±3.96 (b)	6.35±2.95 (a,b)	7.93±3.25	2.00±3.78 (b)	5.63±3.08 (a,b)	3.19±8.85 (b)	9.25±3.95 (a,)	4.62±2.59 (b)
1	11.96±3.38 (c)	5.49±3.90 (b)	7.53±3.35 (b)	4.03±4.06 (c)	3.68±2.76 (a,b)	3.53±2.54 (a,b)	2.11±2.54 (a,b)	13.3±3.01 (a)	2.26±2.27 (b)
2	12.1±4.18 (b,c)	6.67±3.80 (c)	3.55±3.43 (a,b)	6.38±3.61 (b,c)	1.72±3.30 (b)	6.03±3.87 (b,c)	0.475±2.83 (a,b)	12.4±2.50 (a,)	0.480±0.248(b)
4	17.5±3.01 (a,b)	13.2±2.87 (c)	6.96±3.88 (a,b)	10.8±3.92 (b,c)	5.39±2.92 (b,c)	11.8±3.97 (b,c)	1.25±2.77 (a,b)	17.0±2.59 (a)	7.47±2.60 (b,c)
6	17.8±3.13 (b,c)	14.1±4.43 (b,c)	8.05±3.50 (b,c,d)	13.3±3.49 (c,d)	6.65±3.26 (c,d)	7.75±3.31 (a,b)	1.50±2.36 (b)	19.1±242 (a)	4.28±3.00 (d)
8	16.4±3.38 (b,c)	7.37±2.38(b,c)	10.5±3.72 (b,c)	12.6±3.36 (c)	7.43±3.52 (b,c)	15.8±3.48 (b)	3.86±3.21 (c)	19.7±2.76 (a)	2.70±3.77 (e)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-11. Mean and standard deviation for b* values of Spanish mackerel white muscle stored at 4°C for 8 days using the Minolta.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	-0.1125±2.23 (b)	0.8±1.74 (b)	1.86±0.687 (a,b)	-0.738±1.41 (b)	2.25±0.578(b)	0.438±0.827(a,b)	2.92±1.53(b)	1.05±0.598a)	1.85±1.66(b)
1	-0.125±0.678 (c)	0.425±1.19 (b)	2.45±0.657 (b)	-1.56±0.980 (c)	2.90±0.996(a,b)	-1.00±0.763(a,b)	2.90±1.56(a,b)	1.18±1.37(a)	2.44±0.829(b)
2	0.600±1.43 (b,c)	1.78±0.836 (c)	2.10±0.794 (a,b)	1.35±0.719 (b,c)	2.90±1.12(b)	-0.600±0.727(b,c)	2.11±0.840(a,b)	1.65±0.611(a)	2.21±0.713(b)
4	2.24±1.15 (a,b)	2.61±0.869 (c)	2.14±0.997	-0.725±0.874 (b,c)	3.69±1.54(b,c)	-0.55±0.540(b,c)	2.53±1.38(a,b)	2.76±0.789(a)	2.03±0.713
6	7.93±0.869 (b,c)	6.50±2.00 (b,c)	2.10±1.07 (b,c,d)	0.188±0.684 (c,d)	3.00±1.49(c,d)	1.20±1.67(a,b)	1.41±0.758(b)	4.18±0.968(a)	3.34±0.528(d)
8	1.58±1.79 (b,c)	2.53±0.667 (b,c)	2.19±1.33 (b,c)	0.338±0.650 (c)	3.10±1.26(b,c)	1.95±0.852(b)	2.29±1.85(c)	6.01±1.65(a)	2.33±1.01(e)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-12. Mean and standard deviation for b* values of Spanish mackerel white muscle stored at 4°C for 8 days using the color vision machine.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	6.22±2.31 (b)	7.9±2.36 (b)	-0.15±2.60 (a,b)	6.76±2.09 (b)	2.28±3.16(b)	4.51±2.89(a,b)	-1.28±3.14(b)	16.3±2.21(b)	0.520±2.82(a,b)
1	3.36±1.68 (c,)	-0.21±3.45 (b)	-0.5±2.40 (b)	-4.01±2.44 (c)	0.77±3.38(a,b)	2.42±2.27(a,b)	0.295±2.49(a,b)	10.8±3.75(a)	0.345±2.52.(b)
2	4.41±3.47 (b,c)	3.31±3.65 (c)	-2.03±2.45 (a,b)	-2.86±2.47 (b,c)	1.96±3.13(b)	6.59±2.63(b,c)	-1.76±2.43(a,b)	12.4±2.50(a)	2.95±3.49(b)
4	16.5±2.63 (a,b)	15.37±3.31 (c)	1.22±2.87 (a,b)	7.54±1.79 (b,c)	3.58±2.85(b,c)	9.47±2.57(b,c)	1.83±2.59(a,b)	19.5±3.30(a)	8.00±3.66(b,c)
6	18.4±2.56 (b,c)	16.3±3.13 (b,c)	1.45±3.06 (b,c,d)	8.71±1.96 (c,d)	5.72±4.33(c,d)	11.05±3.69	1.14±3.24(b)	17.9±2.25(a)	7.87±3.95(d)
8	15.9±2.73 (b,c)	17.7±3.94 (b,c)	3.68±2.47 (b,c)	11.2±2.29 (c)	7.46±3.68(b,c)	13.27±3.11(b)	4.74±3.08(c)	21.4±2.53(a)	6.99±4.49(e)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-13. Heme means (nm) and standard deviation for Spanish mackerel stored at 4°C for eight days.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	412.5±0.707(a)	412.5±0.707(a)	412.5±0.707(a)	412.5±0.707(a)	412.5±0.707 (a)	412.5±0.707(a)	412.5±0.707(a)	412.5±0.707(a)	412.5±0.707(a)
1	411.0±1.41(b)	416.3±1.77(a)	417.0±2.83(a)	416.0±2.12(a)	415.5±0.707 (a)	418.5±0.707(a)	418.5±0.707(a)	410.0±0(b)	410.0±0(b)
2	409.5±0(b)	417.0±2.83(a)	418.0±0(a)	416.3±1.77(a)	418.0±0(a)	416.8±0.354(a)	418.0±0(a)	10.5±2.12(b)	410.0±1.41 (b)
4	409.8±1.06(b)	413.5±1.06(a)	415.3±2.47(a)	416.0±2.12(a)	417.8±0.354 (a)	413.5±0.707(a,b)	419.0±0(a)	409.5±0.707(b)	410.0±0(b)
6	409.5±0.707(c)	410.5±0.707(b, c)	417.3±0.354(a,b)	409.3±0.353(c)	418.0±0(a)	414.0±0(a,b,c)	419.0±0(a)	409.0±0(c)	409.0±0(c)
8	409.8±0.353(b)	408.5±0 (b)	417.3±0.354(a)	411.0±2.83(a)	417.5±0.707 (a)	414.0±0(a)	419.0±0(a)	409.0±0(b)	409.0±0(b)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-14. Malonaldehyde means and standard deviations of Spanish mackerel red muscle stored at 4°C for eight days.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	18.4±2.50(a)	18.4±2.50(a)	18.4±2.50(a)	18.4±2.50(a)	18.4±2.50(a)	18.4±2.50 (a)	18.4±2.50(a)	18.4±2.50 (a)	18.4±2.50(a)
1	69.5±8.08(a)	53.9±19.7(a)	53.9±19.7(b)	75.8±13.4(b)	75.8±13.4(b)	37.5±8.29(a)	37.5±8.29(b)	70.6±0.434(a)	70.6±0.434(b)
2	102.1±3.91(a)	87.1±8.81(a)	62.7±5.42(b)	69.4±3.91(b)	26.1±0.311(b)	70.6±15.6(a)	28.2±2.04 (b)	102±31.3(a)	51.5±2.84(b)
4	102.1±24.3(a)	98.9±26.3(a)	65.7±0.904(b)	82.1±17.4(b)	44.0±18.5(b)	50.3±5.67(b)	50.3±5.64(b)	106±12.2(a)	63.5±3.59(b)
6	75.3±7.72(a,b)	85.4±4.74(a)	108±22.1(a)	90.8±11.3(a)	16.1±0.960(c)	117±5.31(a)	36.0±4.46(b,c)	107±10.4(a)	80.5±0.0490(b,c)
8	96.4±18.2(a)	84.4±14.4(c)	91.6±12.0(c)	112.8±28.1(c)	68.7±2.32(c)	85.5±11.5(b)	87.4±0(c)	93.0±5.43(a)	44.9±11.3(c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-15. Malonaldehyde means and standard deviations of Spanish mackerel white muscle stored at 4°C for eight days.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	14.4±3.02(a)	14.4±3.02(a)	14.4±3.02(a)	14.4±3.02(a)	14.4±3.02(a)	14.4±3.02 (a)	14.4±3.02(a)	14.4±3.02(a)	14.4±3.02(a)
1	14.0±5.53(a)	18.9±4.22(a)	18.9±4.22(b)	16.1±1.07(b)	16.1±1.07(b)	7.73±0.447(a)	7.73±0.447(b)	8.69±7.63 (a)	8.69±7.63 (b)
2	14.0±3.28(a)	32.8±2.10(a)	18.9±4.22(b)	15.4±1.92(b)	15.3±0.0333 (b)	14.5±4.26(a)	11.8±2.32(b)	25.6±2.51(a)	15.3±4.65 (b)
4	14.8±3.71(a)	43.3±10.9(a)	13.0±2.63(b)	17.5±1.60(b)	9.31±2.57(b)	14.3±4.32(b)	14.3±4.32(b)	25.6±9.08(a)	19.9±5.80(b)
6	30.8±2.49(a,b)	45.1±2.43(a)	43.3±10.9(a)	26.7±2.34(a)	7.49±0(c)	14.3±7.87(a)	13.6±2.61(b,c)	28.0±0(a)	15.1±3.49(b,c)
8	34.5±7.69(a)	39.5±1.66(c)	39.6±2.7(c)	30.3±7.38(c)	23.9±2.96(c,)	20.2±4.37(b)	44.6±5.15©	41.3±10.1(a)	7.82±1.31(c)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-16. Mean aerobic plate counts log(cfu/g) and standard deviations for Spanish mackerel stored at 4°C.

Storage day	n	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	6	1.97±0.423(a)	1.97±0.422(a)	1.97±0.423(a)	1.97±0.42(a)	1.97±0.423(a)	1.97±0.423(a)	1.97±0.423(a)	1.97±0.423(a)	1.97±0.423(a)
1	6	3.02±0.206(a)	3.02±0.191(a)	2.90±0.140(a)	2.75±0.237(a)	3.07±0.0348(a)	2.94±0.138(a)	3.05±0.196(a)	3.04±0.150(a)	2.84±0.156(a)
3	6	4.90±0.272(a)	4.48±0.139(a)	3.03±0.114(d)	3.42±0.102(c)	3.12±0.198(d)	3.78±0.199(c)	3.55±0.275(c)	4.15±0.275(b)	4.28±0.214(b)
5	6	6.20±0.0140(a)	5.20±0.0133(b)	4.17±0.0055(b)	4.86±0.168(b)	3.24±0.145(c)	5.16±0.120(b)	3.41±0.0946(c)	6.22±0.0946(a)	5.52±0.205(a)
7	6	8.15±0.0618(a)	6.73±0.034(c,d)	4.19±0.0260(e)	6.56±0.202(d)	3.94±0.153(e)	6.79±0.264(c)	4.20±0.0186(e)	7.78±0.890(a,b)	7.14±0.0167(b)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

Table A-17.Mean histamine and standard deviations for Spanish mackerel stored at 4°C.

Storage day	No treatment	Filtered Smoke	Filtered Smoke in gas	18% CO	18% CO in gas	100% CO	100% in gas	Nitrogen	Nitrogen in gas
0	0.908± 0.0069(a)	0.908±0.0069 (a)	0.908± 0.0069 (a)	0.908± 0.0069 (a)	0.908±0.0069 (a)	0.908±0.0069 (a)	0.908± 0.0069(a)	0.908±0.0069 (a)	0.908±0.0069 (a)
1	0.850±0.0069 (c)	0.928±0.0345 (a,b,c)	0.904±0.0276 (a,b,c)	0.938±0.0069 (a,b)	0.982±0 (a)	0.904±0.0138 (a,b,c)	0.865±0 (c)	0.884±0.0414 (b,c)	0.845±0 (c)
3	0.908± 0.0069(a,b,c)	0.840±0.0069 (c)	0.855± 0.0276(c)	0.928±0.0484 (a,b)	0.957±0.0207 (a)	0.923±0.0276 (a,b,c)	0.855±0 (b,c)	0.908±0.0069 (a,b,c)	0.874±0 (a,b,c)
5	1.10±0.0069 (a)	0.879±0.0207 (e)	0.865±0.0276 (e)	0.967±0.0622 (c,d)	0.992±0.0138 (b)	0.972±0.0276 (b,c)	0.884±0(d.e)	1.11±0.0138 (a)	0.904±0 (c,d,e)
7	1.09±0.0414 (a)	0.884± 0.0138(e)	0.884±0.0138 (e)	1.01± 0.0276(b)	0.933± 0.0414 (b,d)	0.894± 0.0138(d,e)	1.01±0 (b,d)	1.01±0.0345 (b,c)	0.918± 0.0069(d)

Statistical difference ($P<0.05$) between treatments on each day are represented by different letters.

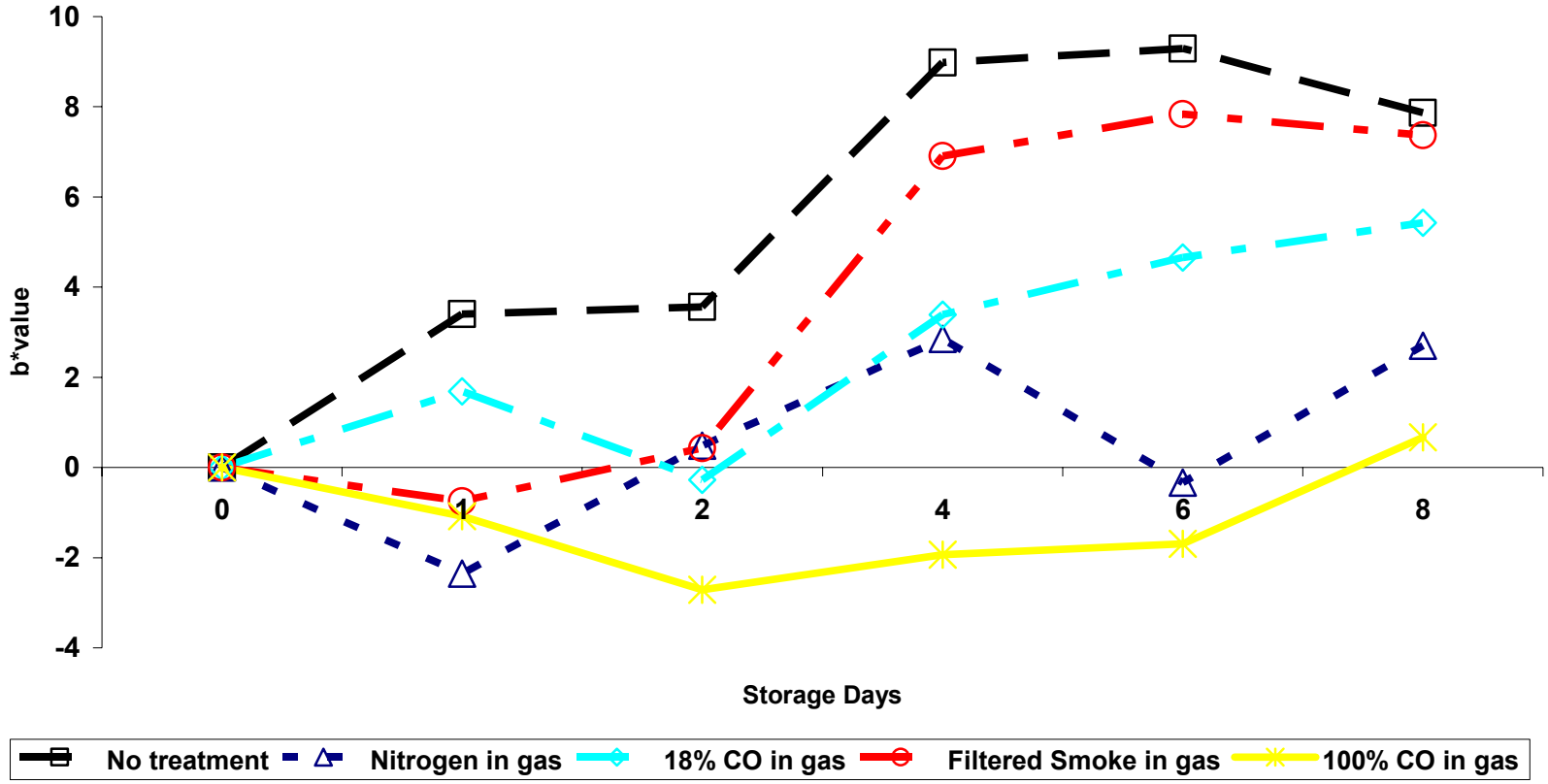

Figure A-1. Change in b*values for the red muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Color Vision Machine

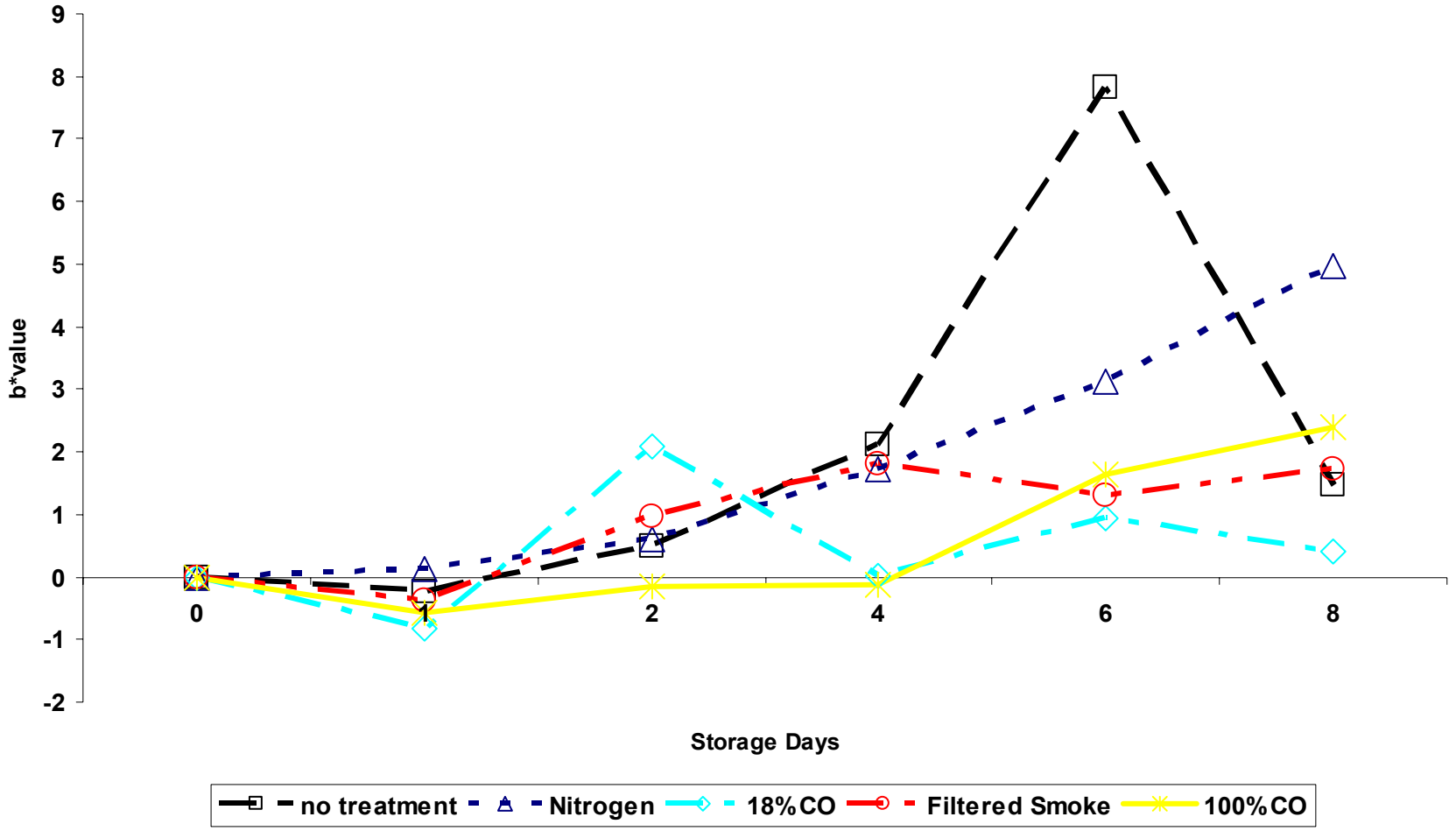

Figure A-2. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Minolta colorimeter.

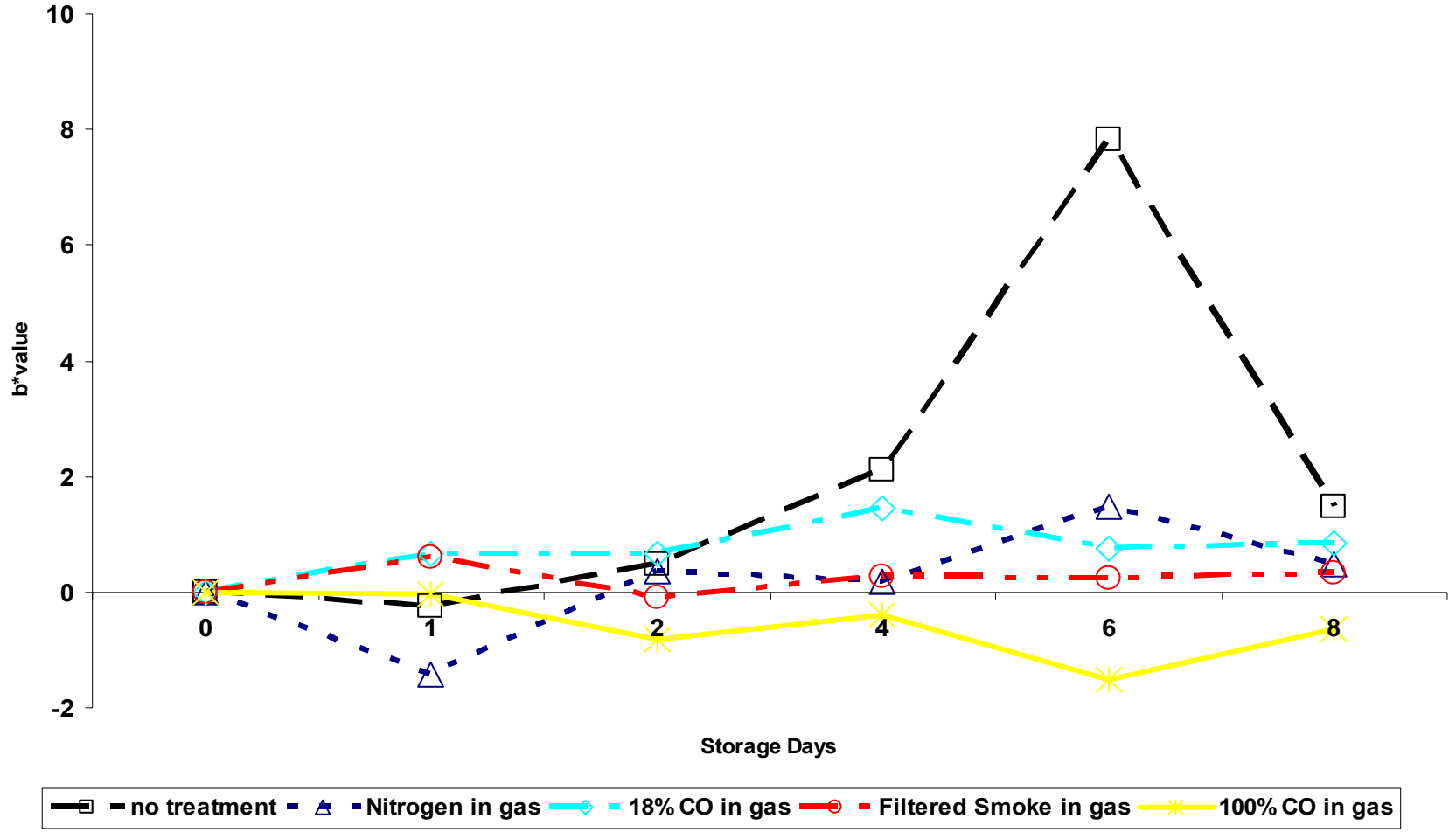

Figure A-3. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Minolta colorimeter.

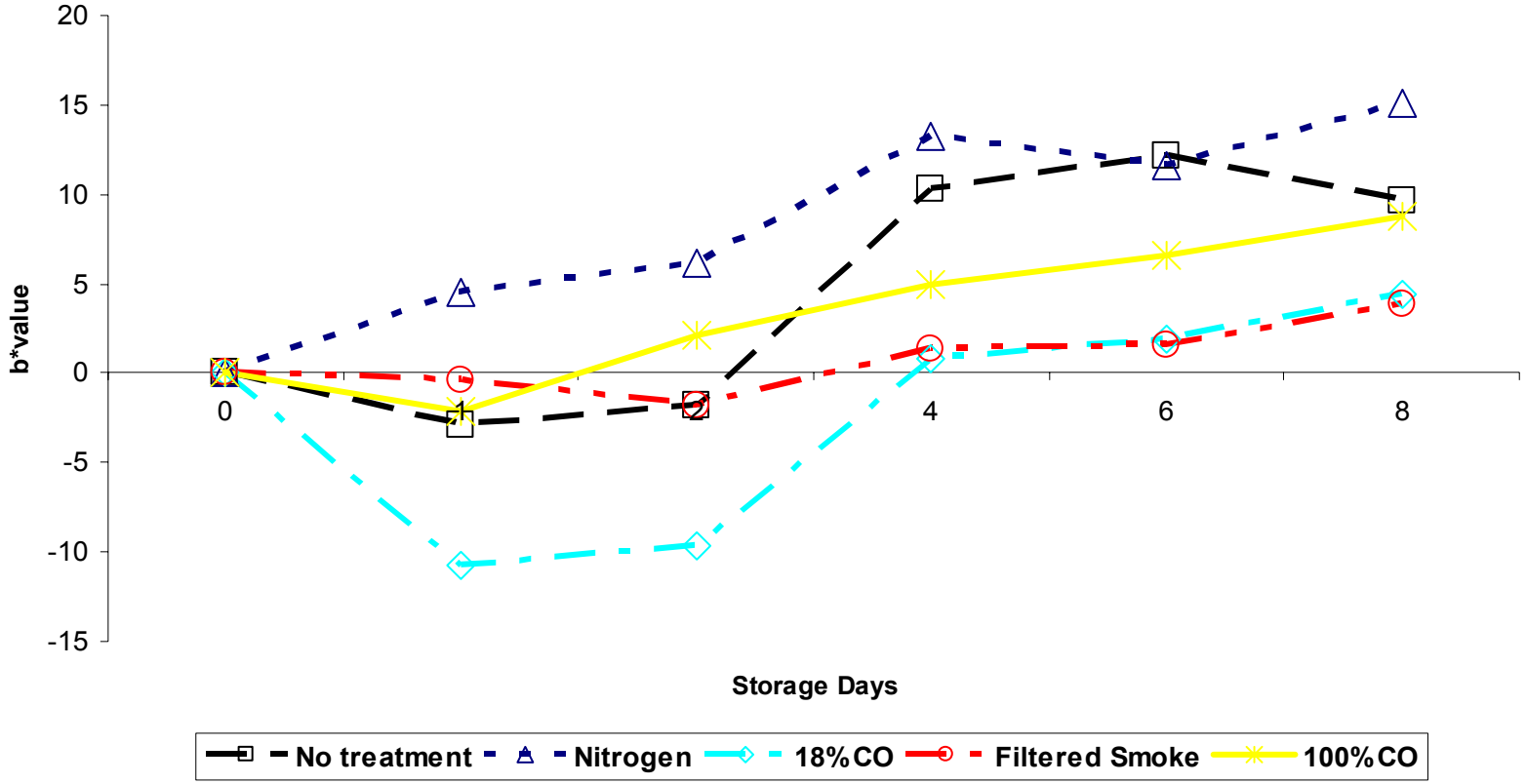

Figure A-4. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h using the Color Vision Machine.

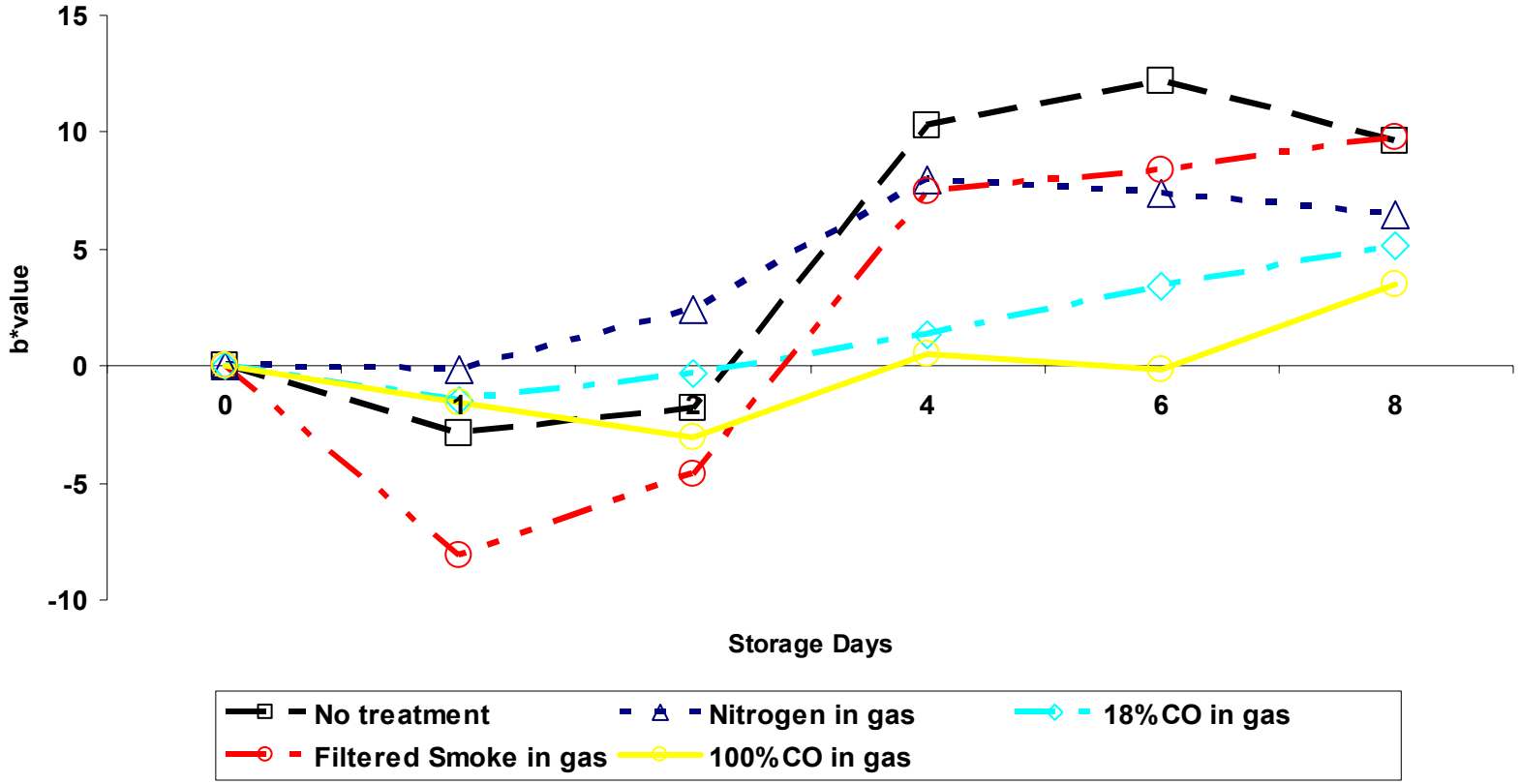

Figure A-5. Change in b*values for the white muscle of Spanish mackerel stored at 4°C for 8 days while staying in the different gas blends using the Color Vision Machine.

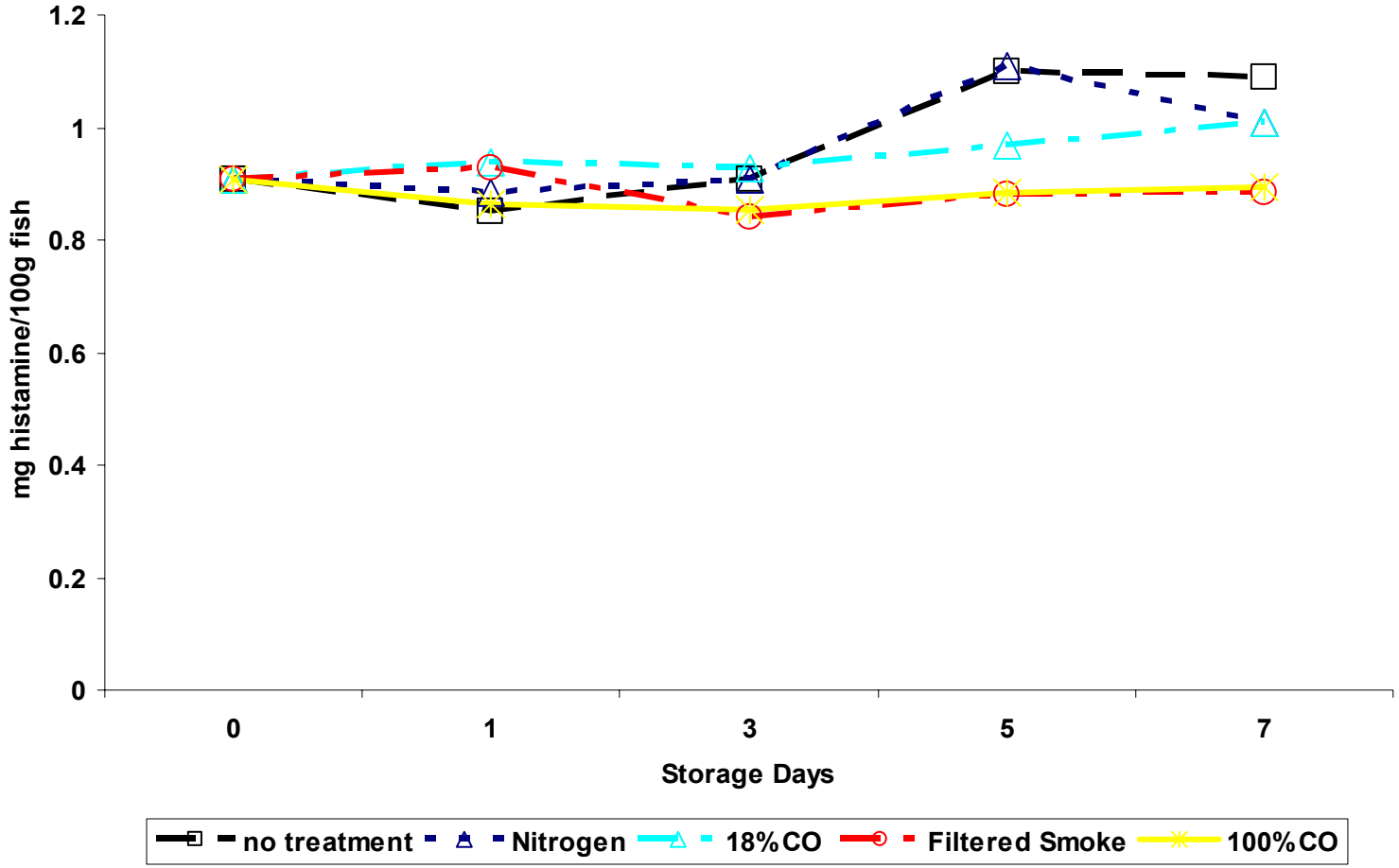

Figure A-6. Mean histamine formation for Spanish mackerel stored at 4°C for 8 days after being treated with different gas blends for 24 h.

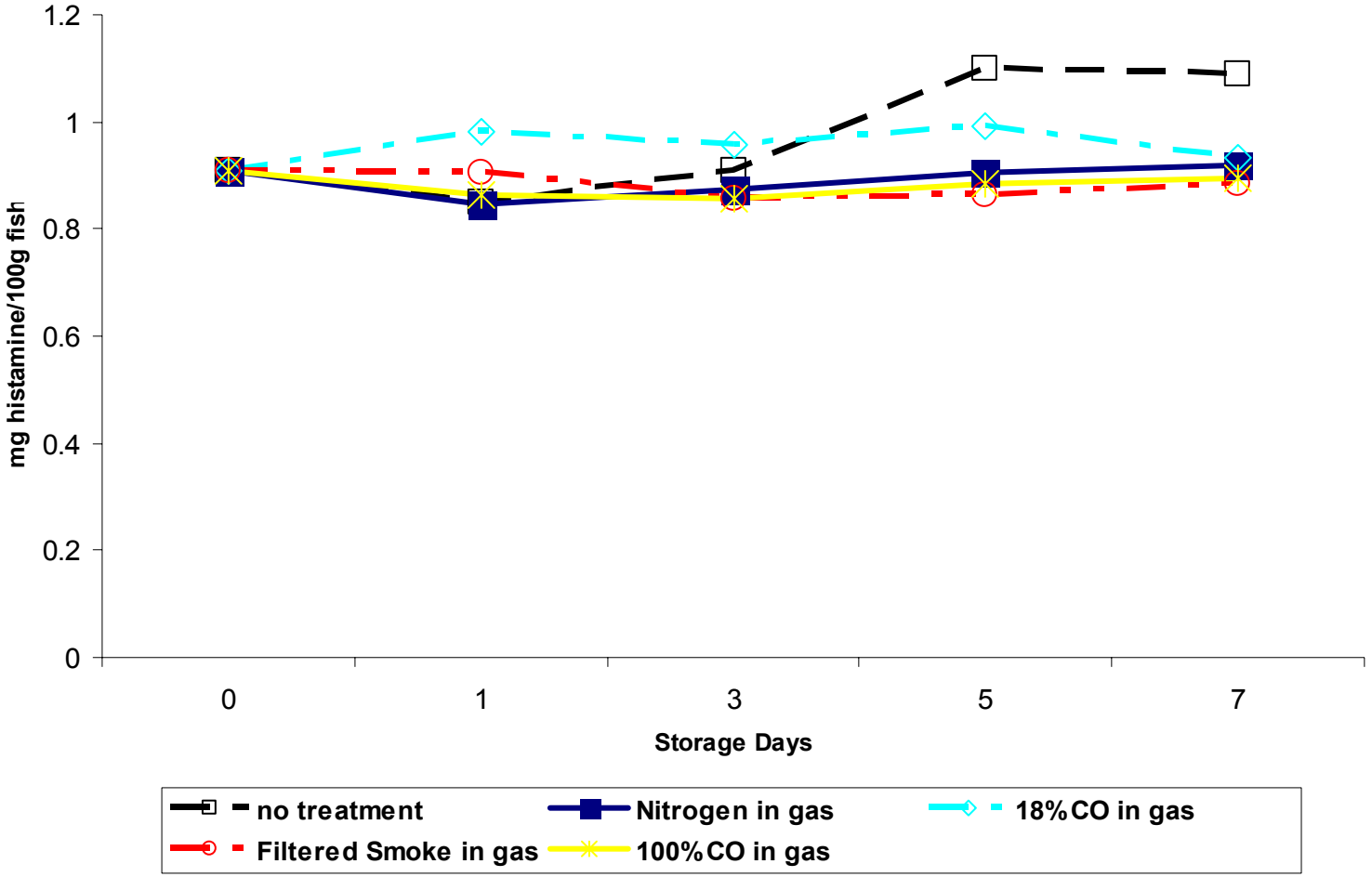

Figure A-7. Mean histamine formation for Spanish mackerel stored at 4°C for 8 days after being stored in the different gas blends.

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BIOGRAPHICAL SKETCH

Kristina Garner was born on July 3, 1979, in Flushing, New York, to Yvonne and Victor Garner. She attended Pembroke Lakes Elementary School and Walter C. Young Middle School, followed by graduation from Cooper City High School on June 7, 1997. Kristina graduated on December 15, 2001 from the University of Florida with a Bachelor of Science degree in Microbiology and Cell Science. As an undergraduate, Kristina was a College of Agricultural and Life Sciences Ambassador. She was awarded the Bertha Marie Cornett Assistantship to study for her Master of Science degree in the Food Science and Human Nutrition Department in the College of Agricultural and Life Sciences. Kristina received her Master of Science degree from the Food Science and Human Nutrition Department on May 2004. Her future plans are to pursue a Ph.D. in the Department of Food Science and Human Nutrition.