INFLUENCE OF INORGANIC IRON, LACTATE, AND SUCCINATE ON MYOGLOBIN REDOX STABILITY, COLOR, AND MITOCHONDRIAL RESPIRATION OF RAW BEEF

by

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(Under the Direction of Anand Mohan)

ABSTRACT

Enhancing beef with metabolic intermediates lactate and succinate is known to improve raw beef color. This work examined the effect of inorganic redox active iron in combination with the named metabolites on the color and myoglobin redox stability of raw beef homogenates and their effect on the respiration of isolated beef heart mitochondria. Homogenates treated with the combination of redox iron and metabolites had significantly lower redness values ($p<0.05$). Inorganic redox active iron ions adversely affected mitochondrial structure and function. The rates of mitochondrial oxygen consumption driven by either of lactate or succinate were significantly reduced in the presence of redox active iron ions. The results indicate that inorganic redox active iron is deleterious to color of raw beef muscles and also to mitochondria which are a medium of color stabilization in fresh beef. Variation in concentration of heme and nonheme iron forms in two muscles of differing color stability and how it correlated to color and lipid oxidation was monitored over a nine day period of refrigerated storage. Heme iron concentration correlated strongly with redness and lipid oxidation and moderately with discoloration. Nonheme iron concentration correlated poorly with redness and discoloration and moderately with lipid

oxidation. Variation in concentrations of different iron forms did not completely explain changes in raw beef color and associated quality parameters, regardless of muscle type. Thus, factors along with iron form concentrations need to be holistically considered when developing and marketing products of raw beef muscles of differing color stabilities.

INDEX WORDS: Inorganic iron, lactate, succinate, redox myoglobin

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DEDICATION

I dedicate this work to my family. I greatly value and respect them for their constant love, guidance, and support.

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CHAPTER 1

INTRODUCTION

The visual color of packaged muscle foods on display is an important quality cue for consumers, which determines their decision of purchase at the point of sale. Although discoloration in fresh beef is a quality defect which has been shown to have poor correlation with eating quality, consumers still prefer bright red meat products (Troy & Kerry, 2010). Discolored meat products present difficulties in sales forcing retailers to discount the prices of such products. Such discounting leads to annual retail losses of about fifteen percent costing about a billion dollars to the beef industry (Smith et al., 2000). Myoglobin is the main red color imparting pigment in muscle foods and the bright cherry red color of fresh beef products depends on the concentration and chemical redox state of myoglobin. Intrinsic to fresh beef, the factors which primarily determine the color and its stability are age and sex of the animal, muscle type, rate of pH decline, and biochemical activity (Bekhit & Faustman, 2005).

In post mortem muscle with lack of blood circulation, the oxygen supply ceases and aerobic metabolism in the Krebs Cycle and electron transport chain ceases. The substrates for all the reactions involved in these two biochemical pathways are depleted with time even though the enzymes remain active. Anaerobic metabolism taking place glycolysis produces lactic acid from the muscle glycogen breakdown. The low oxygen partial pressure in the tissue combined with

declining pH facilitates the formation of metmyoglobin, the ferric redox form of myoglobin which imparts the undesired brown color to muscle foods (Aberle, 2001). The mentioned conditions along with depletion of biochemical substrates also cease formation reduced Nicotinamide adenine dinucleotide (NADH) which is necessary for enzymatic and non-enzymatic metmyoglobin reduction, thus increasing the metmyoglobin concentration (Bekhit & Faustman, 2005).

Lactate is added to packaged case ready meat products due to its antimicrobial and color stabilizing effects. Through the activity of lactate dehydrogenase, lactate produces NADH which can participate in metmyoglobin reduction (Kim et al., 2006; Mbandi & Shelef, 2002). Metmyoglobin reduction has also been demonstrated by a succinate and mitochondria system through electron transfer chain mediated oxygen consumption (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005). Furthermore, sodium succinate addition to raw ground beef patties was shown to stabilize their color and increase metmyoglobin reduction (Zhu, Liu, Li, & Dai, 2009).

Iron is the most abundant transition metal found in muscle foods. In meat, it is present in two broad distinct forms – organic iron is iron present in the heme configuration co-ordinately bonded within a porphyrin ring and inorganic iron – which is covalently bonded to inorganic groups or chelated by relatively small organic molecules (Kanner, 1994). Owing to its transition metal nature, atomic iron by itself or in a coordinately bonded configuration can readily accommodate extra electrons in its penultimate d-orbitals and release them making it a good catalyst for oxidation-reduction reactions. This redox catalyst nature of iron is utilized by the muscle oxygen carrier myoglobin with heme iron and by enzyme molecules such as succinate dehydrogenase having inorganic iron sulfur clusters (Hederstedt & Rutberg, 1981) or cytochrome c oxidase and cytochrome P450 having heme iron (Solomon, Decker, & Lehnert,

2003; Yoshikawa, 1997) for shuttling electrons. The iron storage protein ferritin in muscle foods has been known to release inorganic iron under post mortem biochemical conditions existing in muscle foods which can have deleterious effects through oxidation of lipids, phospholipids, proteins, and nucleic acids which can subsequently affect the color of raw beef (Kanner, 1994; Kanner, Hazan, & Doll, 1988). More recently, it has been shown that inorganic iron can catalyze the oxidation of myoglobin either through products of lipid oxidation (Gorelik & Kanner, 2001) or directly by itself (Allen & Cornforth, 2006). These studies were carried out in purified model systems with isolated components such as microsomes or purified buffered myoglobin. The mentioned conditions preclude the effects of natural pro and antioxidants present in meats, on the oxidative and color stability of raw meat. Hence, the main objectives of the current work are –

- To determine the effect of metabolic intermediates – lactate and succinate in the presence of redox active iron ions on the color and myoglobin redox stability of raw beef homogenate and their effect on the respiration of isolated mitochondria.

- To study the variation in concentration of the heme and non-heme forms of iron in intact steaks from muscle of differing color stability (*Longissimus dorsi*- color stable muscle and *Psoas major*-color unstable muscle) with time and how it relates to visual color, and associated qualitative changes like surface discoloration and lipid oxidation.

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CHAPTER 2

LITERATURE REVIEW

In this chapter, an examination of the biochemistry involved behind red meat colors and related topics is presented. This includes notes on structure and forms of myoglobin, role of biochemical pathways and their intermediates in color stabilization, trafficking of iron in cells and its storage and distribution, means to measure mitochondrial respiration and its implications and some major effects of iron catalyzed oxidation on properties of red meat.

Myoglobin and Meat color

The color of red meats is imparted principally by the muscle pigment myoglobin. Other heme proteins such as hemoglobin and cytochrome C have minor contributions to color in red muscle foods such as beef and pork. Myoglobin is an intracellular water soluble globular protein found in skeletal and cardiac muscles. It has a molecular weight of about 17 kDa and is made up of 8 sub-units in the form of a-helices. It is the oxygen carrier pigment of muscle tissues. The prosthetic group of this molecule is the heme ring made up of iron coordinated to porphyrin ring at the 4 planar nitrogens central to the tetrapyrrole structure within the hydrophobic pocket of the protein. The fifth and sixth positions are perpendicular to the plain of porphyrin (Aberle, 2001). A histidine residue at position 93 is the ligand at the fifth iron position. A distal histidine residue at position 64 and the valence of iron and ligand at sixth position of myoglobin iron determine

the tertiary structure as well as color of myoglobin and the meat at large (Mancini & Hunt, 2005). As the animal is slaughtered and exsanguinated, a major portion of the blood is drained though traces of it persist in meat, which contain hemoglobin which has a minor contribution to the color of red meat.

Being a transition metal, iron has incompletely filled penultimate d-orbitals which can accommodate extra electrons. It is this nature of iron which makes it conducive for complexation by ligand molecules which are electron pair donors, such as the nitrogens in porphyrin and also oxidation-reduction reactions (Baker, Anderson, & Baker, 2003). Depending on the ligand to heme iron and its valence, the three major forms of redox forms of myglobin (Mb) imparting meat its characteristic colors are deoxymyoglobin, myoglobin, metmyoglobin. The deoxymyoglobin form of Mb persists when the heme iron is in ferrous (+2) state and no ligand is present in the sixth position. This form is readily seen in vacuum packaged meats or in the interiors of freshly cut meats which have a typical purplish red appearance. A very low oxygen partial pressure (< 1.4 mm Hg) is necessary to keep Mb in the deoxyomyglobin form (Brooks, 1935). When deoxymyoglobin is exposed to oxygen or air, it is slowly oxygenated by molecular dioxygen, which complexes to the sixth vacant position of heme ferrous iron to form oxymyoglobin. This form of Mb imparts raw meat its typical bright cherry red color which is deemed most pleasing by the typical meat consumer. The process of oxygenation of deoxymyoglobin to form oxymyoglobin is called bloom. The rate formation of oxymyoglobin is dependent on pH, temperature, oxygen partial pressure and post mortem age dependent biochemical processes inherent to meat competing for oxygen. The oxidation of both ferrous myoglobin forms gives metmyolgobin which has ferric iron and a water molecule ligand in the sixth position. Metmyglobin has a brown color and its surface appearance is associated with

discolored meat, though it may be present between a superficial oxymyoglobin layer and interior deoxymyglobin zone. The formation and movement of metmyoglobin layer depends on temperature, pH, oxygen partial pressure, inherent reducing activity of meat and also microbial growth (Mancini & Hunt, 2005).

Measuring raw meat color

The chief methods used to evaluate surface discoloration of beef include human visual color test which is the gold standard as well as instrumental tests that measure tristimulus color space values (eg. CIE *L**, *a**, and *b**) and absorbance or transmission of electromagnetic waves in the visible or near infrared spectra. The tests involving human subjects give better information in terms of understanding or predicting consumer behavior and preferences towards meat. On the other hand, instrumental methods are more objective, repeatable and immune to psychological or cultural factors which greatly vary in human testers. Of the instrumental methods related to beef evaluation, measuring *L** and *a** give fairly straightforward information regarding the lightness or paleness and redness, respectively of the beef samples. Another important instrumental method for evaluating surface discoloration involves quantification of the redox myoglobin forms using absorbance spectrum of the sample in the visible region. Using wavelengths isobestic to 100% deoxy, oxy or metmyoglobin redox forms, the absorbance is converted into percent redox forms. This method is fairly easy to apply and has been shown to have good repeatability (AMSA, 2012). Thus, it was chosen for quantifying the surface relative metmyoglobin content in the present work.

Factors affecting raw meat color

The color of beef meat is affected by both pre-harvest and post mortem factors (Vestergaard, Oksbjerg, & Henckel, 2000). The housing system and pre-harvest physical and psychological state of beef cattle influences the metabolic processes occurring immediately post slaughter. The gender of the animal affects the levels of fast versus slow twitching muscle fibers, oxidative potential. Inclusion of dietary anti-oxidants such as alpha tocopherol or beta carotene in diets not only affects the oxidative stability but also the color of fat and marbling level of the meat (French et al., 2000).

The post mortem factors which affect meat color can be broadly classified into intrinsic or extrinsic. Intrinsic factors are the ones inherent to the meat structure and may be a result of pre-harvest conditions of the animal, some of these include- breed, sex, rate of fall in pH and ultimate pH, metmyoglobin reducing activity which can be enzymatic or non-enzymatic, endogenous antioxidants, muscle type and metabolism. The extrinsic factors are those that the finished or fabricated form of meat is externally subjected to and includes packaging material and type, exposure to air and modified atmosphere, wavelength and temperature of illuminating light, temperature of storage and distribution, type and extent of fabrication and processing, addition of external ingredients, and microbial load (Bekhit & Faustman, 2005).

Packaging type and material directly affects the rate of formation of metmyoglobin since it is sensitive to partial pressure of oxygen. Four main packaging strategies for raw beef preservation are - high oxygen modified atmosphere packaging (MAP), low oxygen MAP, vacuum packaging, and controlled atmosphere packaging (Toldrá, 2010). High oxygen packaging (70- 80% O_2) promotes prolonged retention of the bright red oxymyoglobin but also

promotes oxidation of membranal lipids and phospholipids which adversely affects color, flavor, and nutrition through products of primary and secondary oxidation reactions (Ladikos & Lougovois, 1990; Toldrá, 2010). Breakdown of phospholipids is generally associated with the typical off-flavor and "warmed-over" flavor of reheated muscle foods which are cooked and refrigerated. Vacuum packaging seems to be the most conducive packaging with respect to preventing growth of spoilage micro-organisms and oxidative reactions. The deoxymyoglobin formed under such conditions can bloom to oxymyoglobin on sufficient air exposure. However, this type of packaging presents a serious threat due to the growth of the obligate anaerobe *Clostridium botulinum* which produces the potent botulin neurotoxin. A common packaging type used in supermarkets for fresh meat is meat placed on soaker pads in polystyrene trays and over wrapped in polyvinyl chloride films. The PVC films are permeable to oxygen allowing for the meat to bloom and the process of over wrapping is inexpensive and easy to operate (Toldrá, 2010). This method however, leads to obvious discoloration within a 5-7 days under the typical conditions of retail display for steaks and roasts, and much faster for ground beef by both biochemical and microbial causes (Cornforth & Hunt, 2008). A more recent packaging strategy is anaerobic with 20 – 30% CO_2 with traces of CO (about 0.5%) and the remainder being nitrogen. This has several advantages such as desirable red color (from formation of carboxymyoglobin), no oxidized flavors, less bone discoloration, retarded microbial growth (due to carbonic acid formation in the presence of water), no premature browning and more tenderness due to reduced protein oxidation (Cornforth & Hunt, 2008). However, consumers till date have a negative opinion about carbon monoxide and the stable red color of carboxymyoglobin in meat may mask the presence of high microbial load and the associated spoilage issues.

The illuminant used for display of packaged meat has an important effect on the consumer's perception of meat. The same meat sample when displayed under LED or fluorescent lights of different temperatures, for example 3100 K, 4500 K, and 6500 K appears to have a different hue which greatly shapes the consumers' opinion and purchasing decision. Color temperature, color rendering index and luminous intensity are some important parameters used to distinguish different illuminating lights. High energy wavelengths such as those in the ultraviolet region tend to accelerate discoloration as well as promote photocatalyzed reactions in meat (AMSA, 2012).

Changes in beef color as influenced by microbial growth are dependent on the species and concentration growing in the meat. There are mixed results reported in literature about influence of microorganism growth on the color deterioration with some *Lactobacillus* strains retarding discoloration and other genera such as *Pseudomonas* and *Brochothrix thermospacta* contributing to it at low populations (Faustman & Cassens, 1990). Microbial growth is also dependent on the gas composition in the packaging and inherent oxygen in meat, as well as on initial load, and all these factors are considered together as a whole rather than individually when assessing the effect of microbial growth on packaged raw beef (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Leisner, Greer, Dilts, & Stiles, 1995; Lücke, 2000).

Any form fabrication or processing such as tumbling and injection on beef causes mechanical damage to the tissue structure which leads to enzyme substrate decompartmentalization as well as loss of cellular fluid and nutrients. The surface area per unit volume of meat also increases with fabrication making it more vulnerable to oxidation and microbial growth (Toldrá, 2010).

Though the main intention of addition of antimicrobials to case ready packaged meat is retarding microbial growth, research in the recent years has also evaluated how antimicrobials affect color of raw meat. Many of the commonly used antimicrobial agents are acidulants which reduce the meat pH and can directly or indirectly influence myoglobin. Applying 5% acetic acid on ground beef made from lean beef trimmings was shown to have a negative effect on redness (Stivarius, Pohlman, McElyea, & Apple, 2002). Trisodium phosphate, Cetylpyridinium chloride, and Chlorine dioxide water have all been shown to not only retard microbial growth but also improve redness and myoglobin color stability during display of treated ground beef (Pohlman, Stivarius, McElyea, Johnson, & Johnson, 2002; Pohlman, Stivarius, McElyea, & Waldroup, 2002).

Two important extrinsic factors influencing color of raw beef are pH and temperature since they are the main controlling factors of enzymatic and nonenzymatic reactions occurring in meat which are responsible for metmyoglobin reducing activity. Under physiological conditions, a small percent of myoglobin undergoes oxidation to form the physiologically inactive metmyoglobin and complete oxidation of all myoglobin may occur if not regulated. Hence, the concept of metmyoglobin reducing activity holds solid ground since metmyoglobin does not accumulate in live muscles. Its formation in meat is the chief basis of discoloration and thus the biochemistry behind metmyoglobin reduction warrants exploration (Bekhit & Faustman, 2005). Metmyoglobin reducing activity (MRA) was first reported in 1960 (Dean & Ball, 1960) and has been extensively studied since then. Broadly, MRA can be categorized into enzymatic and non-enzymatic type and the two distinct mechanisms have certain common traits.

Metabolic intermediates and Metmyoglobin reduction

NADH - cytochrome b5 metmyoglobin reductase with the cofactor Nicotinamide adenine dinucleotide reduced (NADH) and the intermediate cytochrome b5 is a well characterized enzymatic system involved in reduction of oxidized heme proteins. Both the enzyme and its intermediate are found in a variety of tissues on the surface of mitochondria, endoplasmic reticulum, and golgi and nuclear membranes and the system mainly transfers electrons from NADH, the reduced form to metmyoglobin and methemoglobin (Shirabe et al., 1992). Based on the location, the NADH - cytochrome b5 reductase is either in the soluble form in red blood cells or membrane bound on endoplasmic reticulum and outer membrane of mitochondria. Since meat is almost devoid of blood, the latter form is of importance in metmyoglobin reduction in meat (Bekhit & Faustman, 2005). A schematic for the transfer of electrons is shown below and is adopted from the work of Arihara and colleagues (Keizo Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995; Bekhit & Faustman, 2005).

Other nonspecific enzymatic systems such as DT diaphorase (NADPH quinone reductase), D diaphorase, (NADH dehydrogenase (quinone)), and NADPH quinone oxidoreductase too have been reported to contribute to metmyoglobin reduction (Schomburg, Chang, & Schomburg, 2014).

Apart from enzymatic involvement in MRA, non-ezymatic reduction or metmyoglobin has been reported as well. NADH has been shown to directly reduce metmyoglobin in the absence of the reductase enzyme (Bekhit, Geesink, Morton, & Bickerstaffe, 2001). Ascorbate is reported to reduce metmyoglobin and this effect was catalyzed by pyridine and pyridine derivatives (Mikkelsen & Skibsted, 1992). An apparent nonenzymatic reduction of

metmyoglobin by Vitamin E in the form of alpha tocopherol was reported in the presence of liver extract (Lynch, Faustman, Chan, Kerry, & Buckley, 1998). As mentioned earlier, certain species of bacteria isolated from the environment including a lactic acid bacteria species were shown to stabilize red color of myoglobin by producing oxymyoglobin or nitric oxide myoglobin (K Arihara et al., 1993).

pH has a major influence on metmyoglobin reduction and higher pH facilitates MRA. Optimal pH for MRA depends on the species producing meat, test conditions, concentration of substrates and cofactors as well as source of myoglobin – crude extract versus purified preparation; and variation in each has been reported to give different results in terms of metmyoglobin reducing activity (Bekhit & Faustman, 2005). Generally, enzymes are optimally active at and nearby physiological pH (near neutral) and deviation from this would be expected to reduce their activity. However, at saturation concentrations of NADH, a work has shown that MRA increases with decrease in pH in the range of 7 to 5 at high enzyme concentrations (Mikkelsen & Skibsted, 1992). Chilling rate and temperature of meat influences pH changes as faster chilling lead to slower pH decline. This leads to darker colored meat with higher water holding capacity (Tarrant & Mothersill, 1977).

Lactic acid natural accumulates in post mortem muscle due to the anaerobic metabolism in the form of glycolysis occurring in meat until its buildup causes a pH drop which becomes unconducive for some of the glycolytic enzymes. The glycolytic pathway proceeds in the cytoplasm of muscle cells and generates the reducing equivalent NADH anaerobically (Kim et al., 2006). Lactate salts such as those of calcium, potassium, and sodium have been utilized in injection enhancement and in marinades for a variety of purposes. Calcium lactate has been reported to improve raw and cooked meat quality in terms of color life, microbial inhibition, and

sensory characteristics like shear force and flavor (Lawrence, Dikeman, Hunt, Kastner, & Johnson, 2003). Potassium and sodium lactate solutions are commonly used for carcass washing and meat decontamination due to their bacteriostatic effect on spoilage and pathogenic microorganisms (Toldrá, 2010). The antimicrobial effect of lactate salts arises from their ability to bind water and reduce water activity as well as to lower pH of the aqueous phase making it hostile for microbial growth (Papadopoulos, Miller, Acuff, Vanderzant, & Cross, 1991). The lactate salts also are known to enhance flavor of meat by enhancing fresh beef flavor and reducing off notes (Papadopoulos, Miller, Ringer, & Cross, 1991). Beef steaks enhanced with lactate injection tend to have a darker redder color and reduced greying. The mechanism behind the color stabilization involves the enzymatic oxidation of lactate to pyruvate in meat in the presence of Lactic dehydrogenase. The proton lost is taken up by NAD+ to form the reducing equivalent NADH which can take part in metmyoglobin reduction through the enzymatic or non-enzymatic pathways aforementioned (Kim et al., 2006).

Succinate serves as an intermediate in the mitochondrial Krebs Cycle and is reported to be involved in metmyoglobin reduction via mitochondrial activity (Tang, Faustman, Mancini, et al., 2005). Unlike the lactate involving metmyoglobin reduction pathway which is cytosolic, the succinate pathway represents the mitochondrial contribution towards metmyoglobin reduction activity. Even though enzymes of the Krebs Cycle and electron transport chain (ETC) are present in post mortem meat, the prevailing conditions of pH (5.6 to 6.4) and temperature (usually 4^{o}C or below) as versus the physiological conditions (7.2 and 27^{o}C respectively) retard enzymatic activity, but not completely inhibit it. Enzymatic activity is also limited by depleted substrates. Thus, external addition of substrates to resume activity which is responsible for metmyoglobin reduction via mitochondrial ETC can also be used as a strategy to reduce

discoloration. Specifically, succinate oxidation in the complex II of ETC has been proposed to provide electrons for metmyoglobin reduction. The proposed pathway is

succinate --> complex II --> ubiquinone --> complex III --> cytochrome c --> OM cytochrome b5 --> MetMb ;

where OM stands for outer mitochondrial membrane.

Under normal ETC regimen, electrons are transferred through a series of carriers to finally oxygen. Thus, exhausting oxygen and reversing ETC would lead to accumulation of electrons which would increase the reducing potential of the mitochondrial environment. The pool of accumulated electrons can be used to reduce metmyoglobin present in the proximity in the cytoplasm of muscle cells. Through a series of enzyme inhibitors which exclusively block complex II, III, and IV of ETC such as malonate, myxothiazol (MY) and azide/CO/cyanide respectively; Tang and co-workers have proposed that electrons become available for metmyoglobin reduction between complex III and complex IV (Tang, Faustman, Mancini, et al., 2005).

These tests were carried out on isolated mitochondria and purified myoglobin and thus may seem to be narrow in scope. However, based on the proposed pathway, succinate was shown to be involved in metmyoglobin reduction in raw beef patties (Zhu et al., 2009). A concentration dependent positive effect on visual color (redness and chroma) and relative myoglobin redox forms was reported with patties enhanced with succinate. The succinate dependent metmyoglobin reducing activity was also shown to be more stable and immune to oxygen as compared to the lactate dependent anaerobic metmyoglobin reducing activity by the same group of workers. Based on these previous reports, in the present study, the substrate lactate was

chosen to examine the cytosolic pathway involved in MRA and succinate, for the mitochondrial pathway involved in MRA.

Iron in muscle foods

Iron is the most abundant trace metal in muscle foods and is necessary for several biochemical functions such as electron transfer, oxygen transport, and catalysis. Transition metals such as iron and copper are important catalysts in biological systems due to their incompletely filled penultimate d orbitals which can accommodate and donate free electrons. The two stable oxidation states of iron in aqueous systems are the +2 and +3 – called ferrous and ferric iron respectively. Iron in these 2 oxidation states, either as ionic iron or iron complexed within macromolecules can catalyze several biochemical oxidation reduction reactions. In muscle foods, the major form of iron is the organic heme iron which is the prosthetic group consisting of porphyrin complexed iron found in proteins such as myoglobin, hemoglobin, certain catalases, and cytochromes (Papanikolaou & Pantopoulos, 2005). The other smaller proportion of iron – the nonheme iron is present in different forms. It is present in metaloproteins in the form of iron-sulfur clusters eg. in aconitase or iron-oxo clusters eg. in ribonucleotide reductase. A very small percent of cytosolic iron (<5%) is present in low molecular weight complexes such as iron complexed to citrate, ascorbate, amino acids, or nucleotides and is extremely reactive as a pro-oxidant (Decker E. & Hultin H., 1992).

The mitochondria are the hub of iron trafficking since the heme assembly is completed inside mitochondria. The major portion of the iron that is absorbed in the intestines and iron from the tissue reserves is transported to the mitochondria for heme assembly (Rouault & Tong, 2005). The plasma iron carrier – transferrin transports iron from the site of absorption in the

small intestines via blood circulation to the cells of various tissues. Specific receptors for inorganic iron – the transferrin receptors receive this iron which is internalized into the cytoplasm through the endosomes. Once in the cytoplasm, this iron is either stored in the cellular reserve ferritin or directed towards mitochondria (Papanikolaou & Pantopoulos, 2005). In the ferritin molecules, iron is stored in the metabolically inactive ferric state which is also much less soluble in aqueous phase at the cytosolic pH. The ferritin molecules are cytosolic sequesterants of free iron and each molecule can accommodate up to 4000 iron ions (Arosio, Ingrassia, & Cavadini, 2009). In the mitochondria, inorganic iron is 'fixed' into the protoporphyrin structure, which is assembled elsewhere in the cytoplasm, to form heme. Owing to the labile and pro-oxidant nature of iron ions, the iron trafficking in heme assembly is tightly regulated. However, about 1-2 % iron does leak and is present in the cytoplasm, and is usually chelated to small molecules such as organic acids, amino acids or nucleotides. Even though chelated, this redox active iron pool is pro-oxidant and can generate reactive oxygen species through Fenton chemistry and the Haber-Weiss reaction (Eaton & Qian, 2002). Of the reactive oxygen generated, the hydroxyl radical ($HO^{\bullet}$) is the most potent pro-oxidant, though other free radicals from the breakdown of lipids and peptides too are generated by reactions such as the following where ROOH and RSH are generic lipid and organic molecules respectively (Papanikolaou & Pantopoulos, 2005).

The cytosolic pH is near neutral under physiological conditions but drops due to glycolysis post mortem. Although ferritin has a remarkable stability towards heat denaturation, it is susceptible to reducing agents when binding of iron is concerned. The ferritin shell is impermeable to reducing agents. However, the reducing agents transfer electrons across the ferritin shell into its interior cavity containing the ferric iron core, where the ferric ions are

reduced to ferrous ions. The ferrous ions so formed can easily exit the ferritin shell through hydrophilic channels (Arosio et al., 2009). The drop in pH as well as reducing agents like ascorbic acid or cysteine which are inherently present lead to ferritin denaturation and subsequent release of ferrous iron from ferritin in post mortem muscle. Decker and Welch reported that beef stored at 4^{o}C for 11 days had a pH and reducing agent concentration dependent iron release which occurs in situ (Decker & Welch, 1990). This iron is available for catalyzing oxidation reactions of lipids, proteins, and nucleotides. The oxidation products in general are deleterious for characteristics of visual, nutrition, texture, and flavor properties.

Products of lipid and phospholipid oxidation tend to have negative impact on flavor imparting rancid, warmed-over or cardboard like flavor to meat. Lipid oxidation releases primary breakdown products like free fatty acids and secondary products like aldehydes, ketones, and alcohols which are associated with unpleasant or unusual odors. Also, oxidation of lipid to lipid free radicals is a problem since the radicals can charge towards any oxidizable substrate. Color deterioration too occurs as a result of auto-oxidation of deoxymyoglobin (Fe^{2+}) to metmyoglobin (Fe^{3+}) and ferryl myoglobins (Fe^{4+}). Oxidized lipids interact with proteins through non-covalent complexes, or radical type oxidation products or reaction with secondary oxidation products, all of which have been implicated with protein polymerization and cross-linking which adversely affects the texture. The free radicals from lipids and other oxidizable organic substrates co-oxidize compounds like Vitamin A and carotenoids, C, E, as well as cholesterol- which reduces the nutritive value of raw meat (Kanner, 1994).

Iron is also externally introduced into the meat via contact surfaces during various stages of storage and processing. Even though austenitic and martensitic stainless steels are considered to be fairly inert and widely used in the food industry, they have been reported to leach iron into

the food or beverage they are in contact with, at very low concentrations. This effect is more severe in acidic and high moisture environment, both of which are presented by muscle foods (Kuligowski & Halperin, 1992; Weblink1). However, a considerable amount of inorganic iron is introduced into meat products as a contaminant in ingredients such as salt, spices, marinade, as well as packaging materials (Pradeep, Geervani, & Eggum, 1993; Reilly, 2008; Weblink2).

Mitochondria and Raw Beef Color

As mention earlier, the mitochondria are the hub of cellular iron trafficking and the major oxygen consumers (>80%) in live tissue. Of the various factors affecting color and oxidative stability of different beef muscles, oxygen consumption and mitochondrial activity has been extensively studied (Lanari & Cassens, 1991; Seyfert et al., 2006; Tang, Faustman, Hoagland, et al., 2005). The retail display life of beef semimembranosus muscle was reported to be inversely proportional to the log of oxygen uptake. The same muscle from lamb and pork had higher oxygen uptake and thus lower display life (Atkinson & Follett, 1973). The proposed mechanism behind oxygen consumption and myoglobin redox stability is based on uptake of reducing equivalents. The metmyoglobin reduction system, both enzymatic and non-enzymatic depends on reducing equivalents (mainly NADH) to reduce metmyoglobin to deoxymyoglobin. When mitochondrial oxygen consumption is high, more reducing equivalents are diverted towards donating electrons for reducing molecular oxygen via the electron transport chain making them less available for metmyoglobin reducing activity. This observation was further verified, when ETC inhibitors such as rotenone, low pH, or high oxygen environments in beef homogenates were shown to improve meat color and oxymyoglobin formation (Cornforth & Egbert, 1985; Cornforth, Egbert, & Sisson, 1985; Egbert & Cornforth, 1986). Due to these reasons, the

mitochondria were chosen for iron targeting to get a fundamental understanding of the effect of inorganic redox iron on myoglobin redox stability and color stability of raw beef.

Mitochondrial activity is commonly monitored by oxygen polarography. Firstly, the mitochondria are isolated from freshly harvested tissue – cardiac and liver tissues usually have the highest mitochondrial density and hence serve as good sources of mitochondria. The mitochondria are then suspended in an isotonic buffer containing sugars which can fuel mitochondrial activity and salts and chelating agents to prevent swelling and disruption of the mitochondrial membrane. The desired substrates/ poisons for mitochondrial enzymatic activity are added to the incubation mixture and changes in mitochondrial oxygen consumption are monitored. The oxygen cells such as Clark Electrode are employed, these are sensitive to oxygen concentration changes down to the order of nanomoles and hence are suitable for studies on isolated mitochondria (Frezza, Cipolat, & Scorrano, 2007).

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CHAPTER 3

INFLUENCE OF REDOX REACTIVE INORGANIC IRON, LACTATE, AND SUCCINATE ON MYOGLOBIN REDOX STABILITY, COLOR, AND MITOCHONDRIAL ACTIVITY OF RAW BEEF[1]

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Abstract

Metabolic intermediates of glycolysis and tricarboxylic cycleare known to stabilize beef color through improved metmyoglobin reducing activity. Inorganic redox reactive iron (RRI) forms are pro-oxidants that have been shown to oxidize myoglobin in model systems. This study investigated how RRI, in the presence of added metabolic intermediates lactate and succinate, influence myoglobin (Mb) redox stability and color of beef strip loin homogenate, and how it affects mitochondrial respiration. Homogenates with added RRI and either lactate or succinate had lower ($p < 0.05$) a^* values than control homogenates. Oxymyoglobin increased ($p < 0.05$) as added ferrous ion increased in the lactate and succinate treatments. Inorganic iron had an adverse effect on mitochondrial respiration and reduced mitochondrial oxidation of lactate and succinate ($p < 0.05$). Hence, the benefit of color stability offered by the metabolic intermediates was reduced in the presence of added inorganic iron ions.

Introduction

Color is a major quality cue for consumers for the acceptability of raw beef products at the point of purchase. Among several factors that affect quality of fresh meat, color deterioration alone is responsible to a loss of approximately a billion dollars revenue annually in the US beef industry (Smith et al., 2000). The bright red color of fresh beef is primarily due to oxymyoglobin, which contains heme iron in the ferrous state (Fe^{2+}). Myoglobin is the predominant heme iron containing protein responsible for meat color, although other proteins such as hemoglobin and cytochrome c do contribute to color as well. Oxidation of deoxymyoglobin (DMb) and oxymyoglobin (OMb) to metmyoglobin (MMb) causes a brown color to form in meat products, which is an undesirable quality trait that reduces consumer acceptability of such products (Mancini & Hunt, 2005).

Various strategies to improve meat color and meat color stability have been studied and used in livestock feed management and by the meat industry, among them are supplementation of animal diet with Vitamin E (Faustman, Chan, Schaefer, & Havens, 1998), modified atmosphere packaging (Jayasingh, Cornforth, Brennand, Carpenter, & Whittier, 2002; John et al., 2005), use of antimicrobials (Pohlman, Stivarius, McElyea, Johnson, & Johnson, 2002), and adding metabolic substrates to post mortem muscle tissues (Mohan, Hunt, Barstow, Houser, & Muthukrishnan, 2010; R. Ramanathan, Mancini, & Dady, 2011). In packaged meat, biochemical processes like glycolysis (GL) and the tricarboxylic acid cycle (TCA) are responsible for regenerating reduced NAD (NADH) through enzymatic reactions. NADH can reduce metmyoglobin to deoxymyoglobin (DMb) until the substrate pool for the processes that generate NADH is available for the inherent enzymes. DMb is then converted to OMb on exposure to oxygen (bloom), which gives meat the desired bright red color (Saleh & Watts, 1968). To make

up for the depleted substrates for enzymatic activity, adding metabolic intermediates of the biochemical pathways (GL and/or TCA) to packaged, case-ready meat products is one way to continue MMb reducing activity and thereby stabilize color (Mancini, Ramanathan, Suman, Dady, & Joseph, 2011; Zhu, Liu, Li, & Dai, 2009). Many studies have discussed the role of metabolic intermediates in color stabilization. Kim and colleagues reported that adding lactate to beef strip loins led to the enzymatic conversion of lactate to pyruvate by Lactate dehydrogenase with the concomitant formation of NADH. NADH subsequently contributed to metmyoglobin reduction(Kim et al., 2006). Another study reported that in raw ground beef, adding succinate increased the reduction of metmyoglobin but had no effect on *a** values (Mancini et al., 2011) while another study (Zhu et al., 2009) reported that adding succinate to ground Longissimus dorsi muscle patties enhanced metmyoglobin reducing activity and stabilized the red color as well. Furthermore, adding malate, pyruvate, lactate, and combinations to different beef muscle homogenates stabilized color by increasing metmyoglobin reducing activity (Mohan et al., 2010).

In muscle foods, iron is predominantly in the organic heme form and a small proportion of the total iron in meat tissue is in inorganic forms (Purchas, Simcock, Knight, & Wilkinson, 2003). The proportion of inorganic iron rises during storage and processing (Estévez & Cava, 2004; Purchas et al., 2003). Inorganic iron also leaches into meat through a variety of contact surfaces during storage, handling, distribution, and processing in small concentrations (Kuligowski & Halperin, 1992) and more prominently as a contaminant in various ingredients like seasoning, salt, spices, water, etc (Reilly, 2008). Inorganic iron in cooked meat products has been extensively studied (Love & Pearson, 1974; Sato & Hegarty, 1971; Tichivangana & Morrissey, 1985). Inorganic redox forms of iron cause catalysis of lipid peroxidation and auto-

oxidation (Decker & Hultin, 1992), which in turn causes oxidative changes in proteins, pigments, fats, and vitamins adversely affecting quality characteristics like flavor, color, texture, and nutrition (Kanner, 1994).

A study investigating oxymyoglobin oxidation in a purified model system in a buffer containing microsomes and redox reactive iron ($Fe^{2+}$and/or Fe^{3+}) has shown that inorganic RRI causes significant oxidation of oxymyoglobin through the products of lipid peroxidation (Gorelik & Kanner, 2001). Another study showed that redox active Fe^{2+} ions oxidize myoglobin independent of the effect of lipid oxidation and its products (Allen & Cornforth, 2006). These studies were carried out in purified model systems. Raw meat has inherent antioxidant compounds such as ascorbic acid, glutathione, cysteine, and NADH (Kanner, Salan, Harel, & Shegalovich, 1991) as well as pro-oxidants such as different heme compounds, lipid breakdown products and inorganic iron (Tichivangana & Morrissey, 1985). Hence the roles of several biochemically active components inherent to raw skeletal muscles were not considered in the mentioned studies carried out in model systems.

Myoglobin and mitochondria are closely associated since the former serves as an oxygen reservoir for the latter under physiological conditions. Mitochondrial oxygen consumption persists post mortem for several days (Tang, et al., 2005). Mitochondrial oxygen consumption rates directly influence the redox form and stability of myoglobin with higher oxygen consumption leading to more discoloration (Lanari & Cassens, 1991; Tang et al., 2005). How redox reactive iron ions in the presence of externally added metabolic substrates affects raw beef color warrants further investigation in meat as well as at a more fundamental sub-cellular level. Hence the specific objectives of this work were to investigate how succinate and lactate, in

combination with redox reactive iron ions, affect myoglobin redox stability and color in raw beef homogenates and to investigate how the same combination affects mitochondrial respiration.

Materials and Methods

Raw Materials and Chemicals

Beef strip loin meat was purchased from a local supplier and frozen to -40^{o}C. Beef hearts were donated by White Oak Pastures, Bluffton, GA and used within one day of harvesting. 3-(N-morpholino) propanesulfonic acid (MOPS) (>99.5%), ferrous chloride tetrahydrate (ReagentPlus®, 98%), ferric chloride hexahydrate (ACS reagent, 97%), HEPES(4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), potassium chloride, potassium dihydrogen phosphate, magnesium chloride, bovine serum albumin (BSA), adenosine 5′-diphosphate (ADP), bacterial Nagarse protease, ethylenediaminetetraacetic acid (EDTA), Pierce™ BCA Kit, 500 microliter Hamilton syringes and sodium hydroxide were obtained from Sigma Chemical Co.(St. Louis, MO), potassium lactate from PURAC America, Inc. (PURASAL HiPure P, 60% potassium lactate/40% water; Lincolnshire, IL), anhydrous dibasic sodium succinate from Spectrum Chemical Corp. (Gardena, CA). Glass Wheaton-Potter-Elvehjem grinders- glass tube with polytetrafluroethylene pestle, RNA-ase free D-Sucrose, and lactic acid were obtained from Fisher Scientific (Fair Lawn, NJ), and Ethylene glycol-bis-(beta-aminoethylether)-N'-N'-N'-N'-tetraacetic acid (EGTA) was obtained from from MP Biomedicals (Solon, OH). Kontes Dual Tissue Grinder- glass tube with polytetrafluroethylene pestle, centrifuge tubes (DNase, RNase, and endotoxin free) were obtained from VWR (Suwanee, GA). A heavy duty mechanical drill driver purchased locally was used to drive the pestles for the tissue grinding procedures.

Experiment on raw beef homogenate

Steaks were cut from frozen strip loins, vacuum packaged in Cryovac Durashrink® bags (polyvinylidene chloride) and thawed for 12h at 0-4^{o}C. Pieces of lean were removed from the steaks and mixed with four times (weight by volume) of 0.04M MOPS buffer solution containing either 2% (w/v) lactate using potassium lactate or 2% (w/v) succinate using monosodium succinate or only buffer as control. Homogenates were prepared by blending this mixture for one minute in a Waring blender and visible lumps of connective tissue were removed. The pH of the homogenates was adjusted to 5.6 ± 0.1 and the homogenates were poured into 10x100 mm sterile polystyrene petri plates. To this solutions of ferrous or ferric ions was added in order to get the final concentration from 0 to 8 ppm of the respective ions in the homogenate. The homogenates were gently stirred at with clean glass rods, the petri plates capped and incubated at 37^{o}C for half an hour.

Post incubation, the homogenates were stirred again, using glass rods, and read for instrumental color CIE *L**, *a**, *b** and reflectance from 400 to 700nm using A/10 illuminant (d/8°, LAV, 25mm port) from HunterLab Miniscan EZ portable spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA). The color and reflectance readings were taken in duplicate on each petri plate and averaged for statistical analysis. The relative percent of the three redox forms of myoglobin were determined using the absorption and scattering indices (K/S) values specific to redox myoglobin form wavelengths as described in the AMSA color guidelines (AMSA, 2012).

Mitochondrial isolation

The procedure of Tang and colleagues was followed for mitochondrial isolation and oxygen polarography (Tang, Faustman, Hoagland, et al., 2005). Briefly, 50 g of cardiac muscle free of visible connective tissue and fat was excised and washed twice in the wash solution before mitochondrial isolation. The lean was finely minced and then suspended in 200 ml of isolation buffer while being gently stirred with a magnetic stirrer. Bacterial Nagarse protease (25 mg/ 50 g tissue) was added to this suspension while being stirred and the pH was maintained between 7.2 and 7.4. After 20 minutes of proteolysis, the suspension was allowed to stand and the supernatant was gently decanted. The protease treated cardiac tissue was washed twice with the isolation buffer to remove the residual protease and re-suspended in 500 ml of isolation buffer. This suspension was given three passes each of Kontes Dual grinder and the Wheaton Potter Elvehjem grinder. The resulting muscle suspension was centrifuged at 1200 × g for 20 minutes. The supernatant so obtained was filtered through two layers of ultrapure water washed cheese cloth. The clear solution so obtained was then centrifuged at 20,000 g for 20 minutes in a Sorvall RC 6 centrifuge (Thermo Fisher Scientific, Waltham, MA) to get pellets of mitochondria. The resulting pellets were washed twice with suspension buffer and re-suspended in the same. The temperature of the contents during the entire operation was maintained between 0 and 4 °C. The protein concentration of the mitochondrial suspension was measured using the procedure of Bicinchoninic acid assay (BCA).

Experiment on isolated mitochondria

The device used for measuring mitochondrial oxygen consumption was a Clark Electrode attached to a Model 20 controller (Rank Brothers- Cambridge, England) and PicoLog ADC 20

USB data logger. The data logger was attached to a computer for real time data acquisition and storage. The temperature of the electrode cell was maintained at 25°C using a recirculating water bath. A 1 cm magnetic flea stirrer was used to keep the cell solution agitated while measuring the oxygen consumption. Iron solutions of suitable concentrations to get final concentrations of 5 or 60 nanomole of iron per milligram of mitochondrial protein were added to 1 ml of incubation buffer in the electrode chamber and it was sealed with a stopper. These levels respectively represent the normal iron concentration and exceptionally high concentration of mitochondrial iron reported in viable cells suffering from mitochondrial dysfunction as in the case of Friedreich's ataxia (Foury & Cazzalini, 1997). Though iron accumulation of such high level in beef muscle mitochondria is unlikely, the level was tested as a realistic extremity to understand the iron mediated changes in mitochondrial respiration.

Additions of mitochondrial suspension, substrates - lactate and succinate, and ADP solutions were made to the cell using Hamilton syringes through the port in the stopper while being continuously stirred. The mitochondrial suspension was added such that the protein concentration in the cell chamber was 1 mg/ml which is sufficient to exhibit measurable oxygen consumption (Estabrook, 1967). The state IV oxygen consumption rate was calculated as the rate of consumption of atomic oxygen per mg of mitochondrial protein per minute in the presence of substrate alone, and state III oxygen consumption rate in the presence of both the substrate and ADP. The respiratory control ratio was calculated as the ratio of state III to state IV oxygen consumption rates. The time of calculation of the slopes varied according to the response given by mitochondria to the substrate and ADP additions.

Design of experiments and statistical analysis

For the experiments on homogenates, the experimental design was balanced factorial with three levels of substrate treatment (control, succinate, and lactate), and five levels each of Fe(II) and Fe(III) ion concentrations (0,2,4,6,8 ppm). Data were collected in duplicate samples on 3 replicates. Type-3 tests of fixed effects were performed using the MIXED procedure of SAS. Least square means for F-tests ($p< 0.05$) were separated using the diff option (least significant differences) and were considered significant at $p < 0.05$. For the mitochondrial experiments, a complete randomized block design was employed on three replicates with the two substrates lactate and succinate serving as the blocks and within each block, the combination of different iron ion concentrations served as a treatment level. Tukey's Honestly Significant Difference test was used to separate means when significant difference ($p<0.05$) was found.

Results and Discussion

Instrumental color

The redness as indicated by a^* values of homogenates significantly varied with different substrates ($p < 0.05$; Figure 3.1). Average a^* values of samples with no added substrates in the presence of RRI ions were significantly higher than for samples with added lactate or succinate. Average a^* values did not differ significantly between samples containing RRI with added lactate and added succinate. However, in the absence of added RRI ions, the average a^* values were significantly higher ($p < 0.05$) for lactate than for succinate and control. The average a^* value decreased with added Fe(II) ions ($p < 0.05$) and added Fe(III) ions ($p < 0.01$; Fig 3.2).

(Lindahl, Lundström, & Tornberg, 2001) found that the overall pigment content and fraction of MMb were most important in explaining the variation of *a** in pork M. Longissimus dorsi and M. Biceps femoris muscles. Metabolic substrates, lactate and succinate, increase MMb reducing activity by generating NADH and electrons respectively which reduce MMb to DMb. On exposure to oxygen, the DMb converts to OMb which gives the desired bright cherry red color to meat thereby stabilizing redness (Mohan et al., 2010; Zhu et al., 2009). In this study, the average *a** for homogenates treated with lactate and succinate, in the absence of added redox iron ions, was higher than control. However, in the presence of RRI, the homogenates with added lactate and those with added succinate had lower *a** values than the control. This reduction of *a** may be due to oxidation of oxymyoglobin directly or through other mechanisms such as lipid oxidation might also contribute to reducing the redness indirectly.

Of the two redox iron forms used in this work, Fe (II) ions are pro-oxidants with well documented roles in lipid peroxidation in raw muscle foods leading to a series of detrimental qualitative changes (Kanner, 1994). Fe (III) ions are not soluble at post mortem meat pH, which is approximately pH 5.2 or more (Feig & Lippard, 1994). In the presence of reducing agents such as ascorbate, NADPH, or cysteine which are commonly present in raw meat (Kanner, et al., 1991) and in an aerobic environment, ferric ions are reduced to ferrous ions, which contribute to lipid peroxidation through reactions 1 to 3 where AH is an arbitrary reducing agent:

$$Fe^{3+} + AH \;\text{---}> Fe^{2+} + AH^{\bullet} \qquad (1)$$

$$Fe^{2+} + O_2 \;\text{---}> Fe^{3+} + O_2^{\bullet\,--} \qquad (2)$$

$$Fe^{2+} + ROOH \;\text{---}> Fe^{3+} + RO^{\bullet} + OH^{--} \qquad (3)$$

Further, in an acidic environment, the superoxide radicals combine to form hydrogen peroxide through reaction 4, which further reacts with RRI ions to generate hydroxyl radicals through Fenton reactions 5 and 6. The hydrogen peroxide can further knock out iron from porphyrin in myoglobin increasing the inorganic iron content. Overall the oxidation reactions are self-catalyzing:

$$2\ O_2^{\bullet -} + 2H^+ \text{---> } H_2O_2 + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \text{ ---> } Fe^{3+} + HO^{\bullet} + OH^{-} \quad (5)$$

$$Fe^{3+} + H_2O_2 \text{ ---> } Fe^{2+} + HO^{\bullet} + HOO^{\bullet} + OH^{-} \quad (6)$$

Variation in myoglobin redox forms:

The interaction effect of Fe(II) concentration and substrate significantly affected OMb content ($p < 0.01$; Fig 3.3). For homogenate samples with added lactate and succinate, OMb content increased with ferrous ion concentration ($p < 0.05$). However, OMb content reduced with ferrous ion concentration in the absence of lactate or succinate. Ferric ion concentration directly influenced ($p < 0.05$) the relative content of all three myoglobin redox forms in the homogenate system (Fig 3.4). The relative deoxymyoglobin content and oxymyoglobin content decreased with Fe(III) concentration ($p < 0.01$), and relative metmyoglobin content increased with Fe(III) addition ($p < 0.01$), averaged over all the substrate treatments and all ferrous ion treatments.

Allen & Cornforth (2006) have reported that the oxidation of OMb to MMb increased when ferrous ions were added in their purified myoglobin model system. In this study, in the presence of ferrous and ferric ions, the relative OMb content actually increased with the addition of lactate and succinate with increasing ferrous ion concentration. A plausible explanation for

this observation is that lactate produced NADH through the activity of lactate dehydrogrenase (Kim et al., 2006) which can participate in metmyoglobin reduction to deoxymyoglobin. Since the homogenates were an open system in contact with air, the deoxymyoglobin is oxygenated to oxymyoglobin. Succinate on the other hand does not directly produce any reducing equivalent (NADH) but instead produces electrons through succinate driven mitochondrial respiration (Zhu et al., 2009). This makes the local environment more reducing in nature, thus lesser metmyoglobin formation, either directly, or through products of lipid oxidation (Mészáros, Tihanyi, & Horváth, 1982; Takayanagi, Takeshige, & Minakami, 1980). In either of the situation, OMb relative content increased with ferrous ion concentration and hence, how ferrous ions influence oxidation of succinate and lactate or how ferrous ions reduce metmyoglobin formation warrants further investigation. The results suggest that the combination of metabolic substrates (lactate and succinate) and ferrous ions promote OMb formation. This is beneficial since OMb is less prone to oxidation into MetMb as well as it imparts the desired bright red color to raw beef.

A previous study in a buffered model system containing microsomes, purified myoglobin, and redox iron ions has shown that redox iron along with membranal lipid oxidation causes most oxidation of OMb to MMb, but the oxidation activity attributed to redox iron alone was very small (Gorelik & Kanner, 2001). The present study involved ground beef homogenate containing the cell membrane lipids as well as other components inherent to skeletal muscles which can have both pro and antioxidant activities.

The relative percentage of OMb decreased ($p<0.05$) with ferric concentration (Figure 3.4) while the percentage of MMb increased, so the results of this study suggest that the extent of

OMb oxidation increased with ferric ion concentration averaged over all ferrous ion concentrations, possibly by the following reaction:

$Mb\text{-}Fe^{2+} - O_2$ (oxymyoglobin) + Fe^{3+} ---> $Mb\text{-}Fe^{3+}$ (metmyoglobin) + Fe^{2+} + O_2 (7)

However, increasing the ferric ion concentration may lead to increased ferrous ion production via reaction 1, which could lead to subsequent formation of hydrogen peroxide, hydroxyl radicals, and perhydroxyl radicals (reactions 2, 4, 5, and 6). These can oxidize oxymyoglobin through reactions 8 and 9:

$Mb\text{-}Fe^{2+} + H_2O_2$ ---> $Mb\text{-}Fe^{4+}=O + H_2O$ (8)

$Mb\text{-}Fe^{2+} + OH^{\circ}$ ---> $Mb\text{-}Fe^{3+} + OH^{--}$ (9)

Hence, it would be difficult to pinpoint the exact route of action of ferric ions on oxidation of deoxymyoglobin and oxymyoglobin. What is also worth mentioning is the underlying presence of ferrous ions in these results. Investigation of the direct reactions between ferrous and ferric ions on the matrix components of raw beef, either alone or in combination with myoglobin would shed light on the plausible interaction between the two iron forms, the related free radicals and their combined oxidative effect, either on myoglobin specifically or in general on other oxidizable substrates present in beef. Also, it would be worth investigating how redox reactive iron ions affect lactate dehydrogenase, succinate dehydrogenase, and other enzymes present in the cells which contribute towards metmyoglobin reduction activity.

Mitochondrial oxygen consumption

In the absence of any iron, the addition of succinate to mitochondria resulted in a much greater state III consumption rate (oxygen consumption in the presence of substrate and ADP)

than that for lactate ($p<0.05$) as seen in Table 3.1. Tang and colleagues have reported that respiratory control ratios (RCR) above 2.2 are indicative of functional integrity and active respiration of beef heart mitochondria (Tang et al., 2005). In the absence of iron, the respiratory control ratios for both succinate and lactate addition observed here indicate that the mitochondria isolated during the present experiments were structurally intact and thus actively respiring. Iron loading has been reported to negatively affect mitochondria by causing oxidation of membrane polyunsaturated fatty acids, loss of activity in thiol dependent-enzymes, and increased fragility of lysosomes (Eaton & Qian, 2002).

When succinate was the substrate for oxidation, mitochondria treated with 5 nmole/mg of ferrous ions, either alone or in combination with same concentration of ferric ions showed a respiratory control ratio of near unity. A similar respiratory control ratio was observed for 60 nmole/mg ferrous ion treatment. The theoretical minimum respiratory control ratio of 1 implies that the mitochondria are structurally compromised and unresponsive to added ADP, which would otherwise increase oxygen uptake i.e. the state III oxygen consumption rate.

For lactate driven mitochondrial respiration, either of ferrous or ferric ions at 60 ppm alone or ferrous and ferric ions together at 60 ppm had similar effect on both state III and state IV oxygen consumption rates- these were significantly lower than those for no iron control treatments. The three treatments reduced the respiratory control ratio with respect to the lactate control though the value was much greater than 1 indicating the mitochondria were actively respiring under the mentioned treatments. Ferric ions at 5 ppm level significantly reduced the state III OCR and RCR ($p<0.05$).

The treatment combination of succinate with 60 ppm each of ferrous and ferric ions and the combination of lactate with 5 ppm of both ferrous and ferric ions when treated to mitochondria showed respiratory control ratios above 2. Both the treatments however significantly reduced the State IV oxygen consumption rates with respect to their respective controls, with the lactate and iron combination having a significantly lower State III oxygen consumption rate as well.

Lactate can drive mitochondrial respiration since it can enter mitochondria through monocarboxylate shuttles. Mitochondria contain an isoform of lactate dehydrogenase which can oxidize lactate to generate NADH. NADH participates in the electron transfer chain and thus leads to oxygen consumption (Brooks, Dubouchaud, Brown, Sicurello, & Butz, 1999; Pallotta, Valenti, Iacovino, & Passarella, 2004). The mitochondrial oxygen consumption rates obtained with lactate driven respiration, in the absence of iron addition are similar to those reported in literature for mitochondria isolated from different sources (Ohnishi, Kawaguchi, & Hagihara, 1966; Ranjith Ramanathan, Mancini, & Konda, 2009). Lactate driven mitochondrial activity competes for oxygen with the myoglobin redox forms leading to formation of more deoxymyglobin leading to darker meat. This mode of action, along with generation of the reducing equivalent NADH through lactate dehydrogenase by oxidation of lactate to pyruvate, leads to metmyoglobin reduction. The lactate driven metmyoglobin reduction is also active in the cytosol owing to the cytosolic lactate dehydrogenase isoform which is related to glycolysis (Kim et al., 2006; Ramanathan et al., 2009).

In the present work, presence of any level of redox iron significantly reduced the state III oxygen consumption rates ($p<0.05$) and only the combination of both ferrous and ferric ions at 5 or 60 nmole iron per mg protein significantly reducing the state IV oxygen consumption rate

($p<0.05$). The main implication of this finding is that redox iron ions hinder the process of oxygen uptake during lactate metabolism making the environment more oxidizing in nature, and this will negatively affect myoglobin redox form and color stability. An interesting observation was that even though oxygen consumption rates were negatively affected by redox iron, the structural integrity of mitochondria treated with lactate and a 5 ppm concentration of ferrous and ferric ions each was maintained considering the respiratory ratio of more than 2. However, owing to the significantly low state IV oxygen consumption rate of mitochondria undergoing this treatment, there is no direct benefit of iron loading to mitochondria. A possible explanation for this observation is redox iron may have compromised the activity of monocarboxylate transporters or mitochondrial lactate dehydrogenase which deliver lactate to the mitochondria and catalyze its oxidation, respectively, without structurally damaging the mitochondria. This way, the mitochondria have hindered lactate utilization even though structurally sound.

Succinate has a long history of being used to drive mitochondrial respiration since it can directly enter enzyme complex II in the mitochondrial electron transfer chain and get oxidized to fumarate. The hydrogen atoms lost as protons and electrons participate in electron transport chain (Ramanathan et al., 2009). In the present work, the combination of succinate with 60 nmol/mg ferrous ions or mixture of 5 nmole/mg each of ferrous and ferric ions was seen to cause mitochondrial disruption due to lower RCR values. The 5 nmole/mg combination of ferrous and ferric ions however caused a significant increase ($p<0.05$) in state IV oxygen consumption rate with respect to the zero iron control. The finding is interesting since the same treatment which compromises the structure of mitochondria also leads to higher mitochondrial oxygen consumption. Increased oxygen consumption can reduce oxygen partial pressure in meat leading

to more MMb formation and damaged mitochondria cannot further participate in reducing MMb thus generated.

Mitochondria treated with redox reactive iron were observed to be compromised in terms of oxygen consumption and possibly structurally as well, from the reduced RCR values. Thus, RRI were observed to be deleterious to color and also towards mitochondria which play an important role in MMb reduction and color stability.

Conclusion

Beef homogenates treated with 2% lactate or succinate had higher *a** values in absence of added inorganic iron. However, redox iron ions decreased *a** values of homogenates treated with lactate or succinate. Both ferrous and ferric ions showed a concentration dependent reduction in *a** values. Relative oxymyoglobin content of homogenates increased with ferrous ion concentration in the presence of lactate or succinate. Ferric ions exhibited a concentration dependent increase in relative metmyoglobin percent and concomitant decrease in oxymyoglobin and deoxymyoglobin content. Redox reactive iron ions caused a significant compromise to the mitochondrial respiration. In limited situations where mitochondrial respiratory control ratios and thus structural integrity were retained in the presence of redox iron ions, the oxygen consumption driven by both lactate and succinate was significantly lower. The results suggest that redox reactive iron ions have a negative impact on color of raw beef. Redox reactive iron ions also damage mitochondria which are modes of metmyoglobin reduction, and thus compromise color stability in raw beef. Thus, entry or formation of inorganic ions needs to be checked in fresh beef via all possible modes to ensure better color and more color stability of processed raw beef products.

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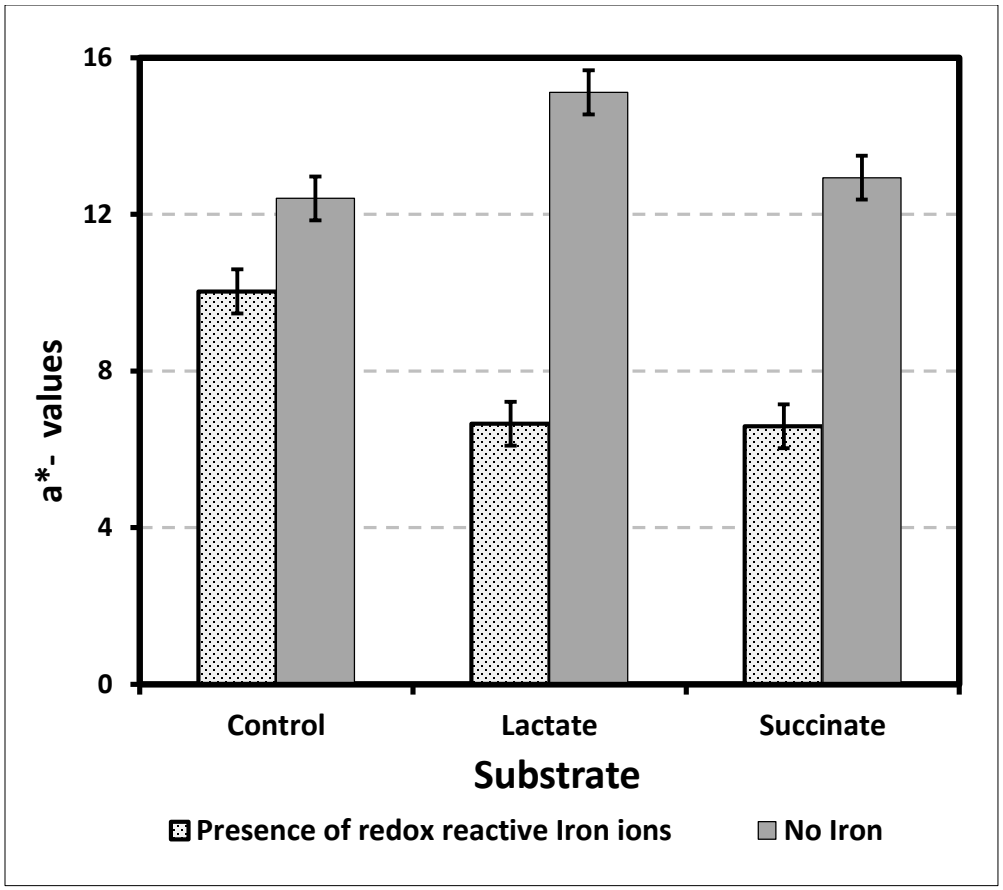

Figure 3.1 Variation of *a** values with metabolic substrates, with and without the presence of redox iron ions.

Values expressed as least square means with standard error bars.

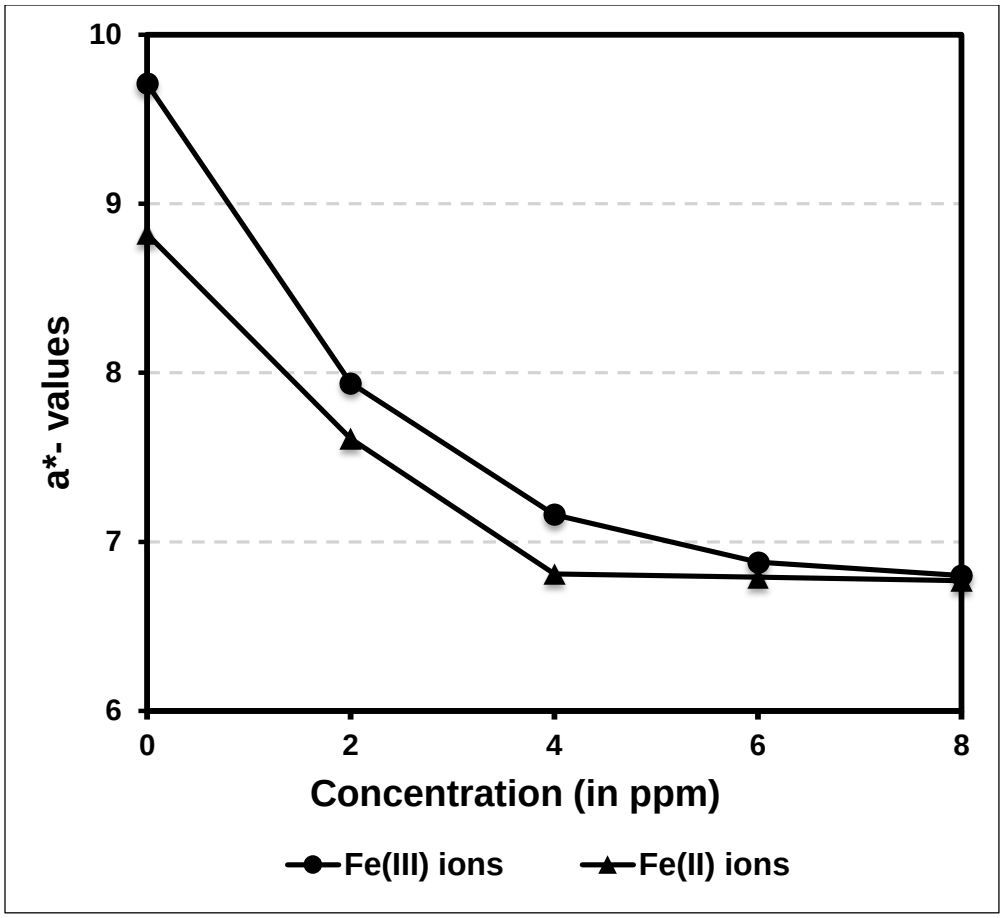

Figure 3.2 Variation of *a** values with ferrous and ferric ion concentration.

Values are expressed as least square means with pooled SEM = 0.471.

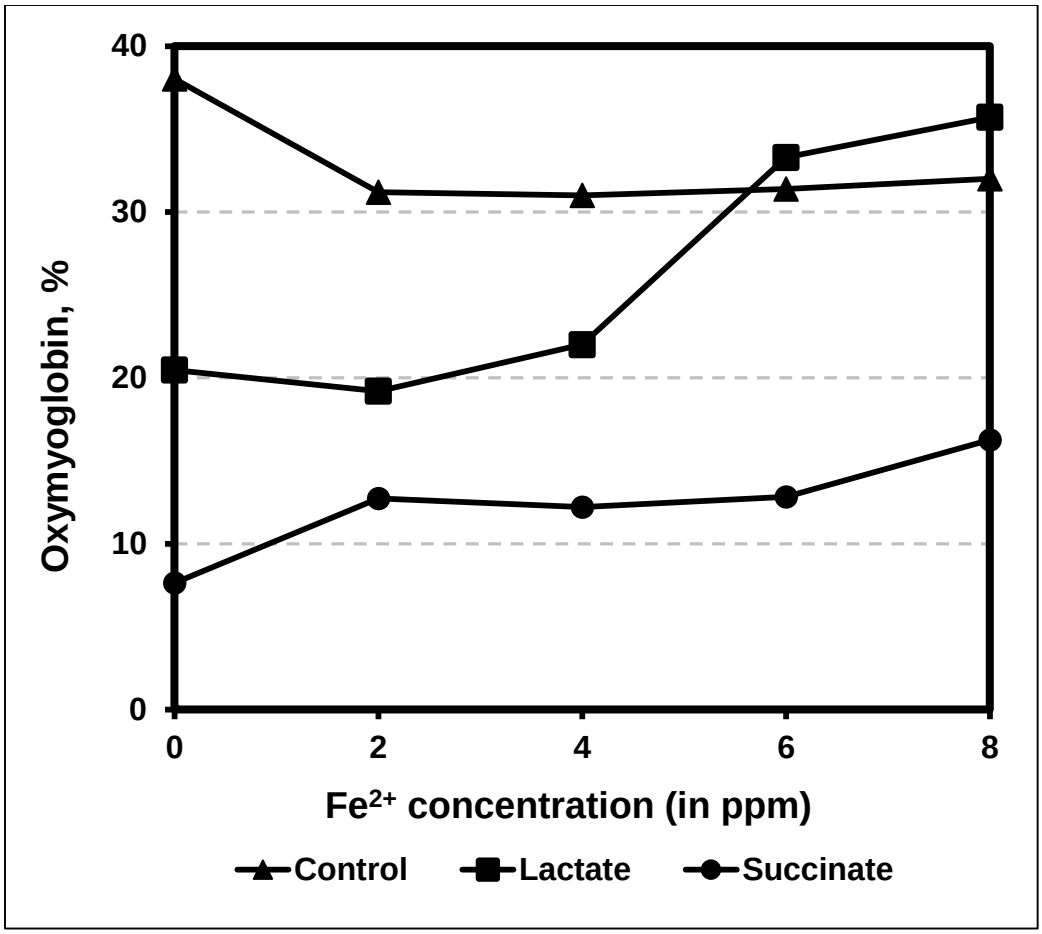

Figure 3.3 Interaction effect of ferrous ion concentration x substrate on percent oxymyoglobin. Values expressed as least square means with pooled SEM = 6.31

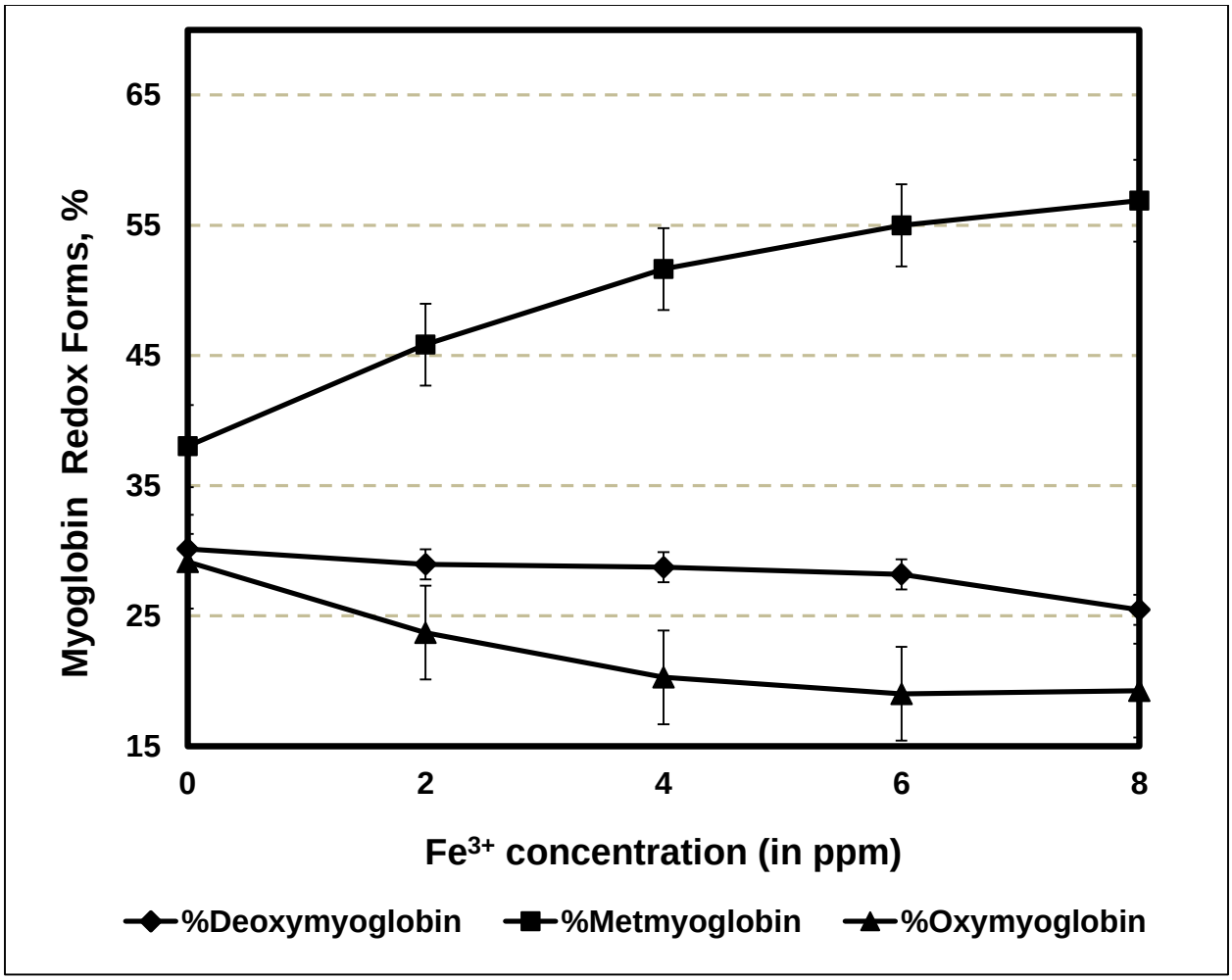

Figure 3.4 Variation in the relative contents of myoglobin redox forms with ferric ion concentration.

Values expressed as least square means with SEM - Deoxymyoglobin: 1.5, Oxymyoglobin: 3.6, Metmyoglobin: 3.15

Table 3.1 Effect of inorganic iron and metabolic substrates on the oxygen consumption rates and respiratory control ratios of beef heart mitochondria, observed at pH 7.2 and 25 ^{o}C (n=3)

Fe(II) concentration nmole/mg protein	**Fe(III) concentration nmole/mg protein**	**State III OCR**	**State IV OCR**	**RCR**
Substrate = 8mM succinate				
0	0	132.48 ax	46.16 cx	2.87 ax
5	0	65.42 cx	60.01 bx	1.14 cdx
60	0	14.82 ex	17.48 ex	0.89 dx
0	5	41.05 dx	42.31 cdx	1.02 cdx
0	60	47.6 dx	31.49 dx	1.53 cx
5	5	78.55 bx	78.31 ax	1.00 cdx
60	60	23.98 ex	11.21 ex	2.14 bx
Substrate = 8mM lactate				
0	0	79.92 ay	34.08 ax	2.38 abx
5	0	33.62 bcy	21.32 aby	1.60 cx
60	0	42.18 by	27.3 abx	1.54 cy
0	5	26.16 cx	36.19 ax	0.72 dx
0	60	46.17 bx	24.91 abx	1.86 bcx
5	5	45.36 by	16.21 by	2.81 ay
60	60	33.26 bcx	18.85 bx	1.80 bcx

The iron ion concentrations are expressed as nmole of iron per milligram of mitochondrial protein. State IV OCR is the oxygen consumption rate of mitochondria in the presence of substrate alone, while State III OCR is oxygen consumption rate in the presence of both substrate and adenosine diphosphate. The oxygen consumption rates are expressed in nmol of atomic oxygen/ mg protein/ min. RCR is the ratio of State III OCR divided by State IV OCR. Each combination of ferrous and ferric ions is considered as a level of treatment. Within a substrate, values in each row not sharing a common letter (a-e) are significantly different ($p<0.05$). Within an iron treatment level, values in each row not sharing a common letter (x,y) are significantly different ($p<0.05$).

CHAPTER 4

VARIATION OF DIFFERENT FORMS OF IRON AND THEIR CORRELATION WITH COLOR AND LIPID OXIDATION IN BEEF MUSCLES OF VARYING COLOR STABILITY[2]

[2] Anuj Purohit, Anand Mohan, Rakesh Singh, and William Kerr. To be submitted to the *Journal of Food Quality.*

Abstract

Iron in the heme and nonheme forms is known to be a catalyst for oxidative reactions in raw muscle foods. The concentration of the two iron forms varies in different muscles and is also altered during storage. Two beef muscles of varying color stability – *Longissimus dorsi* and *Psoas major* were analyzed for their total iron concentration, heme and nonheme iron concentration to evaluate how the iron form concentrations relate to instrumental color and lipid oxidation over a period of nine days in refrigerated storage. Concentrations of both the heme and nonheme forms of iron were significantly lower ($p<0.05$) on day 9 as compared to day 1. There was a moderate positive correlation found between heme iron concentration and redness in both the muscles whereas nonheme iron correlated poorly with redness. Heme and nonheme iron correlated negatively in a strong and moderate manner with lipid oxidation, respectively in both the muscles. The correlation between discoloration and heme iron was negative and moderate, and correlation between discoloration and nonheme iron was positive and moderate in both muscles. These results suggest that concentration of iron forms alone cannot be used to predict time dependent color and associated quality changes in fresh muscles. Hence, other intrinsic and extrinsic factors need to be considered together with concentration of different iron forms while developing and marketing products from beef muscles of varying color stability.

Introduction

Raw meat with bright red color is considered to be of fresh quality at the point of purchase (Renerre & Labas, 1987). The color of fresh meat is determined by intrinsic factors such as age, sex, breed, and nutrition of the animal pre-slaughter, location of the tissue in the body and its physiological function, and by extrinsic factors such as packaging material, temperature, fabrication, and processing (Mancini & Hunt, 2005). These factors are usually considered when developing, merchandizing, and marketing case ready fresh beef products from different muscles. Meat discoloration in fresh beef is the formation of a visible surface layer of metmyoglobin which imparts it brown color. Discoloration may be only a quality defect although microbial growth and prolonged storage are known to cause discoloration too (Troy & Kerry, 2010).

Myoglobin and specifically the oxymyoglobin redox form is the primary pigment offering fresh beef its characteristic cherry red color (Mancini & Hunt, 2005). Myoglobin is also a major reserve of heme iron in the muscle. The inorganic forms of iron consist of iron stored in bound forms such as in ferritin or hemosiderin, as iron-sulfur clusters in several enzymes, and iron that is present in the cytosolic pool chelated to low molecular weight peptides, amino acids and nucleotides(Gorelik & Kanner, 2001). The cytosolic pool of inorganic iron is catalytically active and can generate reactive oxygen species through Fenton chemistry. Both the organic and inorganic forms of iron are known to cause and catalyze lipid oxidation in raw and cooked muscle foods (Estévez & Cava, 2004; Gorelik & Kanner, 2001; Kanner, 1994; Ladikos & Lougovois, 1990; Tichivangana & Morrissey, 1985).

There is inherent variation in the concentration of both the organic and inorganic forms during storage due to factors such as muscle type, pH, reducing agents, protein and lipid oxidation, and drip losses (Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002; Purchas, Simcock, Knight, & Wilkinson, 2003; Rhee & Ziprin, 1987). Although information about the variation in concentration of iron forms and total iron in various muscle foods is available in several food composition tables (Purchas et al., 2003), there is limited information on how the different forms directly relate to color and related qualitative changes in raw beef during refrigerated storage. Hence, the objective of this study was to analyze the concentrations of heme and non heme iron in two beef muscles of varying color stability and to correlate those to raw color, discoloration, and lipid oxidation. The muscles chosen were the striploin (*M. Longissimus dorsi*) known to be a color stable muscle and the tenderloin (*M. Psoas major*) known to be a color unstable muscle.

Materials and Methods

Steaks from *M. Longissimus dorsi* (NAMP#180) and *M. Psoas major* (NAMP#1190A) were fabricated from roasts aged for two weeks post slaughter and were obtained from a local market (NAMP, 2004). The steaks were cut to about half inch thickness, placed in polystyrene trays containing soaker pads and overwrapped with polyvinyl chloride film. The packaged steaks were put on display for up to 9 days at to 2 ^{o}C under fluorescent white light and on the designated days (1,5, and 9), the steaks were removed from the packaging, a sample sufficient for measuring lipid oxidation (described later) was cut out, vacuum packaged in PE-nylon vacuum bags (Sealed Air Corp., Duncan, SC) and frozen to -40 ^{o}C. Samples for proximate composition and total iron content were cut from the steaks on day 0, vacuum packaged and frozen at – 40 ^{o}C till analysis. The remaining portions of all the steaks were freeze dried, vacuum

packaged and stored in a refrigerator at 4 ^{o}C till further analysis. All the analyses were performed on three replicates with samples analyzed in duplicates within each replicate except in cases where mentioned otherwise.

Proximate analysis

The proximate compositional analysis of samples was performed by the University of Georgia's Feed and Environmental Water Laboratory. Moisture analysis was performed by AOAC method 930.15, ash by method 942.05, crude protein by method 990.03 (AOAC, 1996). Fat was measured using Ankom™ XT 15 Extraction System using the manufacturer's instructions.

Total iron

Total Iron was measured by the *aqua regia* method using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) at the Analytical Chemistry Laboratory, Odum School of Ecology at the University of Georgia.

pH

The pH of steaks was measured using a pierce probe pH meter (IQ Scientific, Carlsbad, CA) in three different locations on each of the steaks and averaged for statistical analysis. The pH meter was calibrated using buffers of pH 4 and 7 before taking measurements on the designated days.

Instrumental color and absorbance

Color measurements on the steaks were made using Hunterlab® MiniScan EZ portable spectrophotometer using A/10 illuminant (d/8°, LAV, 25mm port, 14.3 mm diameter of

aperture, 10° standard observer; Hunter Associates Laboratory, Inc., Reston, VA, USA). CIE *L**, *a**, and *b** as well as absorbance in the 400-700 nm range were measured at three different locations on the steaks with a polyvinyl chloride film stretched over the aperture, and averaged. The instrument was calibrated with black and white tiles provided by the manufacturer with a polyvinylchloride film over the aperture, before making measurements on the steaks. Hue angle and saturation were calculated as $\tan^{-1}(b^*/a^*)$ and square root of $(a^{*2} + b^{*2})$, respectively. The relative percent of the metmyoglobin was determined using the absorbance and scattering indices (K/S) method as described in the AMSA meat color measurement guidelines (AMSA, 2012).

Heme iron

The method of Hornsey (1956) as modified by Purchas, et al. was employed with minor modifications (Purchas et al., 2003). Briefly, quarter of a gram of lean portion was removed from freeze dried steak and powdered in a centrifuge tube with clean glass rod. To this, 2 ml of deionized water, 0.25 ml of concentrated HCl and 10 ml of Acetone were added, the content was vortexed for 15 seconds and allowed to stand at room temperature for 15 minutes. The content was then centrifuged at 3000 g for 20 minutes using a Sorvall RC 6 centrifuge. The supernatant was passed through a Whatman glass (GF/A) filter paper and its absorbance was read at 640 nm against a reagent blank using a Shimadzu UV- 1800 UV-VIS spectrophotometer. The hematin content was determined by multiplying A_{640} by 680 with a correction factor for dilution and the heme iron content was determined by multiplying the hematin concentration by 0.882 which is the ratio of weight of atomic iron to weight of molecular weight of hematin (Keith, Miles, Bechtel, & Carr, 1985).

Nonheme iron

Nonheme iron was measured by the ferrozine method with minor modifications (Purchas et al., 2003). Briefly, half a gram of freeze dried meat was crushed in plastic centrifuge tubes using clean glass rods and was dissolved in 3 ml of 0.1 M citrate phosphate buffer adjusted to pH 5.5, and 1 ml of 2% ascorbic acid dissolved in 0.2 M HCl. The mixture was vortexed for 15 seconds and allowed to stand at room temperature in the dark for 15 minutes before adding 2 ml of 11.3 % Trichloroacetic acid and centrifuging at 3000 g for 10 minutes. The supernatant was passed through a Whatman glass filter paper. To 2 ml of supernatant, 0.8 ml of 10 % ammonium acetate and 0.2 ml of ferrozine reagent were added. Absorbance of the purple color developed solution was measured at 562 nm against a standard curve prepared using reagent grade ferrous chloride tetrahydrate.

Lipid oxidation

The products of lipid oxidation, expressed in terms of malondialdehyde (MDA) were measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay as described by John and co-workers (John et al., 2005) with minor modification. Briefly, a 1 gram sample was withdrawn from the center of the steaks, minced finely and added to centrifuge tubes containing 5 ml of 0.375 % thiobarbituric acid, 15% trichloroacetic acid both dissolved in 0.25 N HCl. The mixture was heated in sealed tubes in a boiling water bath for 30 minutes till a pink color developed. The tubes were then cooled with tap water and centrifuged at 5500 g for 25 minutes. The absorbance of the resulting supernatant was measured at 532 against a reagent blank and expressed as milligram of MDA per kilogram of meat.

Statistical analysis

For proximate composition and total iron analyses, a two sample t-test was employed. For all the other indicated responses, the experiment had a factorial design with the muscle and number of days as the main effects. R version 3.0.1 was used for statistical analysis (R-Core-Team, 2013). Means were separated by Tukey's Honestly Significant Test wherever significant difference was observed. Correlation coefficients were calculated between the various measured parameters to understand the direction and association of linear relation between each and tabulated in a correlation matrix.

Results

Proximate composition

Results of the proximate composition analysis (Table 4.1) showed that the crude protein percent was the only parameter which significantly differed in the two muscles, with strip loin having a higher protein content ($p<0.05$). The difference in moisture, ash, and fat content was not significantly different.

pH

pH was significantly affected by a muscle type x time interaction ($p<0.05$). The pH on day 1 between the two muscles was not significantly different. On day 5, Psoas major had a significantly higher pH whereas on day 9, Longissimus dorsi had a significantly higher pH. In both the muscls, pH significantly reduced with time ($p<0.05$).

Instrumental color and discoloration

Each of *L*, a*,* and *b** was significantly affected by a muscle type x time interaction ($p<0.05$) and, all the three parameters were higher in *Longissimus dorsi* through the storage period. LD samples became significantly darker and less redder with a decrease in *L** and *a** values, respectively over the nine day display as seen in figure 4.1. PM samples became progressively less redder, however had increased lightness on day five which then decreased on day nine. Chroma was also significantly affected by a muscle type x time interaction ($p<0.05$) as seen in figure 4.2. The relative metmyoglobin percent was significantly affected by the main effects of muscle type and time ($p<0.05$) as seen in figure 4.3. On days 1 and 5, there was no significant difference between the metmyoglobin percents in both the muscles, however, on day 9, *Psoas major* samples had a significantly higher metmyoglobin percent ($p<0.05$) and were thus more discolored.

Total iron, variation in heme and nonheme iron

The total iron of Striploin (*Longissimus dorsi* -LD) was found to be 91 ± 7.55 ppm and that of tenderloin (*Psoas major* –PM) was found to be 115.67 ± 10.84 ppm, the difference was statistically significant ($p<0.05$).

The heme iron (Table 4.2) was directly affected by the main effects of days and muscle type ($p<0.05$). The heme iron concentration significantly decreased with time ($p<0.05$) in both the muscles. An interaction between days and muscle type had a significant effect ($p<0.05$) on the nonheme iron concentration. The nonheme content of both muscles at day 9 was significantly lower on day 9 as compared to day 1 ($p<0.05$). PM, the nonheme iron increased on day 5, as compared to day 1 and then decreased from day 5 to day 9. The relative percentage of heme iron

(Table 4.3) in both the muscles was highest on day 1, least on day 5 and intermediate on day 9. Conversely, the relative percent of nonheme iron was least on day 1, highest on day 5 and intermediate on day 9.

Lipid oxidation

Lipid oxidation expressed in terms of milligram malondialdehyde (MDA) per kilogram meat (Figure 4.4) was significantly affected by the muscle and time main effects ($p<0.05$). On days 5 and 9, Psoas major had significantly higher lipid oxidation compared to Longissimus dorsi. The MDA content of both the muscles significantly increased with time ($p<0.05$).

Discussions

In the proximate composition the protein content differences can be explained by the histochemical properties of the two muscles. LD is considered to be a white fiber with equal to or more than 40% of the muscle fibres being of the alpha-white type whereas PM is considered a red fiber with equal to or more 40% of the muscle fibers being of the beta-red type (Hunt & Hedrick, 1977). These classifications are based on the staining properties of fibers based on their ATP-ase or NADH tetrazolium reductase activities. Muscles with white fibers have a higher content of connective tissue and lesser intramuscular fat than those with more red fibers which explains the higher protein content in LD (Kirchofer, Calkins, & Gwartney, 2002). However, in the present work, the difference in fat content of the two muscles was not significantly different and may have been due to background factors such as animal maturity, sex, animal nutrition, etc. which were not considered while purchasing the samples.

The the beta-red type fibers of red muscles have higher content of mitochondria and myoglobin and both are reserves of iron as compared to white muscles which have less myoglobin and more glycogen reserves (Kirchofer et al., 2002). This also explains why samples of PM had consistently higher concentrations of both heme and nonheme iron (Table 4.2). Since LD has higher glycogen reserves, there is greater glycolytic potential which involves breakdown of glycogen to glucose and further breakdown of glucose to lactic acid. This partly explains the significantly lower pH of LD samples versus that of PM samples on day 5 (Figure 4.5). Glycolysis is a self-limiting process since the lactic acid generated reduces the pH of meat, which in turn causes protein denaturation and reduces the glycolytic enzyme activity (Aberle, 2001). The faster drop in pH of LD samples may have retarded glycolysis beyond day 5 whereas a slower drop in pH in PM samples from day 1 to 5 may have enabled the glycolytic process to continue, causing a significant drop in pH from day 5 to 9. This would explain PM having lower pH on day 9 as compared to LD.

Considering the objective color characteristics, the lightness of LD samples consistently reduced with time, however, L^* value of PM increased on day 5 and then decreased on day 9. The a^* values were significantly higher for LD samples on days 1 and 5. On day 9, however, the redness of both the muscle types had greatly decreased from before and not significantly different from each other. The correlation obtained between pH and redness was positive and quite high (0.92) for LD but low for PM (0.3) as seen in tables 4.4 and 4.5. The chroma values which are indicative of color saturation (figure 4.2) for LD were consistently higher than those for PM except on day 9. The color results from this study support the view that LD is more color stable than PM which is documented before (Joseph, Suman, Rentfrow, Li, & Beach, 2012; McKenna et al., 2005; Seyfert et al., 2006).

Owing to the greater beta-red type fiber composition of PM which is more abundant in myoglobin as well as mitochondria, the tissue in general has higher iron content than LD as mentioned before. The total iron analysis performed in this study confirms this observation. Myoglobin is a reserve of organic heme iron and the heme iron values were consistently significantly higher in PM than in LD ($p<0.05$) at all days in this study. For inorganic or nonheme iron, a muscle x day interaction effect was found to be significant ($p<0.05$). In LD samples, the nonheme iron was similar on days 1 and 5 and reduced on day 9. However, in PM samples, the inorganic iron concentration significantly increased on day 5 and then reduced on day 9.

In both the muscle types, the concentration of measurable heme iron reduced with time. With a drop in pH over time, the meat exudes fluids due to reduced water holding capacity (Aberle, 2001). Myoglobin is a water soluble globular protein and is the major fraction of water soluble heme iron. Hence, a likely explanation for loss of heme iron is myoglobin loss via drip. The loss of heme iron during refrigerated storage has been documented before (Purchas et al., 2003). However, the heme iron lost as a percent of the initial heme iron in this study was much higher than that reported in literature with LD having lost almost 40% of initial heme iron on day 9 and PM having lost almost 50%. In an aerobic environment with a decreasing pH, hydrogen peroxide is inherently generated within the tissue which can cause oxidative cleavage of porphyrin and release iron from heme (Osawa & Korzekwa, 1991). Another more likely reason for decreased heme iron concentration is its precipitation which reduces its extraction and further analysis (Purchas et al., 2003). The correlation coefficient between meat pH and heme iron concentration was moderate (0.63) for LD and high for PM (0.92). With a drop in pH, as beef meat approaches its isoelectric point of near 5.2, more proteins are precipitated which may limit

the extraction of heme from myoglobin during analysis (Purchas et al., 2003). This would partly explain why such high heme iron losses were reported in samples on day 5 and day 9.

The correlation between redness value (*a**) and heme iron concentration was moderate with the correlation coefficients being 0.72 and 0.44 for LD and PM respectively. It has previously been shown that myoglobin content largely correlated to *a** in pork meat (Lindahl, Lundström, & Tornberg, 2001). In this study, heme iron concentration and consequently the myoglobin content accounted for 48% and 19% of the variation in *a** in LD and PM respectively, as obtained by squaring the correlation coefficients.

As seen in table 4.2, PM had significantly higher concentration of nonheme iron on days 1 and 5. In both LD and PM, the nonheme iron concentration significantly increased on day 5. The main reservoir of nonheme iron in raw beef is ferritin which has been shown to release nonheme iron during refrigerated storage. The iron release is due to the denaturation of ferritin mediated by drop in pH as well as inherent reducing agents in the meat (Decker & Welch, 1990), both of which facilitate the release of iron bound within the ferritin core (Mazur, Baez, & Shorr, 1955). Iron released from oxidative cleavage of porphyrin also leads to increase in the inorganic iron pool as mentioned before (Osawa & Korzekwa, 1991).

In the present study, the increase in nonheme iron in PM on day 5 over day 1 was significant though not too high. Previous reports have shown nonheme iron to increase by 1.4 folds in mackerel and almost 3 folds in turkey over a period of 7 days under refrigerated storage (Decker & Hultin, 1990; Kanner, Hazan, & Doll, 1988). The liberated nonheme iron is usually present chelated to low molecular weight peptides or nucleotides and is in the water soluble form (Halliwell & Gutteridge, 1986). Inorganic iron can precipitate with time. The correlation

coefficient for pH and nonheme iron was moderate (0.46) for LD and high for PM (0.92) as seen in tables 4.4 and 4.5. Precipitation causing inadequate extraction is the most likely explanation for decrease in measurable nonheme iron content in both the muscles from day 5 to day 9 (Purchas et al., 2003).

The correlation between non-heme iron and redness was poor (-0.02) for PM and weakly positive for LD (0.31). Both heme and nonheme iron have a pro-oxidant effect in raw beef (Rhee & Ziprin, 1987). The correlation between heme iron and TBARS was found to be strong and negative in LD (-0.61) and PM (-0.82) and correlation between nonheme iron and TBARS was found to be moderate and negative, (-0.46) in LD and (-0.41) in PM in the present work. The negative correlation is likely due to the loss of measurable heme and nonheme iron with time due to precipitation. Strong and negative correlation (LD -0.61, PM -0.82) was observed between heme iron and products of lipid oxidation and moderate negative correlation (LD -0.46, PM -0.41) between nonheme iron and products of lipid oxidation.

Considering the discoloration behavior, heme iron concentration had a moderate and negative correlation (LD -0.34, PM -0.47) with percent metmyoglobin in both the muscles. Nonheme iron had a positive and moderate correlation with metmyoglobin percent in LD (0.38) whereas it had a positive but weak correlation (0.13) with metmyoglobin percent in PM. The positive nature of the correlation is explained by the pro-oxidant nature of inorganic iron which leads to oxidation of myoglobin directly or indirectly, through products of lipid oxidation (Decker & Welch, 1990). However, the small values of the co-efficient of correlation show that non-heme iron concentration is a poor explanatory variable for discoloration in raw beef and its variation in muscles of different color stability.

The results of this study suggest that variation in concentrations of heme and nonheme iron only partly explains the redness and discoloration in two muscles of varying color stability. While developing and distributing raw meat products, other factors along with iron form concentrations need to be holistically considered to predict color changes – such as type and age of muscle, packaging, effect of nonmeat ingredients, and others.

Conclusion

The overall concentration of iron decreased during refrigerated display over nine days for both the muscles. The heme iron content was found to be significantly affected by muscle type and storage time whereas the nonheme iron content was found to be significantly affected by the interaction of muscle type and storage time. On day 5, the nonheme iron significantly increased in the color unstable *Psoas major* samples and subsequently reduced on day 9. Heme iron concentration was observed to be moderately related to redness and discoloration, but strongly correlated to lipid oxidation in both the tissues. Nonheme iron concentration was observed to be poorly related to redness and discoloration, but moderately related to lipid oxidation in both the muscles. The results of this study indicate that concentrations of different iron forms can explain only a part of the variation in the discoloration and lipid oxidation in different muscles. Thus, factors other than the concentrations of different iron forms – both intrinsic and extrinsic, need to be holistically considered while developing and marketing products from muscles of varying color stability.

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Table 4.1 Proximate Analysis of steaks from Strip loin (*M. Longissimus dorsi)* and Tenderloin (*M. Psoas major).* (*n=3*)

Proximate Composition	Muscle Type	
	Longissimus dorsi	***Psoas major***
Moisture	66.7 ± 1.45 a	70.07 ± 2.7 a
Crude Protein	22.03 ± 0.27 a	19.83 ± 0.87 b
Crude Fat	8.76 ±1.37 a	10.86 ± 3.58 a
Ash	1.03 ± 0.05 a	1.08 ± 0.09 a

The values are expressed as means ± SE. Means not sharing a common letter are significantly different ($p<0.05$).

Table 4.2 Variation in heme iron and nonheme iron concentrations expressed in parts per million on dry weight basis in *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*)

	Day 1		Day 5		Day 9	
	LD*	PM**	LD	PM	LD	PM
H-Fe[1]	56.51 (2.75) ax	85.76 (1.42) ay	43.38 (5.39) bx	71.17 (2.44) by	36.53 (1.52) cx	45.64 (4.56) cy
NH-Fe[2]	22.14 (1.17) ax	31.24 (0.72) by	23.95 (1.87) ax	36.37 (1.78) ay	18.54 (0.69) bx	18.27 (0.68) cx

1. H-Fe = Heme iron 2. NH-Fe = Nonheme iron

*LD=*Longissimus dorsi* **PM=*Psoas major*

The values reported are means with standard errors in brackets.

Means not sharing a common letter (a-c) within the muscle type are significantly different at $\alpha = 0.05$. Means not sharing a common letter (x,y) within a day are significantly different at $\alpha = 0.05$.

Table 4.3 Variation in the relative percentages of heme iron and nonheme iron in *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days.

	Day 1		Day 5		Day 9	
	LD*	PM**	LD	PM	LD	PM
% H-Fe[1]	71.85	73.30	64.43	66.18	66.34	71.41
% NH-Fe[2]	28.15	26.70	35.57	33.82	33.66	28.59

1. Heme Iron 2. Nonheme iron **Longissimus dorsi* *** Psoas major*

Table 4.4 Correlation matrix for various parameters measured on *Longissimus dorsi* samples over 9 day refrigerated diplay.

	pH	*a**	%MMb	H-Fe*	NH-Fe**	TBARS
pH	-					
*a**	0.92	-				
%Metmyoglobin	-0.36	-0.61	-			
H-Fe*	0.63	0.72	-0.34	-		
NH-Fe**	0.46	0.31	0.38	0.57	-	
TBARS	-0.93	-0.92	0.38	-0.61	-0.46	-

*Heme iron concentration ** Nonheme iron concentration

Table 4.5 Correlation matrix for various parameters measured on *Psoas major* samples over 9 day refrigerated display.

	pH	*a**	%MMb	H-Fe*	NH-Fe**	TBARS
pH	-					
*a**	0.30	-				
%Metmyoglobin	-0.23	-0.91	-			
H-Fe*	0.92	0.44	-0.47	-		
NH-Fe**	0.91	-0.02	0.13	0.73	-	
TBARS	-0.65	-0.51	0.64	-0.82	-0.41	-

*Heme iron concentration ** Nonheme iron concentration

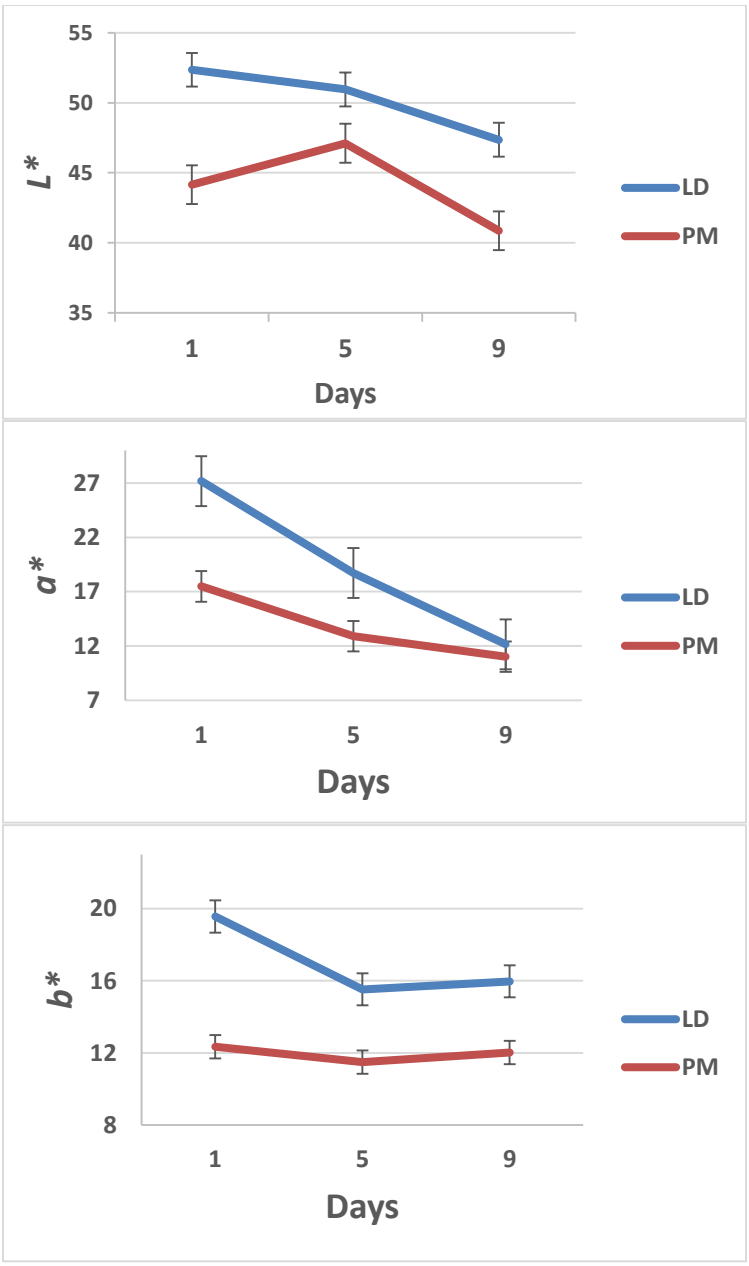

Figure 4.1 Variation in objective color parameters during refrigerated display over 9 days

CIE L, a*,* and *b** for the two muscles *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*)

The data points indicate mean with pooled standard error on mean.

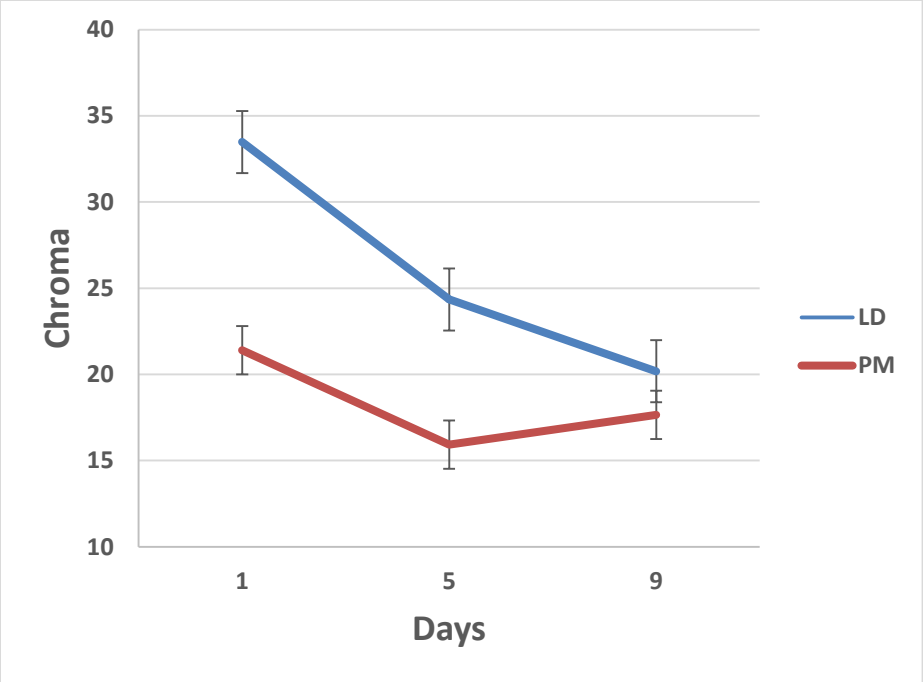

Figure 4.2 Variation in chroma for the two muscles *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*)

The data points indicate mean with pooled standard error on mean.

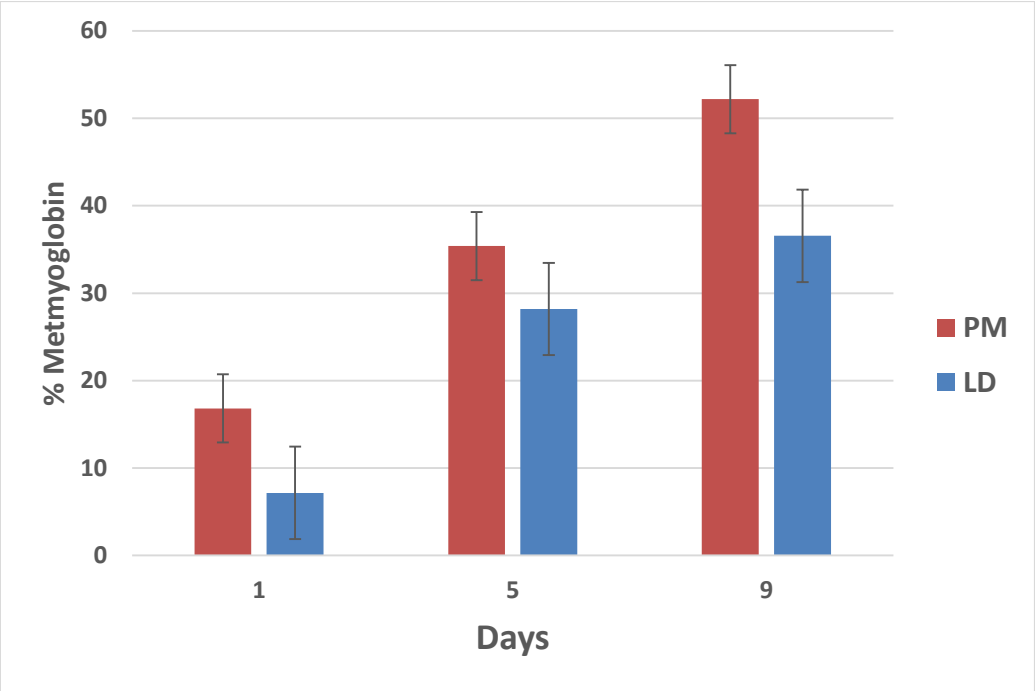

Figure 4.3 Variation in relative metmyoglobin percent in the two muscles *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*)

The data points indicate means with pooled standard error on mean.

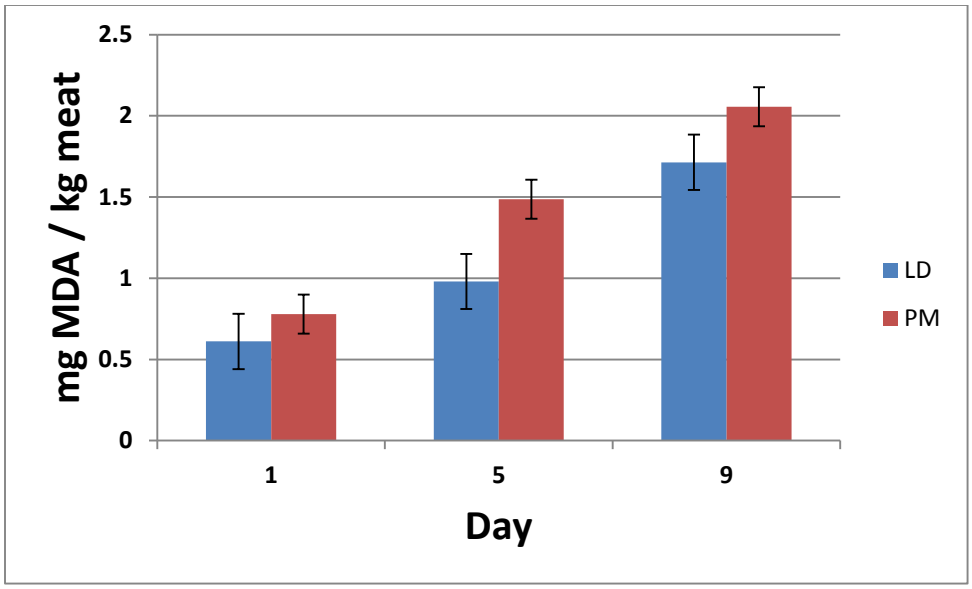

Figure 4.4 Products of lipid oxidation - thiobarbituric acid reactive substances (TBARS) expressed in terms of malondialdehyde (MDA) in the two muscles *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*)

The data points indicate means with pooled standard error on mean.

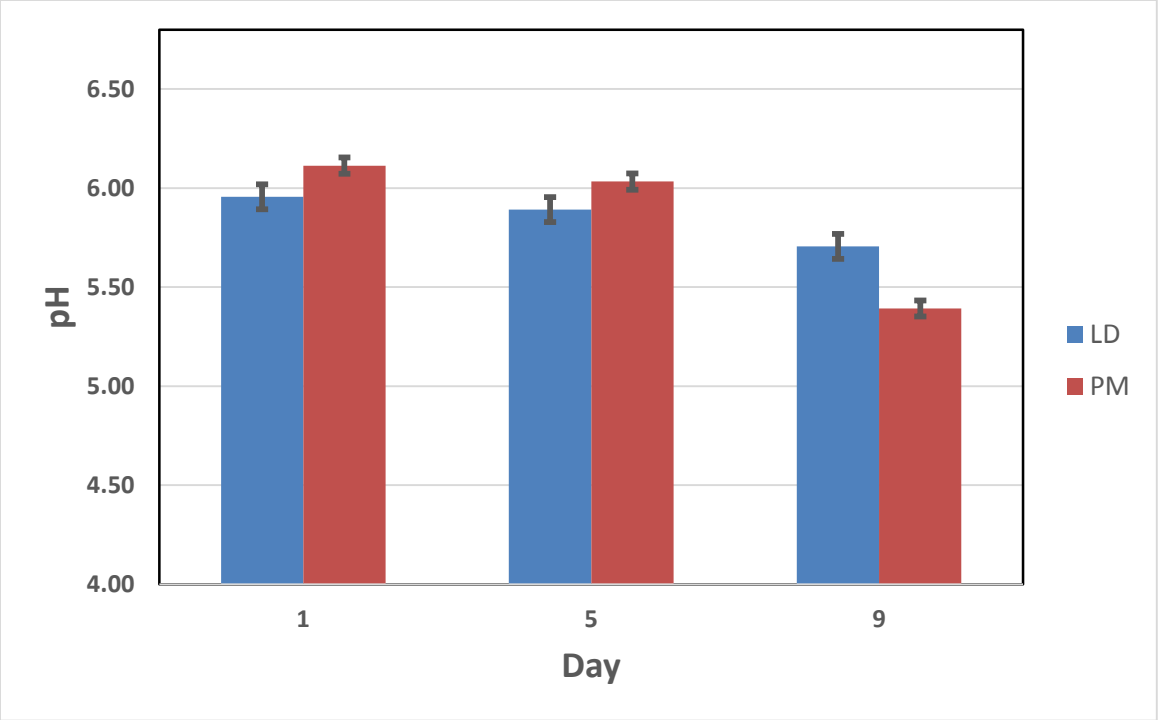

Figure 4.5 Variation in pH of the two muscles *Longissimus dorsi* and *Psoas major* during refrigerated display over 9 days. (*n=3*) The data points indicate means with pooled standard error on mean.

CHAPTER 5

CONCLUSIONS

- Raw beef homogenates treated with either of succinate or lactate and redox reactive iron ions had significantly lower *a** values ($p<0.05$).
- Both ferrous ions and ferric ions showed a significant concentration dependent reduction ($p<0.05$) in *a** values.
- The relative contents of metmyoglobin increased and those of oxymyoglobin and deoxymyoglobin decreased ($p<0.05$) with increasing ferric ion concentration.
- In the presence of redox reactive iron ions, there was a significant compromise in mitochondrial respiratory control rations and thus structure, as compared to control treatments without iron ($p<0.05$).
- Inorganic iron ions are detrimental to the color of raw beef and detrimental to mitochondria which are a medium of color stabilization in fresh beef.
- The overall concentration of iron significantly decreased ($p<0.05$) in both the *Longissimus dorsi* and *Psoas major* muscles on day nine of refrigerated display as compared to day one.
- The variation in concentration of heme iron was specific to muscle and in both the muscles, it significantly reduced over nine days of display.
- The nonheme iron concentration in the two muscles was significantly affected by a muscle x time interaction effect and was lower on day nine as compared to day one.

- Heme iron correlated moderately with redness and discoloration and correlated strongly with lipid oxidation in both the muscle types.
- Nonheme iron concentration correlated poorly with redness and discoloration and moderately with lipid oxidation in both the muscle types.
- Variation in the concentration of different iron forms in raw beef only partly explains the variation in color and associated quality parameters. Thus other intrinsic and extrinsic factors in raw beef need to be considered together along with iron form concentrations while developing and marketing products from muscles of varying color stability.