MYOGLOBIN REDOX FORM STABILIZATION: ROLE OF METABOLIC INTERMEDIATES AND NIR DETECTION

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the
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DOCTOR OF PHILOSOPHY

Food Science

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Abstract

Several Experiments were conducted to evaluate factors affecting myoglobin redox forms stability and detection of myoglobin redox forms using near infrared (NIR) spectroscopy. In experiment 1, we investigated the relationship between metmyoglobin (MMb) reduction and oxidation of malate to α-ketoglutarate with regeneration of reduced nicotinamide adenine dinucleotide (NADH) via malate dehydrogenase (MDH). Our specific objectives for this experiment were: (1) to examine the interaction of malate and MDH to reduce MMb *in vitro*, (2) to determine the influence of pH, temperature, NAD^+, and malate concentration on MDH enzyme activity and MMb reduction, and (3) to determine the effects of malate on NADH generation, metmyoglobin reducing activity, and color stability using beef muscles (*Longissimus lumborum*, *Psoas major*, and *Semitendinosus*) extracts. We observed that, nonenzymatic reduction of horse MMb *in vitro* in a malate-MDH-NADH system increased with increasing NAD^+ and L-malate concentrations. Our findings further confirmed that reduction of MMb in beef extract was NAD^+ and malate concentration dependent ($p < 0.05$). A model system was described for studying mechanisms of enzymatic reduction of metmyoglobin reduction as a means to improve meat color and the results support the hypothesis that malate can replenish NADH via MDH activity, ultimately resulting in stabilizing myoglobin redox chemistry.

In experiment 2, we assessed the ability of mitochondrial and cytoplasmic malate dehydrogenase present in postrigor bovine skeletal muscle to utilize malate as fuel for NADH regeneration and MMb reduction via the malate-NAD-MMb system. Furthermore, addition of lactate to beef mitochondrial and cytoplasmic isolates was evaluated to determine if

interactions between malate and lactate increased MMb reduction. Addition of malate to isolated beef mitochondrial and cytoplasmic isolates at pH 7.2 increased ($p < 0.05$) MMb reduction. MMb reduction resulting from the addition of malate and lactate was equal or greater than MMb reduction resulting from malate alone. The findings from this study provided evidence that mitochondria and cytoplasmic proteins isolated from beef skeletal muscles of different metabolic origin differ substantially in their enzymatic composition. Malate-MDH assisted-MMb reduction using Mitochondrial and cytoplasmic isolates from the three beef skeletal muscles exhibited substantial differences in enzymatic compositions and their ability to reduce MMb *in vitro*. Differences were also observed in the enzymatic characteristics of MDH assisted-MMb among the three beef muscles.

In experiment 3, we investigated the effects of three glycolytic and tricarboxylic acid cycle metabolites on myoglobin redox forms and their influence on meat color stability. Eighteen combinations of malate (M), lactate (L), and pyruvate (P) were added to beef *Longissimus lumborum*, *Psoas major*, and *Semitendinosus* muscle homogenates to study their effect on metmyoglobin formation during incubation at 25 °C. Changes in surface color at 0, 2, 4, 8, and 12 hrs were evaluated using reflecto-spectrophotometry [both $L^*a^*b^*$ and wavelengths specific for MMb]. Results from this study suggests that at 2% concentrations level of the individual metabolites (M, L, or P), the most effective metabolite at retarding MMb formation was L > M > P in the ST, and M > L > P in the PM and LL muscles. MMB was reduced most effectively with combination of metabolites where M+L > M+P > L+P. Enhancement of meat with these metabolites can effectively extend color life of post-rigor meat apparently by providing more reducing conditions for myoglobin, thus increasing myoglobin redox form stability.

Experiment 4 was conducted to determine how near-infrared (NIR) tissue oximeter measurements of post-rigor beef skeletal muscle relate with the more established methods of quantifying myoglobin redox states. Surface color differences were created by packaging

steaks in vacuum (VAC), 80% O_2 and 20% CO_2 modified atmosphere packaging (HiOx MAP), polyvinyl chloride film overwrap (PVC), and HiOx MAP converted to PVC (HiOx-PVC) after 2 days. Changes in surface color and sub-surface pigments during display (0, 2, 4, 10, and 15 days at 2 °C) were characterized by using a reflectance-spectrophotometer and a near-infrared tissue oximeter, respectively. Fiber orientation, storage, and packaging affected ($p < 0.05$) color, total pigment, deoxymyoglobin, and oxymyoglobin content. Tissue oximetry measurements appear to have potential for real-time monitoring of myoglobin redox forms and oxygen status of packaged meat, but fiber orientation needs to be controlled.

In experiment 5, we investigated the response of frequency-domain multidistance (FDMD) NIR tissue oximetry for detecting absolute amounts of myoglobin (Mb) redox forms and their relationship to meat color stability. Four packaging formats were used to create different blends of Mb redox forms and meat colors during display. Changes in surface color and sub-surface pigment forms during simulated display (0, 2, 4, and 10 d at 2 °C) were evaluated using surface reflecto-spectrophotometry (both $L^*a^*b^*$ and specific wavelengths) and FDMD NIR tissue oximetry. Data for both methods of direct measurement of oxymyoglobin and deoxymyoglobin were strongly related and accounted for 86 to 94% of the display variation in meat color. Indirect estimates of metmyoglobin ranged from $r^2 = 59$ to 85%. It appears that NIR tissue oximetry has potential as a noninvasive, rapid method for the assessment of meat color traits and may help improve our understanding of meat color chemistry in post-rigor skeletal muscle.

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Major Professor
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Dedication

Dedicated to MOM and DAD.

Chapter 1

Literature Review

Color is one of meat's most important sensory attributes because once it is deemed unacceptable by consumers, all other attributes loses their importance. Hedrick *et al.*, (1994) and Kropf (1980) suggested that color is the single greatest factor determining retail meat purchase, since consumers use color as an indicator of freshness. However, maintenance of a bright, attractive color becomes challenging during product fabrication, distribution, storage, and display of retail products because that color is relatively short-lived due to inevitable surface discoloration. This chapter therefore will focus on meat color, myoglobin and its chemistry, color stability, metmyoglobin reducing systems, non-meat ingredients, malate dehydrogenase, and varied color-enhancing ingredients.

1.1 Color of Fresh Meat

Fresh meat color is predominantly determined by the concentration and redox status of myoglobin (Mb), typically, distributed uniformly within muscles of vertebrates and some invertebrates; however, variations in Mb content of different types of muscles (and even in some areas within a muscle) may arise from species, sex, breed, and age (Lawrie, 1998).

For example, beef and lamb have a higher concentration of Mb than pork, and steers often have more Mb than heifers. Moreover, meat from older animals is darker because Mb concentration increases as age increases. For instance, Nishida *et al.*, (1976) reported the Mb content in chickens doubled between 6 and 27 weeks. These authors also reported that both raw and cooked turkey meat had higher a^* values (more redness) as animal age increased. Additionally differences in Mb concentration among muscles have a significant impact on meat color and also color stability. In general, red (oxidative) muscles have more Mb than white (glycolytic) muscles and thus are darker (Seideman, 1984). In fresh meat, the redox states of Mb are continuously inter converted and the color of meat is determined by the most predominant redox form (Mancini & Hunt, 2005).

1.2 Myoglobin

Myoglobin is a water soluble, small globular heme protein with 150 amino acid residues and a molecular weight of approximately 18,000 (18 kDa). A porphyrin ring, protoporphyrin IX, is essentially "buried" in a hydrophobic pocket in the interior of the Mb molecule, and a central iron atom is coordinated into this prosthetic group. The prosthetic group is attached to His93 and the heme iron by a coordinated bond which is attached to four pyrrole groups of the heme ring. Meanwhile, the sixth valence position of the heme iron remains available for reversible bonding with ligands. The crucial components of meat color are the ligand attached to the sixth position and the oxidation state of the iron atom. Specifically, the type of ligand bound to the iron atom determines the color and the reactivity of the Mb under most reaction conditions (Livingston & Brown, 1981). The four most common derivatives of Mb redox reactions are as follows: oxymyoglobin (OMb), deoxymyoglobin (DMb), metmyoglobin (MMb), and carboxymyoglobin (COMb).

1.3 Chemistry of Myoglobin Redox Dynamics

1.3.1 Myoglobin oxygenation

Five ligands alter the Mb electronic structure (Livingston & Brown, 1981). When no ligand is present at the sixth position, a deoxygenated form of Mb exits, which results in the purple-red color of freshly cut or vacuum packaged meat (Cornforth, 1989). Addition of molecular oxygen to Mb's sixth position results in formation of OMb, the desired, bright red colored pigment of "bloomed" fresh meat (Figure 1.1). Blooming is an extremely important reaction for meat; however, the rate of oxygenation is dependent upon the oxygen partial pressure and the temperature (lower facilitates oxygenation) of the meat.

$$Mb^{Fe^{2+}} + O_2 \leftrightarrow OMb^{Fe^{2+}}$$

$$(Purple - red) \rightarrow (Bright - red)$$

1.3.2 Myoglobin oxidation

DMb and OMb undergo a nonenzymatic, spontaneous redox oxidation (Figure 1.2). The heme iron (Fe^{2+}) loses one electron and forms the oxidized (Fe^{3+}) pigment metmyoglobin (MMb). Presence of MMb results in discoloration of meat. Although discoloration is most often associated with the amount of MMb present on the meat surface, the thickness and depth of the sub-surface MMb also plays an important role in product discoloration (Mancini & Hunt, 2005). DMb is more susceptible to oxidation than OMb. The presence of molecular oxygen in the prosthetic heme induces a conformational change that stabilizes the electronic structure of OMb and prevents (or delays) Mb oxidation. Factors that cause deoxygenation of the heme make Mb more susceptible to oxidation including low pH, high temperature, and a very low oxygen partial pressure (Mancini & Hunt, 2005; Renerre, 1990). Faustman

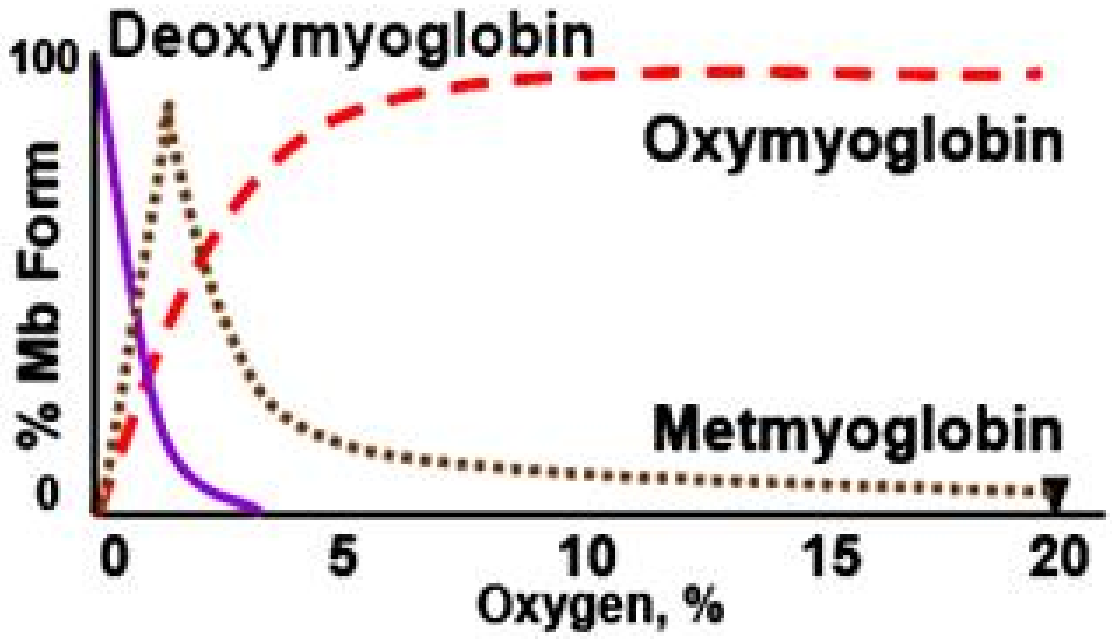

Figure 1.1: *Myoglobin redox forms and relative partial oxygen pressure (Adapted from Kropf, 1980).*

et al., (1999) reported that α- and β-aldehydes, products of lipid oxidation in meat, affect OMb stability by a covalent modification of Mb. Furthermore, Lynch & Faustman (2000) reported that these products will decrease OMb stability and MMb reduction via enzymatic pathways. Additionally, Richards *et al.*, (2002) observed that DMb was a potent catalyst of lipid oxidation in fish muscle and suggested that DMb's high-spin ferrous iron with a less compact structure could contribute to its catalytic behavior in postmortem muscles. Next, Alderton *et al.*, (2003) reported that 4-hydroxy-2-nonenal forms an adduct with the histidine of OMb and induces instability of the Mb redox forms. They concluded that the lipid oxidation and pigment oxidation in meat is likely to be interrelated. Then, Tang *et al.*, (2003) reported that addition of glutathione to bovine skeletal muscles cytosol increased the OMb redox stability. Finally, Mancini *et al.*, (2008) assessed the effects of sodium lactate on equine Mb redox stability *in vitro*. To do so, they incubated OMb at pH 5.6 and pH 7.4

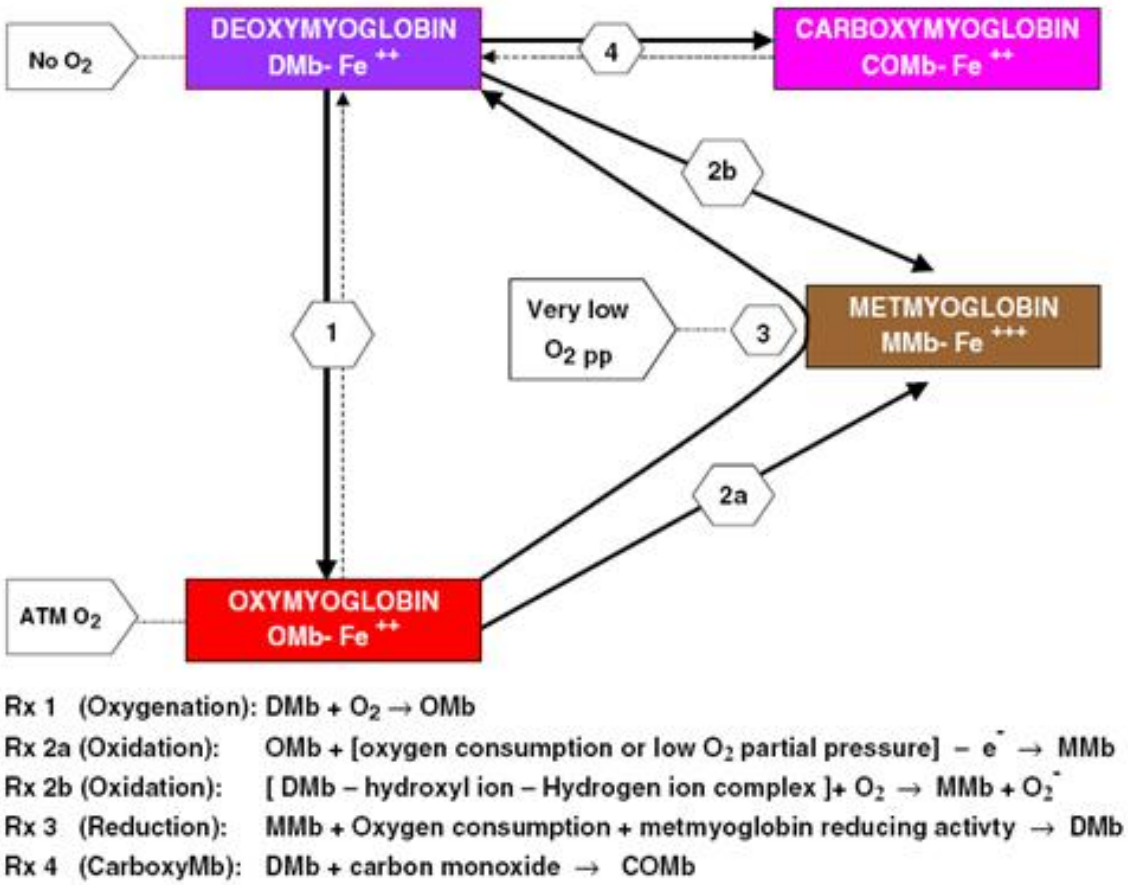

Figure 1.2: *Practical depiction of the visual color and dynamics of myoglobin redox interconversions on the surface of meat. (Adapted from Mancini & Hunt 2005).*

at 4 °C with different concentrations of lactate (0, 5, 10, 100, or 200 mM) and determined myoglobin redox stability using absorbance spectra. These authors reported that lactate (100 and 200 mM) decreased MMb formation at both pH 5.6 and 7.4 and increased OMb redox stability.

1.3.3 Oxidation and Reduction of Myoglobin

During the conversion of muscle to meat and in postmortem muscle, both enzymatic activity and substrate availability are continually being depleted. In fact, postmortem pools of NADH in meat play vital roles in enzymatic or non-enzymatic reduction of MMb. Therefore,

depletion of the postmortem NADH pool significantly affects meat color. Several investigators (Bekhit *et al.*, 2003; Mancini & Hunt, 2005; Kim *et al.*, 2006) have suggested that the discoloration due to surface or sub-surface MMb can be reduced and retarded by regenerating the postmortem NADH pool and that this postmortem pool of NADH will reduce formed MMb via enzymatic or non-enzymatic pathways (Saleh & Watts, 1968; Brown & Snyder, 1969; Madhavi & Carpenter, 1985; Arihara *et al.*, 1996; Mancini & Hunt, 2005). Bekhit *et al.*, (2003) reviewed the dynamics of the enzymes that reduce MMb to DMb. They observed an apparent loss of endogenous reducing capacity in beef patties during storage at 2 °C measured in the presence of 0.1 mL of 1 mM NADH. The capacity, however, was recovered when measured in the presence of 0.1 mL of 2 mM NADH. Therefore, they concluded that the availability of a sufficient amount of NADH is crucial for the full expression of the MMb reductase enzyme.

Mancini & Hunt (2005) described the redox conversion of OMb to DMb as an indirect, two step process (Figure 1.2). The OMb visually appears to first convert to MMb as the muscle consumes oxygen, which creates a low oxygen partial pressure that auto-oxidizes the heme iron. The MMb, then visually appears to convert to DMb depending upon the muscle's reducing capacity and the meat temperature. However, reaction 2a of Figure 1.2 is not thermodynamically feasible. Thus, the OMb likely converts first to DMb, which then very rapidly oxidizes to MMb (the pigment visually seen). The meat then remains brown until the oxygen partial pressure is sufficiently reduced and the ferric iron is reduced to form DMb. This latter reaction is absolutely critical for maintaining color stability.

1.4 Fresh Meat Color Stability

In living animals, skeletal muscles are categorized based on their specialized functional and metabolic activities. As a result, muscles differ in their distribution of muscle fiber types and

metabolic activities. Apart from species, sex, breed, and age, meat color and color stability is dependent on many intrinsic and extrinsic factors, as various researchers have determined. Mancini & Hunt (2005) reviewed several of these factors that are important to color stability. Seyfert *et al.*, (2007) reported that differences in muscle color were linked to muscle's metmyoglobin reducing activity (MRA). McKenna *et al.*, (2005) studied the biochemical properties of 19 beef muscles and then classified the muscles into four groups: high color stability (*M. longissimus lumborum*, *M. longissimus thoracis*, *M. semitendinosus*, *M.tensor faciae latae*), intermediate color stability (*M. semimembranosus*, *M. rectus femoris*, *M. vastus lateralis*, *M. trapezius*, *M. gluteus medius*, *M. latissimus dorsi*), low color stability (*M. triceps brachi*, *M. biceps femoris*, *M. pectoralis profundus*, *M. adductor*) and very low color stability (*M. supraspinatus*, *M. infraspinatus*, *M. psoas major*) muscles. McKenna *et al.*, (2005) also evaluated metmyoglobin reductase activity (MRA) on these muscles and suggested that those with high color stability had highest MRA and those with very low stability muscles had the least MRA. The researchers also suggested that the amount of MMb formed initially on the surface of the muscles was inversely related to color stability and that the initial amount was as good as or a better indicator of color stability than the amount of MMb reduced over time. The following two sections address factors affecting meat color stability, oxygen consumption rate, pH, and packaging.

1.4.1 Factors Affecting Meat Color Stability

Kropf (1980) suggested that meat color stability can be achieved if DMb in meat remain unexposed to light and oxygen. However, Bekhit *et al.*, (2001) evaluated the effects of MRA on ovine *longissimus* muscle color and color stability and indicated that MRA was not the primary determinant of color or color stability in ovine *longissimus* muscle. Moreover, Sammel *et al.*, (2002b) reported that traditional chilling of large, thick beef muscles caused a rapid pH decline while the meat temperature in the deep muscles was "high", causing more

protein denaturation and damage to color stability. More recently, Seyfert *et al.*, (2007) examined three lactic acid salts (calcium lactate, potassium lactate, and sodium lactate) with and without sodium acetate on ground beef color stability and MRA. These authors concluded that use of these salts increased MRA of the ground beef; however, the increase in MRA had no effect on the color stability.

1.4.2 Oxygen Consumption Rate

Oxygen consumption rate (OCR) of postmortem skeletal muscles is a critical factor contributing to meat color stability. The bright-red color of postmortem tissue is determined by the rate of DMb oxygenation and depth of oxygen penetration into the surface of meat. Partial oxygen pressure at the meat surface, rate of oxygen diffusion, oxygen consumption by muscle enzymes, and the product temperature are the primary determinants of oxygen penetration depth (OPD) and saturation of red color (O'Keefe & Hood, 1982). Formation of underlying sub-surface MMb (located between the outer surface OMb and interior DMb) is largely dependent on the oxygen tension above the meat surface (Atkinson & Follet, 1973). The deeper the OMb layer, the longer it takes for the sub-surface MMb to move upward and impact the hue and discolor the meat.

Cheah & Cheah (1971) isolated mitochondria from ox neck muscle at various times from 5 to 144 h after slaughter and reported that at least 96 h of the storage was necessary for significant decrease in OCR. They indicated that although the ADP/O remained constant up to 96 h, significant decline occured in the respiratory control index and state III respiratory rate, respectively. Apparently, a drop in pH from 6.86 at 5 h to 5.7 at 144 h postmortem seems to be the primary factor affecting a reduction in OCR and regardless of storage time, the mitochondria were still capable of oxidative phosphorylation as long as muscle pH did not drop below 5.5. In particular, Bendall & Taylor (1972) determined that mitochondrial respiration was a main factor influencing post-rigor OCR, while Taylor

& MacDougall (1973) reported that OCR was high in the first 2 days after slaughter and that a decline in the OCR occured as postmortem age increased. They suggested that the reduction of OCR postmortem was due to decay of respiration, which was apparently due to depletion of substrate and/or enzyme degradation. Further, DeVore & Solberg (1975) reported a relationship between postmortem muscle OCR and cytochrome *c* reductase activity. Specifically, postmortem muscle OCR seems to be directly related to the respiratory enzyme activity which indicates that a reduction in postmortem muscle OCR could be a result of functional impairment of mitochondrial electron transport chain. Renerre & Labas (1987) reported a similar significant relationship between OCR and cytochrome a. Other researchers have contended that OCR contributes more muscle's color stability than the reducing activity (Atkinson & Follett, 1973; O'Keefe & Hood, 1982; Renerre & Labas, 1987). O'Keefe & Hood (1982) reported that muscles that had a high OCR were also tended to have higher Mb content and were more unstable. They also indicated that beef muscle endogenous enzyme activity appears to be the controlling factor affecting color stability. Sammel *et al.*, (2002a) evaluated the relative role of reducing activity and OCR in beef *M. semimembranosus* muscle. They reported that a positive relationship (r = 0.39 to 0.50) existed between beef muscle's OCR and the reducing activity. Also, McKenna *et al.*, (2005) suggested that OCR and OPD were negatively correlated, indicating that OCR and OPD are not interdependent processes and each can be affected by fluctuations in partial oxygen pressure (O'Keefe & Hood, 1982).

1.4.3 pH

Few factors play a more important role in meat color and color stability than pH. Urbin & Wilson (1958) reported that increasing pH or temperature also increased tissue oxygen uptake. Some other researchers found postmortem muscle storage pH also affected mitochondrial activity (Bendall & Taylor, 1972). Concurrently, Bendall & Taylor (1972) reported

that an with an increase of pH from 5.6 to 7.2 there was an increased muscle oxygen uptake. These researchers also determined that myoglobin tended to be more susceptible to oxidation at lower pH (Gotoh & Shikama, 1974; Ledward, Dickinson, Powell, & Shorthose, 1986). Clearly, as the enzymatic demand for oxygen increases, the harder it is for Mb to maintain color stability.

1.4.4 Packaging

Packaging materials will inherently affect meat color because they provide a barrier to the ready access of oxygen. With the increased preference for case-ready meats, the meat industry continues to find ways to increase color shelf-life of fresh meats without compromising essential sensory attributes and wholesomeness. Packaging options commonly used include a polyvinyl chloride (PVC, oxygen permeable) overwrap, vacuum and no-oxygen modified atmosphere packaging (MAP) with or without CO, and high oxygen (80 %) MAP. In general, high-oxygen modified atmosphere packaging (MAP) has few application technicalities and easily provides a desirable bright, cherry-red color with extended display compared with meat packaged in PVC. Also, vacuum and no oxygen MAP are excellent for shelf life, but the meat is purple, while no-oxygen MAP with CO provides excellent anaerobic shelf life for meat that is bright red.

Behrends *et al.*, (2003) suggested that the increased shelf life of case-ready meats in MAP was due to a differential response of muscles when placed in high oxygen (80%). Therefore, they found that the *M. semitendinosus* was more color stable than the *M. biceps femoris* and *M. semimembranosus*. Specifically, Seyfert *et al.*, (2007) reported that beef muscles packaged in MAP (20% and 80% oxygen) with and without 0.4% CO had no effect on color, reducing activity, and/or OCR. Meanwhile, Grobbel *et al.*, (2008) reported that packaging of beef steaks in vacuum and in ultra-low oxygen MAP with 0.4% CO resulted in better color stability than steaks packaged in high-oxygen MAP. In particular, steaks packaged

in high-oxygen MAP developed an undesirable reddish tan color by day 7 of display and discolored at a faster rate than steaks packaged in ultra-low oxygen and/or in vacuum. Supporting this finding, Sørheim *et al.*, (1999) found that meat packaged in high-oxygen MAP resulted in greater off odor development and faster discoloration of the products than did ultra-low oxygen MAP.

However, Tørngren (2003) reported that beef loin steaks packaged and aged in a vacuum and then displayed in either PVC overwrap or in high-oxygen MAP maintained a desirable red color and had higher a^*-values (intensity of redness). Seyfert *et al.*, (2004a) evaluated color stability of injection-enhanced beef from hot- or cold-boned quadriceps muscles packaged in high-oxygen MAP or ultra-low oxygen MAP and found steaks packaged in high-oxygen MAP were brighter cherry-red and had more color stability, but the ultra-low MAP had excess residual oxygen that resulted in some MMb formation. Mancini *et al.*, (2009) evaluated the color stability of beef *longissimus lumborum* and *psoas major* steak enhanced with lactate in combination with different packaging systems, finding both kinds enhanced with 2.5% lactate and packaged in high-oxygen MAP exhibited improved redness. They also indicated that beef steaks enhanced with 2.5% lactate and packaged in CO did not improve the lactate induced color darkening effects; however, steaks packaged in COMAP had improved color stability compared with those in high-oxygen packaging.

Suman *et al.*, (2009) studied the effects of lactate-enhancement, muscle source, and modified atmosphere packaging (MAP) on the internal cooked color of beef steaks. Beef steaks cooked to an internal temperature of 71 °C and enhanced with 2.5% lactate had darker internal cooked color. Furthermore, the internal cooked color of lactate-enhanced beef steaks from COMAP was more stable than that of steaks packaged in vacuum or high-oxygen MAP. They suggested that a combination of lactate-enhancement and COMAP was more effective in minimizing premature browning of whole-muscle beef steaks.

MacDougall & Taylor (1975) reported that increased levels of oxygen in packages or

a high-oxygen MAP should delay browning of fresh meats better than PVC packaging. Recently, meat packaging in anaerobic MAP (0.4% CO + 20% CO_2 + 79.6% N_2) improved beef color stability with extended retail display life (Luño *et al.*, 1998; Sørheim *et al.*, 1999; Hunt *et al.*, 2004). Jayasingh *et al.*, (2001) evaluated meat products packaged in 5% CO followed by vacuum packaging and reported that meat products remained red in the packaging for at least 5 weeks. Brewer *et al.*, (1994) reported similar findings using 100% CO for about an hour followed by vacuum packing. El-Badawi *et al.*, (1964) evaluated the color stability of fresh meat products using COMAP by exposing packaged beef cuts to 2% CO and 98% air and found that beef color was stabilized for 5 days at 2-3 °C. Beggan *et al.*, (2004) employed a master pack system containing 0.6% residual oxygen and reported that removing all of the oxygen from a low oxygen MAP environment was critical to prevent and delay formation of MMb. They suggested that 0.6% residual oxygen in the master bag was likely responsible for faster surface discoloration. Supporting this claim, Mancini & Hunt (2005) suggested that packages containing 0.15-2.0% oxygen make fresh beef susceptible to browning.

Hunt *et al.*, (2004) evaluated the effectiveness of a COMAP (0.4% CO) master packaging system of PVC overwrapped packages (that were subsequently displayed) based on meat color, shelf-life, and microbial growth of beef steaks (*longissimus*, superficial and deep *semimembranosus*, and *psoas major*) and ground beef. Muscles and ground beef had an acceptable initial cherry red color; however, color stability of *longissimus*, superficial *semimembranosus*, and ground beef exposed to CO increased in comparison to muscles that were not exposed to CO. Additionally, the researchers reported that beef muscles exposed CO did not mask microbial spoilage. Prior to this, Luño *et al.*, (1998) assessed the effectiveness of the MAP packaging system containing 1% CO + 70% or 24% oxygen or only 70% oxygen with no CO on color stability, self-life, and sensory analysis of ground beef and steaks. They reported that beef steaks and ground beef packaged in 24% O_2 and 1% COMAP had reduced

microbial growth and increased color stability for up to 29 days of storage. They found no differences in microbial growth on meat packaged in 70% O_2 or 1% COMAP; however, formation of surface MMB was higher for ground beef and steaks packaged in COMAP than for steaks packaged in no COMAP.

Sørheim *et al.*, (1999) evaluated the color stability, microbiological quality, and development of off-odor from beef steaks, pork chops, and ground beef packaged in high oxygen MAP (70% O_2 + 30%CO_2) and in ultra-low oxygen with COMAP (0.4% CO + 60% CO_2 + 40% N_2) and stored at 4 or 8 °C for up to 21 days. They reported faster development of off-odor in meat packaged in high-oxygen MAP than for that packaged in ultra-low oxygen COMAP and stored at 8 °C compared to storage at 4 °C. Moreover, meat packaged in high-oxygen MAP, though bright cherry-red initially, discolored faster than that packaged in ultra-low oxygen COMAP. Additionally, meats stored at 8 °C had higher microbial growth than meat stored at 4 °C, regardless of the packaging methods used. They suggested that excluding oxygen from COMAP package appears to extend meat color stability.

Martínez *et al.*, (2005) found that fresh pork sausage packaged in 0.3% CO, 30% CO_2, and 69.7% argon had the highest a^*-values (redder) and less lipid oxidation after 20 days of storage than did the pork packaged in modified atmospheres including O_2, N_2, and CO_2. Moreover, Krause *et al.*, (2003) reported greater color stability (higher a^*-values) for injected and non-injected fresh pork chops packaged in MAP containing 0.5% CO than pork chops packaged in PVC overwrap, MAP without CO (20% CO_2/ 80% N_2), and vacuum packaging. However, they also found increased purge in non-injected pork chops packaged in MAP with CO than with other packaging methods. Additionally, Wicklund *et al.*, (2006) reported injection-enhanced pork chops packaged in MAP with CO were redder and had less purge loss than pork chops packaged in high-oxygen MAP with no effect on flavor or consumer acceptability. Wilkinson *et al.*, (2006) compared master-packaged fresh pork in 100% CO_2 or including 0.4% CO and found improved color with the COMAP and no effects

on microbial counts or lipid oxidation.

1.5 Reduction of Metmyoglobin in Meat

Metmyoglobin reduction activity (MRA) in meat has received detailed attention and has been extensively reviewed according to Giddings (1974). Several biochemical factors affect MRA, and it is well-accepted that multiple pathways are involved in postmortem muscle MRA. For example, Ledward (1985) identified reduction of MMb in fresh meats as the primary determinant of meat color stability. Further, Rossi-Fanelli *et al.*, (1957) identified different reducing systems capable of reducing MMb in meats. Hagler *et al.*, (1979) isolated an NADH-dependent specific MMb reducing enzyme and reported that the reducing enzyme requires NADH and ferrocyanide for activity. Later, Güray & Arinç (1990, 1991) purified cytochrome b_5 reductase from sheep lung microsomes, finding that the affinity of MMb reductase for cytochrome b_5 was 6-10 times higher than ferrocyanide. Meanwhile, Arihara *et al.*, (1989a, 1989b) localized and characterized NADH-cytochrome b_5 reductase in the mitochondrial fraction and at a lower level in microsomal fraction. The reductase enzyme had a pH optimum of 6.5 and a molecular weight of 33 kDa.

Shirabe *et al.*, (1992) demonstrated that the enzyme responsible for methemoglobin reduction in erythrocyte was NADH-cytochrome b_5 reductase (NADH-CB5R). Strittmatter & Velick (1956) earlier had demonstrated that NADH-cytochrome b_5 reductase is a flavoprotein tightly bound to endoplasmic reticulum membranes. Takesue & Omura (1970) reported an outer mitochondrial membrane bound NADH-cytochrome b_5 reductase capable of reducing MMb. Enoch *et al.*, (1977) reported that the cytochrome b_5 and NADH-cytochrome b_5 reductase were embedded in the mitochondrial membrane, endoplasmic reticulum, and golgi membranes. Estabrook & Werringloer (1978) suggested that most animal tissue flavoproteins and cytochromes participating in the electron transport chain require oxygen and

NADH. Shirabe (1992) further added that NADH-cytochrome b_5 reductase functions by transferring two electrons from NADH to cytochrome b_5. Reduced cytochrome b_5 then transfers the electrons to MMb. Livingston *et al.*, (1985) presented similar findings and evidence for a mechanism of MMb reduction, stipulating that NADH- cytochrome b_5 reductase first reduces ferricytochrome by transferring electrons from NADH to ferricytochrome and reduced ferrocytochrome then reduce MMb (Fe^{3+}) to Mb (Fe^{2+}) non-enzymatically. Finally, Bailey & Driedzic (1992) provided the evidence for the contention that NADH- cytochrome b_5 reductase was responsible for reducting MMb in vivo.

1.6 Metmyoglobin Reducing Systems in Meat

Bekhit & Faustman (2005) reviewed evidence for possible MMb reduction in fresh meats similar to a mechanism reported in living systems for methemoglobin reduction (Hagler *et al.*, 1979). Giddings (1974) suggested that since MMb does not accumulate in vivo, a reductive system is essential for survival in living conditions. Because presence of MMb on meat surface is very undesirable, its reduction is considered a key factor in meat color life (Mancini & Hunt, 2005). Reduction of MMb in meat depends on several factors including muscle's oxygen-scavenging enzymes' activity, reducing enzyme systems, and availability of cofactors and substrates.

Dean & Ball (1960) made early observations of a reducing system in fresh meat, finding that the loss of the redness of fresh meat was restored when it was vacuum packaged during the initial stages of storage. Cutaia & Ordal (1960) also reported a similar observation of disappearance of formed MMb in ground beef during a two day display under anaerobic conditions. Subsequently, reduction of MMb was reported under anaerobic conditions in minced pork at 37 °C (Walters & Taylor, 1963). To clarify, reduction of MMb in meat occurs through both anaerobic (Stewart *et al.*, 1965a) and aerobic (Ledward, 1985) conditions.

Watts *et al.*, (1966) used sodium nitrite to oxidize meat pigment and suggested that generation of a cytosolic pool of NADH was essential for MMb reduction. Ultimately, different methods have been used (Sammel *et al.*, 2002a) to measure the meat's inherent capability to reduce MMb, including metmyoglobin reducing activity (MRA), aerobic reducing ability (ARA), and total reducing ability (TRA). Giddings (1974) reported that reduction of MMb occurs both enzymatically and non-enzymatically, while Renerre (1990) concluded that the reduction of MMb in meat is primarily enzymatic and indicated that MMb reducing systems play one of most critical roles in maintaining meat color and color stability. Atkinson & Follett (1973) demonstrated that MRA of the postmortem skeletal muscles was not related to the proportions of Mb redox forms present on meat surface. Giddings (1974) further suggested that the aerobic reduction of MMb proceeds via a different mechanism from that of anaerobic reduction because the generation of reducing equivalents would necessarily differ in aerobic and anoxic meat.

Van den Oord (1974) suggested that some types of activator were required for MRA activity in meat. O'Keeffe & Hood (1982) evaluated MRA and ARA in four different beef muscles of varied color stability and concluded that muscles of poor color stability had lower MRA than color stable muscles. Also, they suggested that the accumulation of MMb in meat had no effect on muscle MRA. Ledward (1985) believed that there are other factors regulating the color of fresh meats: a catalytic mechanism that utilizes oxygen present in meat and other was the presence of an enzymatic reducing system being able to convert MMb to DMb. Renerre & Labas (1987) concluded that MRA of meat was one of the most important factors for meat color stability. Their specific analysis indicated that the loss of substrates and cofactors, post-mortem pH decline, and the loss of structural and functional properties of mitochondria were the main limiting factors responsible for loss of MRA in meat during storage.

Echevarne *et al.*, (1990) evaluated MRA using bovine muscle homogenates and methy-

lene as an electron carrier under aerobic and anaerobic conditions. They found that the microsomal and mitochondrial fractions exhibited higher reducing activity than sarcoplasmic fractions. They also reported that the muscles with low color stability exhibited the highest reducing activity and found no differences between aerobic and anaerobic MRA. Therefore, the authors concluded that the color stability of postmortem muscles was not regulated by any MMb reducing system in stored meat. Faustman & Cassens (1990b) presented similar evidence that differences in color stability of the postmortem muscles were not related to aerobic MMb reduction. Finally, Lanari & Cassens (1991) studied the OCR, mitochondrial activity, and MRA of LD (color stable) and GM (color labile) muscles of Holstein and crossbred cattle, and concluded that muscles and breeds with low color stability exhibited the high levels of MRA.

Potassium ferrocyanide as an electron transfer mediator is essential for the reduction of MMb in meat (Hagler *et al.*, 1979; Madhavi & Carpenter, 1993; Mikkelsen *et al.*, 1999; Reddy & Carpenter, 1991). In support of this claim, Reddy & Carpenter (1991) found that Potassium ferrocyanide as an electron transfer agent exhibited 2-fold higher activity with bovine MMb than with equine MMb. They also reported that metmyoglobin reductase enzyme activity was highest at pH 6.4 and 30 °C compared with pH 5.8 or 7 at 4 °C. Livingston *et al.*, (1985) reported that compared to cytochrome b_5, ferrocyanide increased the rate of electron transfer by stoichiometrically forming a complex with the hemeprotein. Madhavi & Carpenter (1993) studied the effects of aging and processing on color, metmyoglobin reductase activity and oxygen consumption of beef muscles. They found that surface MMb accumulation, MRA, and OCR were affected by muscle type, post-mortem aging and fabrication method. They reported that color labile muscle (*psoas major*) appears to have higher MMb accumulation, lower MRA, and greater OCR than color stable muscle (*longissimus dorsi*).

Sammel *et al.*, (2002b) studied beef *semimembranosus* (inner vs. outer) reducing activity

and its relationship with color stability. Their results indicated that MRA of this muscle was sensitive to temperature and pH profile during processing and concluded that reducing activity correlated with visual color acceptability. Sammel *et al.*, (2002a, 2002b) evaluated the conditions damaging to reducing activity of the beef muscle and reported that a rapid chilling of the carcass from hot boning caused less protein denaturation and higher reducing activity. They also found ARA and nitric oxide induced MRA to be highly correlated with visual and instrumental color stability.

1.7 Use of Non-Meat Ingredients in Meat

Meat is a highly perishable food product that becomes unfit for consumption as a result of microbial growth, chemical change, or breakdown by endogenous enzymes. One way of extending the shelf-life of fresh meat products is to add non-meat ingredients, brine, and flavoring compounds during processing; this is called "enhancement". A National Meat Case Study (National Meat Case Study 2004: Product labeling information, branding and packaging trends, 2008)found that 16% of whole-muscle beef cuts in U.S. retail cases were enhanced, generally with injections of liquid compositions to decrease product variability in tenderness, juiciness, and flavor (Lawrence, Dikeman, Hunt, Kastner, & Johnson, 2003a; Lawrence, Dikeman, Hunt, Kastner, 2003b; Vote *et al.*, 2000).

Seyfert *et al.*, (2005) compared enhanced beef round muscles injected with 6% and 10% of a solution containing salt, phosphate, and natural flavoring and reported that muscles enhanced with 10% solution had less oxidation but more non-typical beef flavors than muscles enhanced 6%. Knock *et al.*, (2006) evaluated injection enhancement of beef rib steaks with different combinations of potassium lactate, sodium chloride, sodium tripolyphosphate, and sodium acetate and packaged in high-oxygen MAP. They found that beef steaks injected with potassium lactate with or without the acetate exhibited greater color stability but were

darker than the controls. Beef rib steaks enhanced with lactate or acetate had less surface glossiness (shine) than control steaks. Moreover, the increased salt content of the 10% pump vs. 6% was detrimental to visual color of steaks but did reduce surface shine. Papadopoulos *et al.*, (1991a) evaluated beef top round muscles enhanced with salt, phosphate, and sodium lactate (0 or 3%). They reported that cooked beefy, brothy, and bloody-serumy flavor was associated with muscles that were enhanced with the lactate. They also reported that lactate enhanced steaks had a better overall flavor and texture, but also had increased saltiness and sourness compared to control samples. Additionally, the sodium lactate enhanced steaks had lower aerobic plate counts after 42 and 84 d of storage than the controls. In another study, Papadopoulos *et al.*, (1991b) injected beef round roasts with 0, 1, 2, 3, or 4% sodium lactate and 0.5% salt and evaluated sensory attributes with trained sensory panelists from d 0 to 84 of frozen stored cooked products. They found that as lactate levels increased in enhanced beef muscles, cooked yields increased, but fresh beef flavor and warmed-over flavor scored decreased. Maca *et al.*, (1997) used sodium chloride, phosphate and sodium lactate (3 or 4%) with or without sodium propionate (0.1 or 0.2%) as an enhancement solution for beef top round roasts and stored them from 0 to 84 d. Beefy flavors increased in roasts injected with sodium lactate and propionate compared to non-injected controls. As a result, they also found decreased aerobic plate counts in injected roasts compared with controls. They suggested that lactate concentration should not exceed 3% when combined with 0.1% sodium propionate.

Scramlin *et al.*, (2006) added oregano oil as an antioxidant to enhancement brine of beef steaks stored in high-oxygen environment. This retarded lipid oxidation and increased off flavors, reducing acceptibility overall. Rowe *et al.*, (2009) studied the effects of adding BHA/BHT with salt and phosphate in the enhancement solution and reported that enhancing beef with BHA/BHT increased juiciness and decreased the amount of connective tissue. Lawrence *et al.*, (2004) assessed the effects of injection enhancement of beef *longis-*

simus muscles with phosphate and salt solution containing calcium lactate, beef broth, carrageenan, and/or a rosemary extract. When rosemary was included with calcium lactate in the enhancement solution, display color stability of the beef muscles improved compared with steaks injected with phosphate, salt, and rosemary. The trained sensory panelists found that steaks enhanced with calcium lactate with rosemary were less tender than steaks enhanced with phosphate, salt, and rosemary. They also found that beef steaks enhanced with phosphate, salt, and rosemary were salty and had a metallic off flavor compared with steaks enhanced with calcium lactate and rosemary.

Wicklund *et al.*, (2005) evaluated injection enhancement (phosphate, salt, natural flavoring) of beef strip loins before and after aging (7, 14, 21, or 28 days) in vacuum packaging. Enhanced steaks were saltier and juicier than non-enhanced, but aging time had no effect on salty flavors. They also reported that enhancement prior to aging decreased juiciness as storage time increased compared with steaks enhanced after aging, which had no change in juiciness. Their study also demonstrated that enhanced steaks were tender and reached maximum tenderness by 14 days of aging, whereas non-enhanced steaks reached maximum tenderness after 21 days of aging.

Hoffman (2006) studied the effects of enhancement with salt, sodium and potassium phosphates, and lactate of cow *longissimus* and *semitendinosus* muscles after 7 days of aging (before enhancement) and then after an additional 7 days of aging after enhancement. Enhanced *longissimus* and *semitendinosus* steaks were tender, saltier, juicier, and had less overall beef flavor than non-enhanced steaks.

1.8 Malate Dehydrogenase

Malate dehydrogenase (EC 1.1.1.37) is an enzyme which is widely present in animal, plant, and microbial sources. Recent advances in the meat science research have enhanced the

potential for use of endogenous enzymes and substrates that help stabilize meat color. For example, Kim *et al.*, (2006) established lactate's ability to minimize meat surface discoloration in injection-enhanced beef products, reporting that lactate enhancement in postmortem beef skeletal muscle was related to an increased postmortem pool of NADH via LDH activity. MDH may similarly add to the postmortem muscle NADH pool.

Watts *et al.*, (1966) and Saleh & Watts (1968) suggested a pathway for NADH regeneration whereby electrons are transferred from glycolytic and tricarboxylic acid metabolites to NAD, which was associated with the eventual reduction of MMb in postmortem skeletal muscle. Further, they added that the malate dehydrogenase (MDH) in postmortem muscle can help hydrogen transfer from malate to nicotinamide adenine dinucleotide (NAD), and subsequent NADH production by MDH could couple with MMb reduction in the presence of electron carriers such as enzymes, quinones, or methylene blue. Additionally, Andrews *et al.*, (1952) noted that enzymes involved in glycolysis, the tricarboxylic acid (TCA) cycle, and the electron transport chain (ETC), remain active in postmortem muscle and thus could facilitate the production of reducing equivalents.Malic acid can be derived from food sources as well as synthesized by the body via the TCA cycle. It also helps produce energy under both aerobic and anaerobic conditions via the malate-aspartate redox shuttle (Figure 1.3) in the electron transport chain.

Malate dehydrogenases belong to the NAD-dependent dehydrogenases, which are one of the largest and most widely studied families of nucleotide-binding proteins. Participation of malic acid in the malate-aspartate redox shuttle and the TCA cycle produces NADH, each of which then yields 2.5 ATPs. Malic acid is a metabolite of the TCA cycle which catalyzes the interconversion of malate and oxaloacetate linked to the oxidation/reduction of dinucleotide coenzymes. MDH have been isolated from many diverse sources, including eubacteria, archaea, fungi, plants, and mammals, and from subcellular organelles such as mitochondria, chloroplasts, glyoxysomes, and peroxisomes. Additionally, MDHs are multimeric enzymes

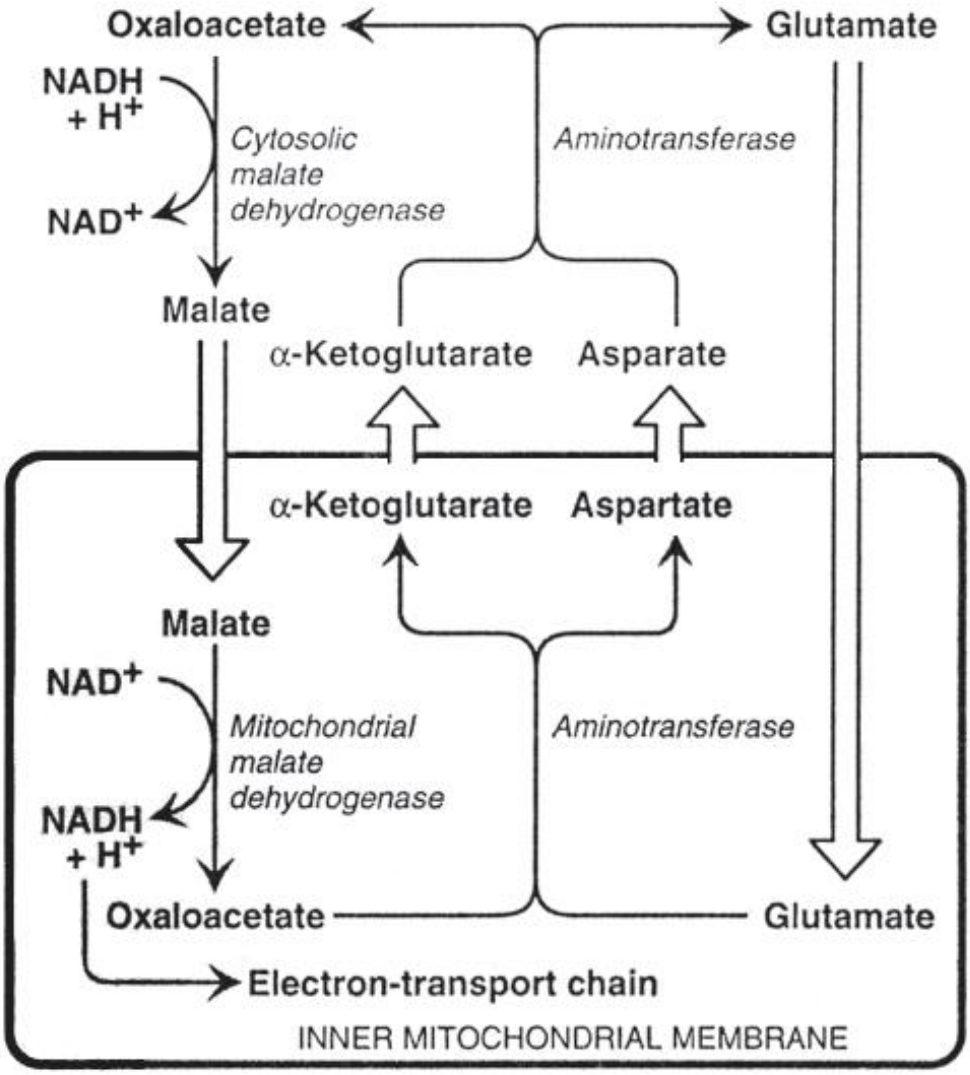

Figure 1.3: *Malate - aspartate redox shuttle (Lippincott's illustrated reviews: Biochemistry 2nd Edition, PC Champe & PA Harvey 1994).*

consisting of identical subunits usually organized as either dimers or tetramers with subunit molecular weights of between 30 and 35 kDa (Banaszak & Bradshaw, 1975; Sundaram *et al.*, 1975). In terms of catalytic mechanism, MDHs have unique properties where studies with mitochondrial MDHs have shown the MDH to be allosterically regulated, for instance. Furthermore, high concentrations of malate stimulate the production of oxaloacetate (Mullinax *et al.*, 1982; Fahien *et al.*, 1988).

Saleh & Watts (1968) tested several glycolytic and TCA cycle intermediates for their ability to increase MMb reduction in ground beef. The addition of intermediates, including glyceraldehyde-3-phosphate, fructose-1, 6-diphosphate, α-glycerophosphate, malate, and glutamate increased MMb reduction (Figure 1.4). Thus, they concluded that the reducing activity of meat can be increased by adding specific substrates.

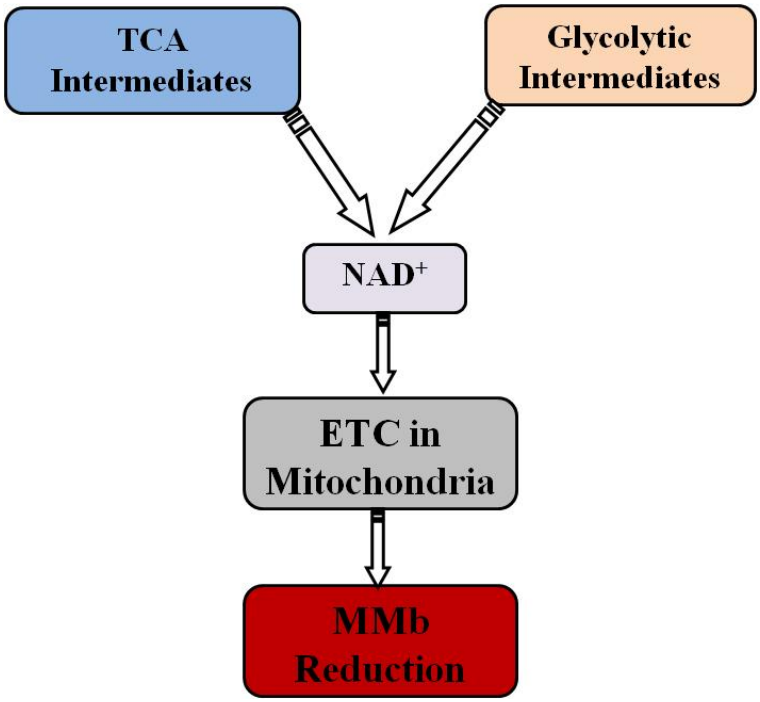

Figure 1.4: *Hypothetical scheme for the role of substrates and intermediates in MMb reduction in meat (Saleh & Watts, 1968).*

1.9 Enhancing Ingredients that Modulate Meat Color Stability

1.9.1 Lactate

Sodium, potassium, and calcium lactate are lactic acid derived salts and listed as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Their usage in

meat products is regulated by the United States Department of Agriculture, Food Safety and Inspection Service (USDA, FSIS). The maximum usage level of sodium/potassium lactate is 4.8% of the solution (USDA, 2004). However, sodium and potassium lactate are allowed at levels up to 4% by weight of total formulation to inhibit the growth of certain pathogens.

Lactates improve color stability, and while some report that color is unaffected, some indicate that meat turns darker (Eckert *et al.*, 1997; Jensen *et al.*, 2003a; Papadopoulos *et al.*, 1991a; Tan & Shelef, 2002). Brewer *et al.*, (1991) reported no differences in L^*, a^*, and b^*-values, but noticed a trend of decrease (indicates color deterioration) in reflectance values (630 nm - 580 nm) over time during 21 days of storage at 1-2% of sodium lactate, but the 630-580 parameter was constant at 3% sodium lactate. Lamkey *et al.*, (1991) found that adding lactate to fresh pork sausage reduced surface discoloration during simulated retail display. In particular, Eckert *et al.*, (1997) noted that ground beef patties enhanced with sodium lactate developed a dark red color that was more stable during retail display. Also, Maca *et al.*, (1999) reported that 4% sodium lactate had a color stabilizing effect on cooked beef top rounds, which turned to a darker red according to trained color panel and Hunter color measurements. Tan & Shelef (2002) used 2% sodium lactate with sodium chloride in refrigerated and frozen fresh ground pork and determined that the lactate enhanced color stability and increased redness (higher a^*-values). Anwar *et al.*, (2004) reported that CIE L^*, a^* and b^* and trained sensory panel values for strip loin steaks enhanced with lactate determined the steaks turned a dark red color. Bradford *et al.*, (1993) reported that 3% potassium lactate increased lean color scores and decreased surface discoloration for low -fat fresh pork sausage. Furthermore, Miller (1998) suggested that sodium lactate stabilizes color and increases the length of time color is maintained during storage.

Jensen *et al.*, (2003) evaluated the sensory and physical characteristics of pork loin chops enhanced with 110% of their original weight with a solution of either (1) potassium lactate and diacetate, phosphate and salt, (2) sodium lactate, phosphate, and salt, (3) potassium

lactate, phosphate, and salt, (4) sodium acetate, phosphate, and salt, or (5) phosphate and salt. Chops enhanced with lactate/diacetate had significantly lower aerobic plate counts than the unpumped control chops after 96 h in display, or those pumped with other solutions. Also lactate/diacetate-enhanced chops maintained higher a^* and b^* -values during display and had less visual discoloration after 96 h display. Chops pumped with lactate, acetate or the lactate/diacetate mixture were more tender and juicy, and had more pork flavor than controls or those pumped with phosphate/salt only. Researchers therefore concluded that an advantage exists in using a lactate-diacetate enhancement over either lactate or acetate alone.

Along these research lines, Mancini *et al.*, (2004) studied the effects of postmortem-injected lactate on meat color stability in beef as a model system. They hypothesized that the endogenous lactate dehydrogenase (LDH) enzyme is involved in the conversion of postmortem-injected lactate to pyruvate, which replenished the reducing equivalent (NADH) pool of postmortem muscle and chemically reduced MMb due to greater metmyoglobin reducing activity (Figure 1.5). Their study concluded that in the model system that used lactate enhanced, LDH was involved in both regeneration of postmortem NADH and MMb reduction.

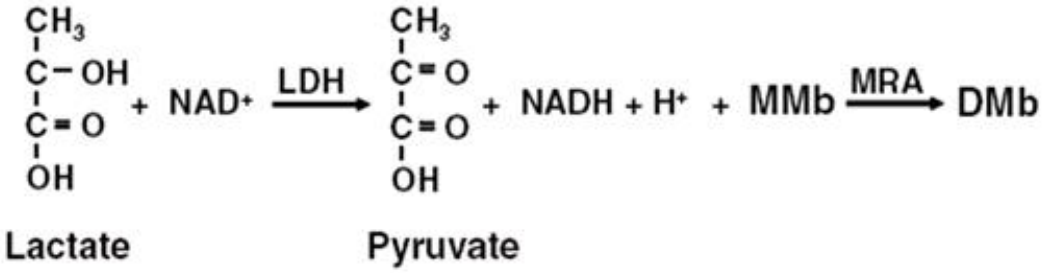

Figure 1.5: *Proposed mechanism for lactate enhanced postmortem regeneration of NADH pool in a beef model system utilizing endogenous LDH enzyme (Mancini et al., 2004).*

Knock *et al.*, (2006) determined the effects of potassium lactate (0% or 1.5%), sodium

chloride (0.3% or 0.6%), STPP (0% or 0.3%), and sodium acetate (0% or 0.1%) on color, color stability, and oxidative properties of injection-enhanced beef rib steaks. Specifically, enhancement solutions were pumped at 8.5% of the green weight and the steaks were packaged in a high-oxygen modified atmosphere. Results from this study revealed that steaks enhanced with potassium lactate or potassium lactate + sodium acetate were darker but more color stable than control steaks. Also, steaks enhanced with acetate and potassium lactate had a less glossy surface. Moreover, both potassium lactate and sodium acetate improved visual appearance of injection-enhanced beef rib steaks, whereas the greater sodium acetate level was detrimental.

In a similar study, Kim *et al.*, (2006) investigated the relationship between MMb reduction and conversion of lactate to pyruvate associated with subsequent regeneration of NADH via LDH. They reported a nonenzymatic reduction of horse MMb using a lactate-LDH-NAD system, and exclusion of any reaction components such as NAD+, L-lactic acid, or LDH resulted in a minimal reduction of MMb. In addition, they injected beef strip loins (longissimus lumborum) with a combination of potassium lactate, STPP, sodium chloride, and/or sodium acetate and stored in high-oxygen MAP for 2-9 days. The results of their study indicated that postmortem enhancement of beef loins with 2.5% potassium lactate increased LDH activity, NADH concentration, MMb-reducing activity, and subsequent color stability during display. These findings clearly support the hypothesis that enhancing beef with lactate replenishes NADH via increased LDH activity, resulting in greater meat color stability.

Mancini & Ramanathan (2008) explored the effects of sodium lactate on equine Mb redox stability *in vitro*. In this experiment OMb at pH 5.6 in 50 mM sodium citrate and pH 7.4 in 50 mM sodium phosphate was incubated at 4 °C with different concentrations of lactate (0, 5, 10, 100, or 200 mM). Lactate at concentrations of 100 and 200 mM decreased formation of MMb and increased OMb redox stability at pH 5.6 and 7.4, respectively. This

study suggested that the lactate induced Mb redox stability could be partially responsible for the improved color stability associated with lactate injection-enhanced beef products.

Mancini *et al.*, (2009) explored the potential for interactions among packaging systems with lactate enhancement of beef *longissimus lumborum* and *psoas major* steak color. The study used 1.25 and 2.5% lactate in the finished product and packaged steaks in either vacuum, high-oxygen MAP, or CO-MAP (0.4% CO/30% CO_2/69.6% N_2) and stored steaks for either 0, 5, or 9 days at 1 °C. Both L^* and a^* -values of the *longissimus* and *psoas* steaks responded similarly to lactate. Specifically, enhancement at 2.5% lactate caused darkening of color packaged in all atmospheres, but it improved the redness of steaks packaged in high-oxygen MAP. Meanwhile, packaging of beef steaks in CO-MAP did not prevent the darkening effects of lactate, but they concluded that CO-MAP was more effective in improving the color stability of beef steaks than high-oxygen packaging.

Kim *et al.*, (2009a) investigated the effects of enhancement solutions containing phosphate and/or calcium lactate on beef strip loins packaged with high-oxygen MAP, with and without irradiation (2.4 kGy), stored for 10 days, and then displayed for 5 days at 1 °C. Beef loins enhanced with calcium lactate and phosphate were color stable, had increased NADH, and were the least discolored. Among irradiated steaks, the calcium lactate with phosphate combination minimized lipid oxidation, increased NADH and had higher a^* -values. The findings suggested that lactate inclusion improves color stability of fresh beef by enhancing the reducing activity and offering superior antioxidant capacity.

In another study (Kim *et al.*, 2009b), beef *M. longissimus lumborum* (LD) and *M. psoas major* (PM) samples were injection-enhanced with solutions containing phosphate and potassium L- or D-lactate and packaged in high-oxygen MAP, stored for 9 days at 2 °C, and displayed for 5 d at 1 °C. Measurements of instrumental color, TRA, LDH activity, and NADH indicated that L-lactate enhancement resulted in less color deterioration, and more redness (higher a^* and chroma values) than non-enhanced control samples of the bovine

muscles. Also, enhancement of beef muscles with L-lactate increased postmortem NADH pool and TRA of LD and PM better than for the non-enhanced control samples. However, inclusion of D-lactate was ineffective in maintaining color. This study presented supportive evidence that the lactate-LDH system remains active for muscles with varying color stability and suggested that it may be a possible mechanism of the lactate-color stabilization effect.

1.9.2 Malate

Sodium malate a natural constituent of many fruits and vegetables, is one of a group of acids known as alpha hydroxyl acids. The molecular structure for sodium malate is depicted below in Figure 1.6.

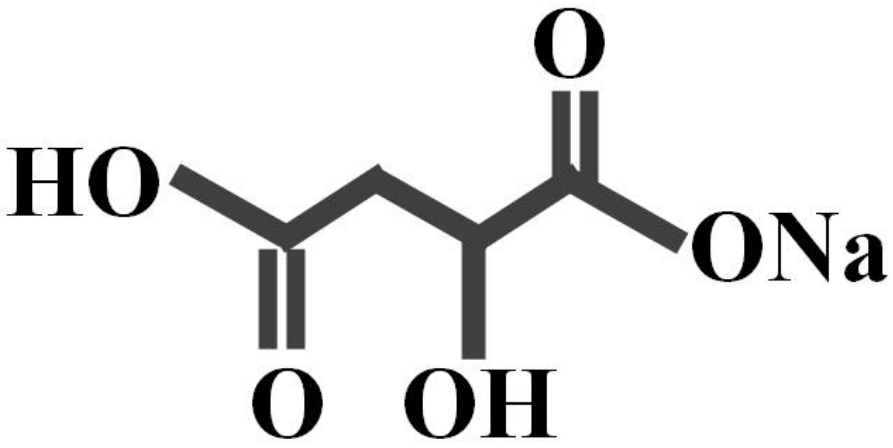

Figure 1.6: *Structure of sodium malate.*

Malic acid is considered to be nontoxic and has GRAS status. Animal studies show it to be free of any reproductive toxicity and to be non-mutagenic across a wide range of genotoxicity tests. Also, it binds and removes aluminum from the brain. Malic acid has a clean, smooth, mellow and persistent tart taste. In supplements and powdered beverages, malic acid enhances fruit flavors by prolonging their release. Taste receptor cells are stimulated by these fruit flavors over a longer period of time, and this prolonged stimulation

is translated by the brain as a stronger fruit flavor. This creates a smoother more natural tasting flavor profile. Additionally, malic acid has a more prolonged sensation of tartness than citric acid and this prolonged tartness, in combination with malic acid's flavor-blending properties, helps it mask the undesirable flavors of some of the nutrients commonly found in nutritional sodium lactate supplements and powdered beverage mixes. Also, it has lower hygroscopicity and forms a more soluble calcium salt. These properties give malate some significant advantages over citrates in a wide range of dietary supplements, as well as other applications in food and beverage products.

Sodium malate plays an important role in generating mitochondrial ATP both under aerobic (Cheeseman & Clark, 1988) and hypoxic (McKenna *et al.*, 1990) conditions. Under aerobic conditions, the oxidation of malate to oxaloacetate provides reducing equivalents to the mitochondria by the malate-aspartate redox shuttle (Cheeseman & Clark, 1988). In the form of malate, reducing equivalents and substrate are transported into the mitochondria where they are utilized for succinate synthesis (Hohl *et al.*, 1987). One mole of ATP is formed for each mole of malate reduced to succinate via fumarate (Hoehl *et al.*, 1987), and 2.5 moles of ATP for, each mole of malate oxidized to oxaloacetate. Bobyleva-Guarriero & Lardy (1986) reported that intraperitoneal injection (7.5 mg/kg body weight) of malate into rats elevated mitochondrial malate followed by increased mitochondrial respiration, increased mitochondrial uptake and utilization of key substrates for ATP formation.

Abraham & Flechas (1992) suggested that chronic malate deficiency could play a role in certain types of hyperlipidemia. They reported that when there was an increased demand for ATP, there was also an increased demand and utilization for malate in humans and animals.

Buchanan *et al.*, (1998) evaluated the effects of pH, malic acid, and sodium malate supplemented in brain heart infusion broth on the inactivation kinetics of Listeria monocytogenes. They reported that inactivation rates were dependent on both the pH and malic

acid concentration and higher pH, appeared to provide some degree of protection with malic acid as compared to control. A concentration-dependent anion effect was observed at lower pHs and higher malic acid concentrations. Thus, they concluded that malic acid's antimicrobial characteristics are similar to those of citric acid, but it is less bactericidal than lactic or acetic acids.

Eswaranandam *et al.*, (2004) studied the effectiveness of partial replacement of glycerol with citric, lactic, malic, and tartaric acids on the antimicrobial activities of nisin incorporated soy protein film against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella gaminara*. Soy protein film containing 2.6% malic acid had the fewest survivors of L. monocytogenes, S. gaminara, and E. coli O157:H7 (5.5, 3.0, and 6.8 log number CFU/mL, respectively) which suggests that malic acid has the potential to inhibit a wide spectrum of microbes in product application.

Raybaudi-Massilia *et al.*, (2009) investigated the bactericidal effects of malic acid against Listeria monocytogenes, Salmonella enteritidis and Escherichia coli O157:H7 inoculated in apple, pear, and melon juices stored at 5, 20 and 35 °C. Bactericidal effects of malic acid were more effective at 35 and 20 °C than at 5 °C in all fruit juices and among bacterial types. However, E. coli O157:H7 appeared to be more resistant to malic acid than S. enteritidis and L. monocytogenes. In addition, transmission electron microscopy showed that malic acid damaged the cell cytoplasm of pathogens without apparently changing the cell membrane.

Malate has potential for providing an alternative to currently used antimicrobial compounds in ruminant feeds according to the study done by Martin & Streeter (1995) who evaluated the effects of malate concentration (0, 4, 8, and 12 mM) on *in vitro* mixed ruminal microorganism fermentation of soluble starch or cracked corn. Incubation of malate with these organisms increased rumen pH, propionate, and total volatile fatty acids, but decreased the acetate:propionate ratio as the concentration of malate increased from 0 to 12 mM. Also adding of sodium lactate with 8 or 12 mM malate in the fermentation of cracked

corn decreased methane production.

Martin (1998) showed that sodium malate was involved in stimulating lactate utilization by *Selenomonas ruminantium*. The study suggested that the ability of *S. ruminantium* to grow on malate in the presence of extracellular hydrogen, may indicate that malate acts as an electron sink for hydrogen in the succinate-propionate pathway used by *S. ruminantium*. In fact, incorporating malate into soluble starch and cracked corn fermentations with mixed ruminal microorganisms changed final pH, CH_4, and volatile fatty acids in a manner analogous to ionophore effects. The study further reported that including malate as a feed additive in the diets of ruminants might serve as a vehicle for providing malate to ruminants.

Callaway & Martin (1996) determined the effects of malate in combination with other organic acids (L-aspartate and fumarate) and monensin on the *in vitro* fermentation of cracked corn by mixed ruminal microorganisms. Specifically, adding malate reduced lactate accumulation. In conclusion, adding organic acids to *in vitro* mixed ruminal microorganisms yielded beneficial fermentation results (decreased acetate: propionate ratio and increased final pH) independently of monensin treatment.

1.10 Other Enhancement Ingredient Effects on Meat Color and Color Stability

1.10.1 Phosphate

Phosphates are used in the meat industry for a variety of reasons: 1) to increase water holding capacity (WHC); 2)to control pH; 3) for chelation of cations (mainly divalent); 4) to increase ionic strength; 5) to bind to meat protein; and 6) to control microbial spoilage. Accordingly, USDA (USDA-FSIS, 1997) allows up to 0.5% of polyphosphates in processed products. Commonly used phosphates in meat and poultry include tetrasodium pyrophos-

phate (TSPP), tetrapotassium pyrophosphate (TKPP), sodium tripolyphosphate (STPP), and hexametaphosphate (SHMP or GLASS; Zheng *et al.*, 2000).

Sodium tripolyphosphate has been used extensively in meat products. Addition of STPP in meat is known to increase the meat pH, which moves the pH further from the isoelectric point, which improves the water binding of meat proteins, which contributes to less scattering of light on meat surface, which makes meat to appear darker in color. Young *et al.*, (1996) reported that STPP treatment in cooked chicken breast resulted in a darker and less red color than controls. Baublits *et al.*, (2005a) suggested that phosphates reduced the desirability of the instrumental color (L^*, a^*, b^*) profile. In a similar study, Baublits *et al.*, (2005b) reported that STPP at 0.4% level was more effective than SHMP or TSPP in maintaining color of beef *biceps femoris* muscle. They also reported that STPP enhanced beef steaks had lower L^*-values but better color stability than the no-phosphate control.

Jun Lee *et al.*, (1998) evaluated the effects of TSPP and STPP in raw and cooked beef. Both phosphates decreased the Hunter L^*, a^*, and b^* values in raw beef but not in cooked beef. Baublits *et al.*, (2006b) evaluated effects of TSPP and NaCl injection in beef triceps brachii steaks and found that the combination produced darker color meat in these steaks. An inverse, linear relationship between $L^*a^*b^*$ values and color vividness with salt concentration (Baublits *et al.*, 2006c).

Pohlman *et al.*, (2002) studied the impact of 10% trisodium phosphate (TSP) or 0.5% cetylpyridinium chloride (CPC) applied to beef trimmings before grinding under vacuum or aerobic conditions on *Salmonella typhimurium*, *Escherichia coli*, coliforms, aerobic plate counts, color and sensory attributes of ground beef through days 0, 1, 2, 3 and 7 of display. The TSP and CPC reduced all bacterial types and improved ground beef redness (a^*), oxymyoglobin stability (630 nm/580 nm), and overall color throughout display.

Finally, Sutton *et al.*, (1997) evaluated the effects of 0% to 2% sodium lactate and/or 0% to 0.4% STPP on the physical properties of whole, boneless pork loins. The color of the

pork loins was not affected by either ingredient, but the lactate enhanced pork flavor, salt intensity, and alkalinity while STPP reduced purge loss.

1.10.2 Salt

For centuries, adding sodium chloride in meat has been associated with its antimicrobial properties. More recently, salt has been shown to improve water holding capacity by lowering the isoelectric pH of meat proteins. In enhanced meats it functions to develop flavor, greater moisture retention, higher ionic strength, and protein binding (Aberle *et al.*, 2001). However, increased concentration of sodium chloride in meat also accelerates lipid oxidation in refrigerated and frozen meats. Furthermore, enhancement of fresh meat with salt decreases color stability (lower a^*-values) during retail display (Robbins *et al.*, 2002; Tan & Shelef, 2002; Trout, 1990). Finally, Boles & Swan (1997) reported that adding salt to meat increased cooking yields, decreased post mortem pH decline, and increased water binding.

Lamkey *et al.*, (1986) demonstrated the effects of salt and phosphate at several levels: 0.0%-0.0% (control); 0.5%-0.0%; 0.0%-0.5%; and 0.2%-0.2%, respectively, on the color and textural properties of restructured beef steaks. Restructured steaks at 0.2% of each ingredient had decreased cooking loss, redness, and oxidation during frozen storage. They concluded that addition of phosphate to restructured beef steaks enhanced with salt had no detrimental effects on color.

Schwartz *et al.*, (1976) investigated the effects of 20 different combinations of salt and STPP on restructured pork. Their study revealed that added salt increased lipid oxidation as measured by 2-Thiobarbituric acid (TBA) values and packaging loss, improved cooked color, aroma, flavor and eating texture, and decreased cooking loss and raw color. STPP also decreased cooking loss and increased TBA values, packaging loss, raw color and juiciness. They reported a synergistic effect of salt and STPP for most traits studied and among the 20 combination levels, 0.75% salt and 0.125% STPP were the most desirable for producing

restructured pork.

Finally, Trout (1989) evaluated the effects of pH (5.5-7.0), sodium chloride concentration (0.0-3.0%), and STPP (0.0 and 0.5%) on the percent Mb denaturation in beef, pork, and turkey muscle when heated to temperatures between 55 and 83 °C. Findings show that salt and STPP increased Mb denaturation at most endpoint temperatures. However, as pH increased Mb denaturation decreased, which indicates that higher pHs increase the temperature for Mb denaturation. The effect of pH on the Mb denaturation was similar for all meat species studied and was sufficient to produce color differences in the cooked muscle.

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Chapter 2

Kinetics of Metmyoglobin Reduction by Malate Dehydrogenase

2.1 Abstract

We investigated the relationship between metmyoglobin (MMb) reduction and oxidation of malate to α-oxaloacetate with regeneration of reduced nicotinamide adenine dinucleotide (NADH) via malate dehydrogenase (MDH). In experiment 1, kinetics of nonenzymatic reduction of horse MMb was observed *in vitro* in a malate-MDH-NADH system and increasing NAD^+ and L-malate concentrations increased MMb reduction. In experiment 2, interaction of malate-MDH-NADH system was investigated in beef muscle extract (ME). The interaction of malate and metmyolgobin reducing activity of beef ME supports the evidence of a possible mechanism of regeneration of postmortem pool of mitochondrial NADH via MDH activity resulting in reduction of MMb. Our kinetic data suggests that reduction of MMb in ME was NAD^+ and malate concentration dependent and served as an excellent model for studying mechanisms of enzymatic reduction of metmyoglobin reduction as a means to improve meat color. These results support the hypothesis that malate replenishes NADH

via MDH activity, ultimately resulting in stabilizing myoglobin redox chemistry.

KEYWORDS: Myoglobin; Malate; MDH; meat color; metmyoglobin-reducing activity; NADH

2.2 Introduction

The color of meat is primarily governed by the concentration and redox states of myoglobin (Mb) present in the meat. Changes in myoglobin redox chemistry and consequent effect on meat color play a vital role in muscle food appearance. Implications of metmyolgobin formation and its inability to bind with oxygen have received numerous attentions in the last decade by meat scientists. Utilization of bio-based ingredients that mediate in postmortem biochemical pathways affecting metmyoglobin reducing activity (MRA) provides new insights into myoglobin chemistry and meat color research. Recently, enzymatic systems that involve regeneration of the postmortem pool of reduced nicotinamide dinucleotide (NADH) have been reported as highly effective pathway for MMb reduction and meat color stability (Mancini & Hunt, 2005; Kim *et al.*, 2006).

In meat, conversion of MMb to deoxymyoglobin (DMb) is mediated in presence of a reductive system or metmyoglobin reducing activity (MRA) that utilizes NADH as the substrate (Kropf, 1980). The interest in using such endogenous enzyme systems that utilize numerous ingredients as metabolite for NADH regeneration has great potential for adding value, product shelf-life extension, and myoglobin redox stability promotion. Andrews *et al.*, (1952) noted that enzymes involved in glycolysis, the tricarboxylic acid cycle (TCA), and the electron transport chain (ETC), remain active in postmortem muscle, and thus could be possible sources of reducing equivalents. Watts *et al.*, (1966) and Saleh & Watts (1968) suggested the possibility of the presence of reductive pathway through which intermediates of glycolytic and tricarboxylic acid (TCA) can be used for the production of reduced NADHs

that is eventually used for MMb reduction. In a similar study, a specific MMb reducing enzyme that required NADH for its activity was purified from bovine heart (Hagler *et al.*, 1979).

Mitochondria maintain metabolic activity in postmortem muscle for a long period of time and may reduce MMb by the enzymes located in mitochondria (Arihara *et al.*, 1995). Several researchers (Renerre & Labas, 1987; Lanari & Cassens, 1991) suggested that meat discoloration was more indicative of muscle mitochondrial enzyme activity and mitochondria content. Muscles with high mitochondrial content were associated with high oxidative metabolism and low color stability. Lanari & Cassens (1991) reported that mitochondrial and sub-mitochondrial particles facilitated the myoglobin redox stability. Giddings (1974) hypothesized that mitochondria may facilitate MMb reduction by supplementing the meat tissue with a postmortem pool of reduced cofactors (NADH) generated by the reversal of electron transport chain.

Mitochondrial enzymes help regenerate NADH, a key component of MRA and meat color stability (Arihara, *et al.*, 1995). Tang *et al.* (2005) suggested that the enzymes responsible for MMb reduction are located within the muscle's mitochondria and that the mitochondria pool of NADH should provide for MRA. However, it is unknown, how such enzymes within muscles mitochondria may relate to MRA.

Additionally, NADH regenerated in the cytoplasm are transferred to the mitochondria through NADH shuttle to be further used in electron transport chain (ETC), which in turn help transport metabolites between mitochondrial and cytosolic compartments. Moreover, numerous cytoplasmic processes will compete and utilize NADH and thus can limit the amount available for MMb reduction. Regeneration of postmortem pool of NADH by feeding the TCA cycle enzyme, specifically malate dehydrogenase (MDH) with depleted substrate such as sodium malate (SM) in meat has not been reported to our knowledge. In this study, the role of MDH, a key member of malate-aspartate shuttle was investigated for the first

time in the replenishment of NADH and concomitant reduction of MMb. Specifically, the objectives of this study is (1) to examine the interaction of malate and MDH to reduce MMb *in vitro*, (2) to determine the influence of pH, temperature, NAD^+ , and malate concentration on MDH enzyme activity and MMb reduction, and (3) to determine the effects of malate on NADH generation, metmyoglobin reducing activity, and color stability using beef muscles extracts.

Additionally, due to the critical role that NADH plays in stabilizing meat color, it is warranted to evaluate the potential roles of mitochondrial enzymes in the regeneration of NADH in postmortem skeletal muscle that can reduce MMb more efficiently.

2.3 Materials and Methods

2.3.1 Experiment 1: Malate - MDH System in Nonenzymatic Reduction of Horse MMb.

Reagents.

Horse heart metmyoglobin, NAD^+, EDTA (disodium), methylene blue, L-malic acid, glutamate-oxaloacetate transaminase (GOT, from bovine heart muscle, 700 units/mg of protein), L-glutamic acid and L-malate dehydrogenase (MDH, from bovine heart muscle, 600 units/mg of protein), were obtained from Sigma (St. Louis, MO). Water was purified through a Millipore-Q-Plus (Millipore, Amsterdam).

Assay Procedures

The roles of malate and MDH in nonenzymatic reduction of horse MMb were assessed by adding reactants of various combinations to 10 mm path length polystyrene cuvettes with 1.8 mL final reaction volume under aerobic conditions at assay temperature of 30 °C. The

standard reaction mixtures at pH 7.5 contained one or more of the following (Table 2.1): 0.1 mL of 100 mM of L-glutamic acid, 0.2 mL of 0.5 mM equine MMb in 40 mM phosphate buffer, 0.1 mL of citrate buffer (50, 80, 100, or 150 mM), 0.2 mL of 25 mM NAD^+, 0.1 mL of 100 mM L-malic acid, 0.1 mL of 0.1 mM methylene blue, 0.1 mL of GOT, and distilled deionized water to make the total reaction volume of the assay 1.0 mL. The reaction was initiated by adding 0.1 mL of MDH to the mixture (1unit will reduce 1.0 μmol/min of malate to oxaloacetate). To measure MMb reduction, absorbance at 580 nm was recorded every 2 s for 5 min in a spectrophotometer (UV-2010; Hitachi Instruments, Inc., San Jose, CA). Nonenzymatic reducing activity was calculated as nanomoles of MMb reduced (equal to nanomoles of OMb formed) per minute during the initial linear phase of the assay, using a difference in molar absorptivity of 12000 $mol^{-1}cm^{-1}$ at 580 nm (the wavelength at which the difference in absorption between MMb and OMb is maximal). Activity is expressed as the mean of triplicate samples. Standard Km analysis of MDH for requirements of cofactor NAD^+ and substrate malate was determined using using different concentrations of NAD^+ and malate. The Michaelis constant (Km) and maximum velocity (Vmax) of the MDH for NAD^+ and malate were determined. To calculate the Km and Vmax, for NAD^+ and malate, the Michaelis-Menten equation was converted into a linear form by taking the reciprocal of both sides of the equation. Initial velocity data were analyzed for Km, and Vmax using Lineweaver-Burk equation.

$$\frac{1}{V_o} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax} \tag{2.1}$$

where, Km : Michaelis constant of uninhibited MDH. It is the substrate concentration at which, the velocity of the reaction is half the maximum velocity (Vmax)

Effects of pH

The effects of assay pH on the rate of nonenzymatic reduction of horse MMb were assessed keeping other reactant's concentration constant. The final assay pH (5.7, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 or 10.0) was varied by altering the concentration of the mono- and disodium phosphate buffer, citrate buffer, and tris/HCl buffer. Nonenzymatic reducing activity at different assay pH was calculated as nanomoles of MMb reduced (equal to nanomoles of OMb formed) per minute during the initial linear phase of the assay. Activity is expressed as the mean of triplicate samples.

Effects of NAD^+ Concentration

The rates of MMb reduction were determined at various concentrations of NAD^+ at assay pH of 5.7. The reduction of MMb was carried out at NAD^+ concentrations of (2, 4, 6, 8, 10, 20, 40, and 80 mM) containing 100 mM L-malic acid in 40 mM phosphate buffer. The absorbance changes at 580 nm were recorded every 2 s for 5 min in a spectrophotometer and nonenzymatic reducing activity was expressed as nanomoles of MMb reduced per minute during the initial linear phase of the assay.

Effects of L-Malic Acid Concentration

L-Malic acid concentration was varied from 1-100 mM (1, 2, 4, 10, 20, 25, 50, and 100 mM) keeping NAD^+ concentration at 25 mM and assay pH at 5.7. The rates of MMb reduction were determined by observing absorbance change at 580 nm recorded every 2 s for 5 min in a spectrophotometer. The nonenzymatic reducing activity was expressed as nanomoles of MMb reduced per minute during the initial linear phase of the assay. Furthermore, 0.1 mL of 200 mM hydroxymalonic acid was added to the mixture to investigate potential inhibiting effects on LDH in the MMb-reducing system. Activity is expressed as the mean of triplicate samples.

Effects of Processing Temperature

The effects of processing temperature (5, 10, 15, 20, 25 and 30 °C) on rates of MMb reduction were determined keeping NAD^+ concentration at 25 mM and L-malic acid at 100 mM concentration at assay pH of 5.7. Tubes containing reaction mixture were incubated at respective temperatures (5, 10, 15, 20, 25 and 30 °C) for 5, 10, 15, 30, and 60 min and placed in a hot water bath at (80 °C) to stop the reaction. After cooling down to room temperature for at least 30 min, samples were assayed at 30 °C to determine MMb reduction by observing absorbance change at 580 nm in a spectrophotometer. The activity is expressed as the mean of triplicate samples.

2.3.2 Experiment 2: Malate - MDH System in Enzymatic Reduction of Horse MMb via MMb Reductase

Metmyoglobin Reductase Extracts from Three Bovine Muscles.

Metmyoglobin reductase extracts were obtained from freeze stored (-80 °C) beef *M. longissimus dorsi*, *M. psoas major*, and *M. semitendinosus* muscles as described by Reddy & Carpenter (1991) with slight modifications. Briefly, 5 g of beef muscle was minced and homogenized in 25 mL phosphate buffer (2.0 mM, pH 7.0) using a homogenizer (Kinematica Polytron benchtop Model PT 3100; Brinkmann, Lucerne, Switzerland) at 13,500 $\times$ g (around 2-3 min). The homogenate was centrifuged at 35,000 $\times$ g for 30 min at 4 °C, and the supernatant was filtered using a 0.45 micron micropore membrane in order to remove the fat layer. Oxyhemoproteins were oxidized with an excess of $K_3Fe(CN)_6$ and the solution was dialyzed (10,000 MW cut-off membrane) at 4 °C with three 1 L per hour changes of phosphate buffer (2 mM, pH 7.0) in order to remove the excess of ferricyanide. The solution was centrifuged at 15,000 $\times$ g for 20 min at 4 °C and collected supernatant volume was adjusted to 25 ml with phosphate buffer (2.0 mM, pH 7.0).

Metmyoglobin Reductase Activity

Metmyoglobin reductase activity (MRA) of the muscle extracts (ME) were measured spectrophotometrically as described by Reddy & Carpenter (1991) by adding reactants in various combinations to a 10 mm path length polystyrene cuvette with 1.10 mL final reaction volume under aerobic conditions. The standard assay mixture contained 0.10 mL of 5 mM EDTA, 0.10 mL of 40 mM phosphate buffer at pH 5.7, 0.10 mL of 3 mM $K_4Fe\ (CN)_6$, 0.20 mL of 0.5 mM MMb in 40 mM phosphate buffer pH 7, 0.30 ml of bovine muscle extract and 0.10 mL of 25 mM NAD^+ , and 0.10 mL of 100 mM malate solution and distilled deionized water to make the total reaction volume of the assay 1.10 mL. The EDTA, $K_4Fe(CN)_6$, NAD^+ , and L-malate solutions were prepared in 40 mM phosphate buffer pH 7.0. The reaction was initiated by addition of NAD^+ and malate. One unit of MRA was defined as the quantity which would reduce one nanomole of metmyoglobin per minute per gram of muscle, during the initial linear phase of the assay, using a difference in molar absorptivity of 12,000 l $mol^{-1}cm^{-1}$ at 580 nm. The assay was conducted at 30 °C. The effects of different amounts of enzyme extract, NAD^+ concentration, malate concentration, and assay pH on the rate of MMb reduction were studied. Each assay was made on the same day in triplicates for each extract and each set of conditions.

2.4 Statistical Analysis

Statistical analyses were performed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). F-Test denominator degrees of freedom were estimated using the Satterthwaite adjustment. Least square means for significant F-Tests were separated using least significant differences.

2.5 Results

2.5.1 Experiment 1: Malate - MDH System in Nonenzymatic Reduction of Horse MMb.

The malate-MDH system could effectively reduce MMb nonenzymatically with NAD^+, however, exclusion of NAD^+, malic acid, or NADH had no effect on MMb reduction (Table 2.1). Replacing malic acid with hydroxymalonic acid, a known MDH inhibitor (Heyde & Ainsworth, 1968; Bernstein & Everse, 1978), to the reaction mixture decreased reducing activity. Replacing L-malic acid with D-malic acid in the assay mixtures decreased MMb reduction due to the selective interaction of MDH with L-malic acid (Heyde & Ainsworth, 1968).

The effects of pH

The nonenzymatic formation of reduced NAD^+ indicated pH dependence (Figure 2.1A). The malate-MDH system produced an increased formation of reduced NADH with increasing assay pH from 5.5 to pH 10. The interaction of MDH with coenzyme NAD^+ has been reported to form reduced NAD^+ under the conditions of mild alkaline conditions (Bernstein & Everse, 1978; Luo *et al.*, 2006). The NADH formation gradually increased from pH 5.5 to 10 and then decreased sharply from pH 10.5 to 11.

The effects of pH from 5.5 to 11 on nonenzymatic reduction of MMb were examined in phosphate buffer at assay temperature 30 °C. An increase in assay pH resulted in an increased nonenzymatic reduction of MMb (Figure 2.1B). The malate-MDH requirement for the cofactor to reduce MMb nonenzmatically is probably a pH dependent interaction. The nonenzymatic reduction of MMb exhibited an increased reduction of MMb from pH 5.5 to 10 but decreased as the assay pH further increased from pH 10.5 to 11. Concentration of NAD^+ is directly related to meat-color stability because, NAD^+, a source of oxidized

substrate, decreases rapidly in post-mortem muscle (Saleh & Watts, 1968). Mild alkaline conditions appear to be more conducive to reduction of MMb within the malate-MDH system.

The effects of NAD^+ Concentration

Addition of increased amount of NAD^+ enhanced the nonenzymatic reduction on MMb at assay pH 5.7 and at temperature 30 °C (Figure 2.2A). For the nonenzymatic reduction to occur there is a 100% requirement of cofactor in addition to other ingredients in malate-MDH system. The increased MMb reduction with malate-MDH system with an increased concentration of NAD^+ suggests that concentration of NAD^+ is directly related to the amount of MMb reduced via malate-MDH reaction pathway. Saleh & Watts (1968) reported that addition of increased NAD^+ to ground beef samples resulted in an increased MMb reduction. The Km for NAD^+ is 3.9 mM, and Vmax is 14.24 nMoles min^{-1}. The reduction of MMb proceeded linearly until exhaustion of malate. The rate of MMb reduction is apparently proportional to the rate of formation of NADH and NADH concentration in the reaction mixture.

Effects of L-Malic Acid Concentration

Addition of increasing amounts of L-malic acid enhanced rate of nonenzymatic MMb reduction at assay pH 5.7 and at temperature 30 °C (Figure 2.2B). The nonenzymatic reduction of MMb reduced via malate-MDH reaction pathway proceeded linearly with increasing concentration of malic acid. Figure 2.2B shows Lineweaver-Burk plot of MDH activity for MMb reduction using malic acid as a substrate. The Km and Vmax of the MDH activity for MMb reduction was found to be 1.1 mM and 2.54 nMoles min^{-1} respectively.

Effects of Processing Temperature.

The reduction of MMb at various assay temperatures was investigated. Nonenzymatic reduction MMb via malate-MDH reaction pathway was very low (0.23 nMoles min^{-1}) and increasing temperature followed a linear trend up to the highest processing temperature of 30 °C (Figure 2.3). Since meat is generally stored at or below 5 °C temperature, it appears that malate-MDH system could reduce MMb via NADH production.

2.5.2 Experiment 2: Malate - MDH System in Enzymatic Reduction of Horse MMb via MMb Reductase.

In this study, we observed enzymatic reduction of horse MMb using muscle extract from three beef muscles (*Longissimus dors*i, LD; *Psoas major*, PM; and *Semitendinosus*, ST) of known color stability. The role of malate-MDH system interaction with muscle reductase activity was explored to characterize bovine muscle's MMb reductive capacity and their relative color stability.

The regeneration of NADH via malate-MDH system is shown in Figure 2.4A. An increase in peak at 340 nm wavelength was used for monitoring the NADH formation. Addition of NAD^+ and malate to the muscle extracts resulted in the regeneration of NADH at different rates in muscles of different color stability. Among the three muscles, PM exhibited the highest rate of NADH regeneration (10.1 nMoles/min/g) as compared with LD (7.6 nMoles/min/g) and ST (5.8 nMoles/min/g). Exclusion of malate and NAD^+ from the reaction mixture resulted in no NADH production. Additionally, substitution of malate with water in the reaction mixture containing cofactor NAD^+ , produced NADH, but at a very low rate. In the absence of malate, muscle extracts of the three beef muscles followed a similar trend as observed in presence of malate for NADH regeneration.

In a similar experiment, effects of NAD^+ reduction with concomitant formation of NADH

was used to characterize the muscle color stability following MMb reduction. We observed that the malate-MDH system could interact with the skeletal MMb reductase enzyme to reduce horse MMb. Low MRA (LD, 0.9; ST, 0.6; and PM, 0.5 nMoles/min/g) was observed in the absence of malate in the assay mixture (Figure 2.4B). Exclusion of malate in the muscle extract obtained from the most color stable muscle (LD) exhibited highest MRA (0.9 nMoles/min/g) compared with ST (0.6 nMoles/min/g), an intermediate color stable, and PM (0.5 nMoles/min/g), the lowest color stable muscle.

Addition of malate in the reaction mixture containing 0.2 mL of ME from different bovine muscles resulted in an increased rate of MMb reduction in the all the three muscles ($p < 0.05$; LD, 3.2; ST, 2.4; and PM, 1.5 nMoles MMb Reduced/min/g, respectively). The MRA of the three beef muscles exhibited significant differences ($p < 0.05$) on the rates of MMb reduction when malate was added to ME (Figure 2.4B). LD and ST had higher ($p < 0.05$) MRA compared to PM, which had a very low reducing activity. Similar trend was observed in ME from the three bovine muscles when standard NADH was added to the reaction. All muscles showed an increased rate of MMb reduction and compared to LD, ME from ST and PM showed no significant differences in the rates of MMb reduction ($p < 0.05$; ST 2.6; and PM, 2.7 nMoles of MMb Reduced/min/g).

Increasing the amount of ME resulted in an increased rate of reduction in a linear fashion ($p < 0.05$). Figure 2.5 shows the rate dependency of MMb reduction with increasing amount of ME. Increasing volume of ME from the three bovine skeletal muscles (0.02-0.2 mL) exhibited an increased level of activity (nmol MMb/min/g). For a given volume of ME, the rate of MMb reduction was found to be higher for high color stable muscle, LD, as compared with ST, and PM. Addition of increasing amounts of NAD^+ and L-malate to the standard reaction mixture affected the initial rates of MMb reduction (Figure 2.6 and 2.7). For the initial rate of reduction of MMb, the effect of NAD^+ concentration showed a saturation kinetics and rate became independent of NAD^+ concentration above

6 mMolesL^{-1}(Figure 2.6). For standard experimental conditions (0.2 mL of ME from LD, ST, and PM, 0.5 mM MMb, pH 7.5), the Michaelis-Menten constant had a value Km = 3.3 $\times$ 10^{-6} mol L^{-1}. However, the initial velocity of the three muscle were different (LD, 4.8; ST, 2.7; and PM 1.2 $\times 10^{-9}$mol/g/min respectively).

The enzymatic conditions under which MMb is reduced are not only limited by the amount of cofactors, but also depended largely on the amount of substrate required for that enzymatic reaction to proceed. Similar behavior was seen with ME obtained from the three bovine muscles of varied color stability. The enzymatic reduction rates of MMb were found to be L-malate concentration dependent. The L-malate addition in the increasing order showed an increased rate of MMb reduction in the ME from bovine muscles (Figure 2.7). Among the three muscles, LD demonstrated the highest rate of MMb reduction. PM exhibited the lowest rate of reduction of MMb under all experimental conditions. For the initial rate of reduction of MMb, the effect of L-malate concentration showed a saturation kinetics and MMb reduction rate seemed to be L-malate concentration independent above 100 mmolL^{-1}. For standard experimental conditions (0.2 mL of ME from LD, ST, and PM, 0.5 mM MMb, pH 7.5), the Michaelis-Menten constant had a value Km = 42.2 $\times$ 10^{-6} mol L^{-1}.

2.6 Discussions

2.6.1 Reduction of Metmyoglobin *in vitro* by Malate-MDH Activity

We report here a new method of nonenzymatic and enzymatic reduction of MMb via malate-MDH system. A linear increase in MMb with increasing concentrations of NAD and malate suggests that MDH requires cofactor and specificity towards the substrate malate to carry

the enzymatic functions and a concomitant reduction of MMb in the malate-MDH system. Kim *et al.*, (2006) demonstrated that non-enzymatic reduction of MMb in the lactate-LDH system was NAD^+ concentration dependent.

There are many examples of commercial applications of natural enzymes in the meat industry where the properties of natural enzymes catalysis have been exploited to improve the quality characteristics of muscle foods. We investigated the potential role of an oxido-reductase enzyme malate dehydrogenase to perform a particular biological role, specifically reduction of NAD^+ to regenerate a postmortem pool of NADH as an application to improve meat color and color stability. Mitochondrial malate dehydrogenase is involved in catalyzing the interconversion of malate and oxaloacetate linked to the oxidation/reduction of dinucleotide coenzymes while cytoplasmic MDH is responsible for shuttling of NADHs across the mitochondrial membrane via malate-aspartate shuttle (Luo *et al.*, 2006). In this study, the role of malate-NAD-MDH system was investigated for the first time for MMb reduction *in vitro* and its ability to enhance MRA.

As shown in Table 2.1, the nonenzymatic reduction of MMb via malate-MDH acts an efficient source of regenerating reducing equivalents. In addition to its capability of MMb reduction, the NADH produced in the reaction is not utilized by the reversible product of the enzymatic reaction because GOT in the presence of glutamate utilizes oxaloacetate and converts it to α - ketoglutarate. Moreover, the NADHs produced via malate-MDH reaction pathway were active and stable at lower pHs (5.5-5.8), pH range at which meat enzymes generally operate postmortem.

Our data suggests a direct relationship between change in pH, NADH regeneration, and MMb reduction via malate-MDH pathway (Figure 2.1B). In the present study, when hydroxymalonic acid was used as substrate, a known inhibitor of MDH, the rate of MMb reduction slowed down primarily due to specificity of MDH for its substrate malate.

Many glycolytic as well as mitochondrial enzymes, remain active in postmortem meat

(Andrews *et al.*, 1958) and may have a crucial role in enzymatic reduction of MMb by providing the postmortem cytoplasmic and glycolytic pool of NADH to be utilized by the NADH-cytochrome b_5 reductase system in muscle (Arihara *et al.*, 1996). Furthermore, Saleh & Watts (1968) tested several glycolytic and Krebs cycle intermediates for their ability to increase MMb reduction in ground beef, and they concluded that the reducing activity of most meat samples can be increased by appropriate substrate addition. However, under physiological conditions the ratio of cytosolic free NAD^+ / NADH is approximately 700 to 1 (Zhang *et al.*, 2002), while the ratio of mitochondrial NAD^+ / NADH is 7-8 to 1 (Williamson *et al.*, 1976). Di Lisa *et al.* (2001) reported that mitochondrial pool of NADH represents a significant portion of the total pool of NADHs. Additionally, NADH pool produced from glycolytic flux is mostly transferred to mitochondria through malate-aspartate shuttle system (Rubi *et al.*, 2004). Therefore, it was our primary interest to investigate the potential role of an oxido-reductase enzyme, MDH prevalent in both cytosol and mitochondria because of its ability to regenerate NADH and concomitant reduction of MMb.

2.6.2 Reduction of Metmyoglobin by Skeletal Muscle Extract

The purpose of this investigation was to provide a direct evidence of malate interaction with myoglobin redox chemistry in preparations of beef skeletal muscle extract. Furthermore, the intent of this study was to support the hypothesis that reduction of horse MMb *in vitro* is related with malate's ability to replenish postmortem pool of NADH via MDH activity.

The kinetic data of malate interaction with MMb reductase of ME reported in this study provides direct evidence that ME obtained from beef skeletal muscles contains enzymes that utilize malate as substrate and its ability to replenish postmortem pool of NADH in ME (Figure 2.4) and that the regenerated postmortem pool of NADH was able to reduce MMb through interaction and electron transfer mediation with metmyoglobin reductase. Additionally, the kinetic data presented (Figure 2.5, 2.6, and 2.7), suggest that MDH present

in ME required NAD^+ and malate for its enzymatic function and that MMb reducing enzymes present in ME required regenerated NADH for subsequent reduction of MMb. Acivity of MDH in ME obtained from three different bovine muscles indicates the validity of the original concept proposed by Watts *et al.*, (1968) that post-mortem muscle can replenish NADH by the reduction of NAD^+ , and that a NADH-dependent reducing system, either enzymatic or non-enzymatic, can reduce metmyoglobin (Figure 2.8A and B).

Since enzymatic reduction of MMb in meat occurs primarily through a NADH-dependent MMb reductase (Arihara *et al.*, 1989), a mechanism that supports the hypothesis that malate-MDH system interacts with metmyoglobin reductase extracted from bovine muscle *in vitro* would provide greater insight into understanding the effects of malate on meat color. It is well-established fact that MMb reduction occurs through both enzymatic and non-enzymatic reducing systems and that the reduced nicotinamide adenine dinucleotide (NADH) is the ultimate reducing substrate for both pathways (Mancini & Hunt, 2005). Giddings (1974) reviewed enzymatic reduction of metmyoglobin in meat. Subsequent investigations of metmyoglobin reduction in meat systems have provided evidence for the phenomenon but without explanation of a mechanism (Lanier *et al.*, 1978; O'Keefe & Hood, 1982; Ledward, 1985; Renerre & Labas, 1987). Evidence for nonenzymatic reduction was presented by Brown & Snyder (1969). NADH and NADPH were active reductants and were enhanced in their metmyoglobin-reducing ability by the addition of flavins and methylene blue. Several metmyoglobin reductases have been described which require NADH and an appropriate mediator to facilitate conversion of ferric myoglobin to its ferrous form (Matsui *et al.*, 1975; Al-Shaibani *et al.*, 1977; Hagler *et al.*,1979; Levy *et al.*, 1985).

Here, the importance of the malate-aspartate shuttle member malate was investigated for the first time in metmyoglobin reduction *in vitro* and in ME from different bovine muscles. The results demonstrate the crucial role of the malate-MDH system in postmortem replenishment of NADH and subsequent mediated electron transfer to and reduction of MMb.

Our data confirm the predominant role of a key Krebs cycle intermediate for its ability to reduce MMb and its apparent role in stabilizing myogobin redox chemistry in postmortem skeletal muscles. Furthermore, addition of malate in ME provides an excellent model for studying metmyoglobin reduction and may provide a means of improving meat color by utilizing components of low commercial value variety meats.

ABBREVIATIONS USED

ME, muscle extract; MMb, metmyoglobin; DMb, deoxymyoglobin; OMb, oxymyoglobin; MRA, metmyoglobin-reducing activity; EDTA, ethylenediaminetetraacetic acid; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; MDH, malate dehydrogenase; FMN, flavin mononucleotide.

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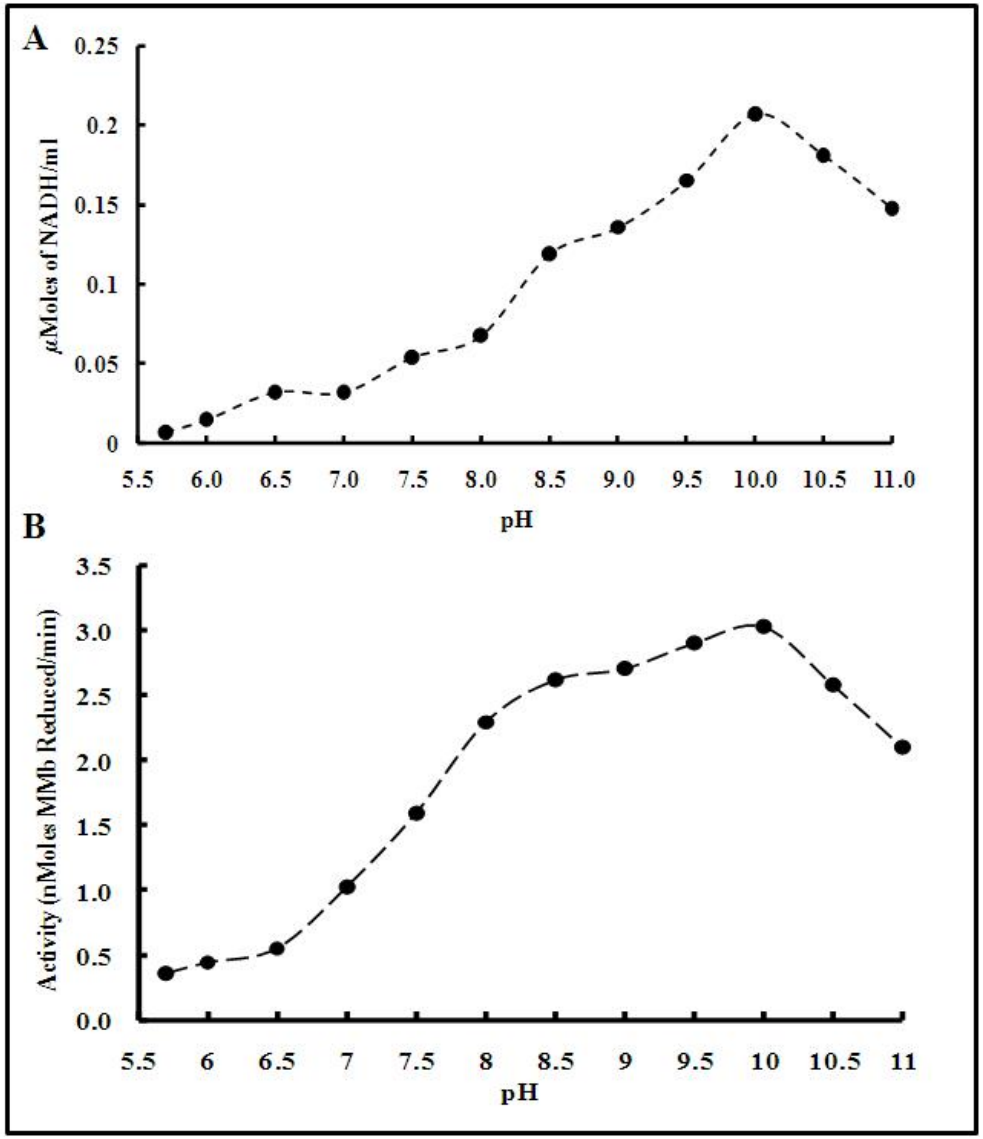

Figure 2.1: *Effects of pH on NADH production and on the initial velocity(v) of MMb reduction via malate-MDH reaction pathway at temperature* $30\,^{\circ}C$.

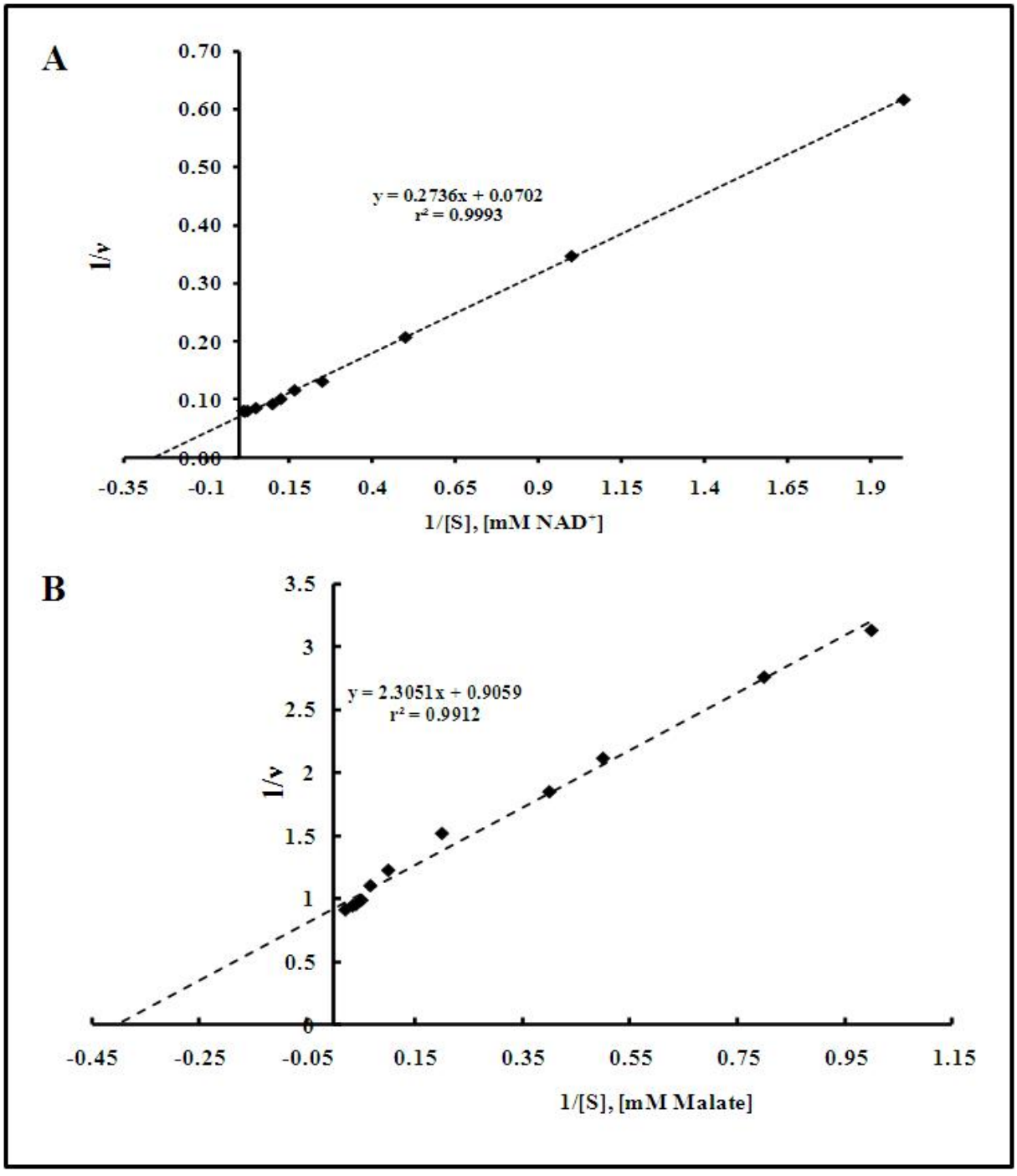

Figure 2.2: *Effect of A) NAD^+; B) malate, as the variable substrate on the initial velocity of the MMb reduction via malate - MDH reaction pathway. The initial velocity ν is expressed as nanomoles of MMb reduced per min; each point is the mean from four determinations.*

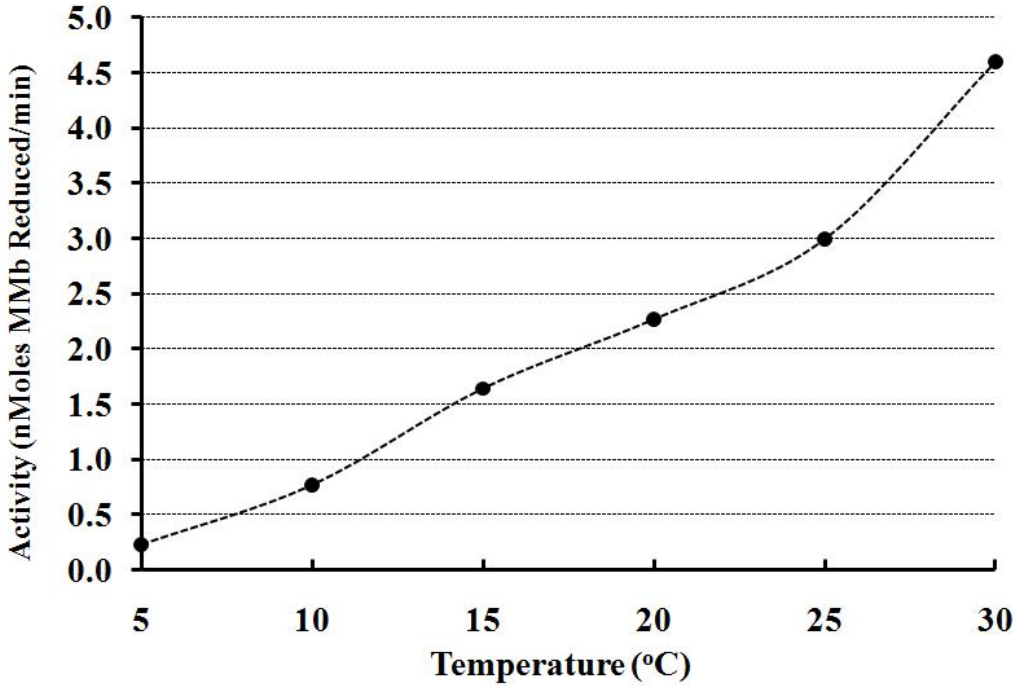

Figure 2.3: *Effect of assay temperature on nonenzymatic reduction of MMb via malate - MDH reaction pathway. The activity is expressed as nanomoles of MMb reduced per min; each point is the mean from four determinations.*

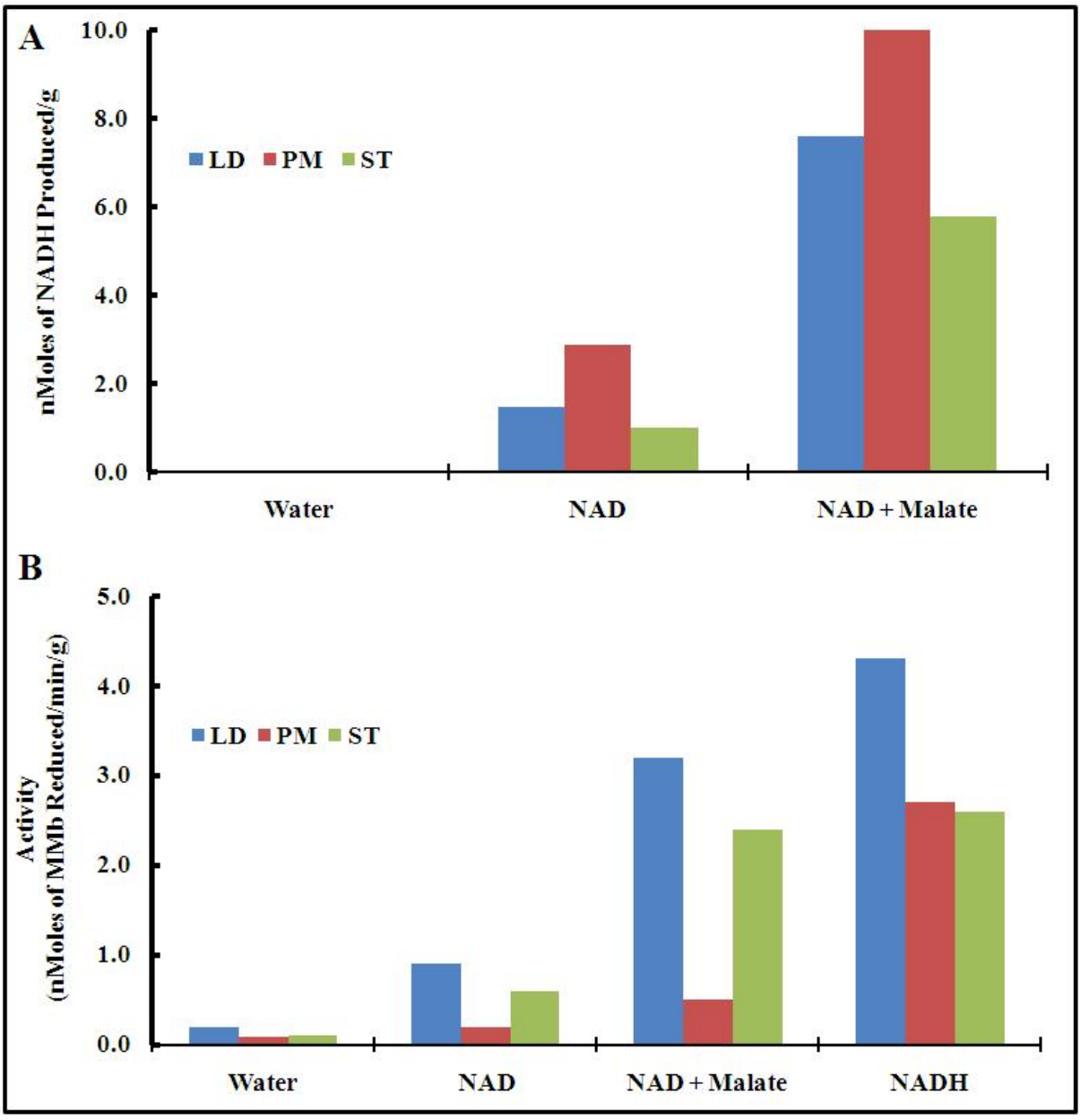

Figure 2.4: *MRA using muscle extracts from three beef muscles w/ and w/o malate and comparison between produced NADH via MDH and std NADH*

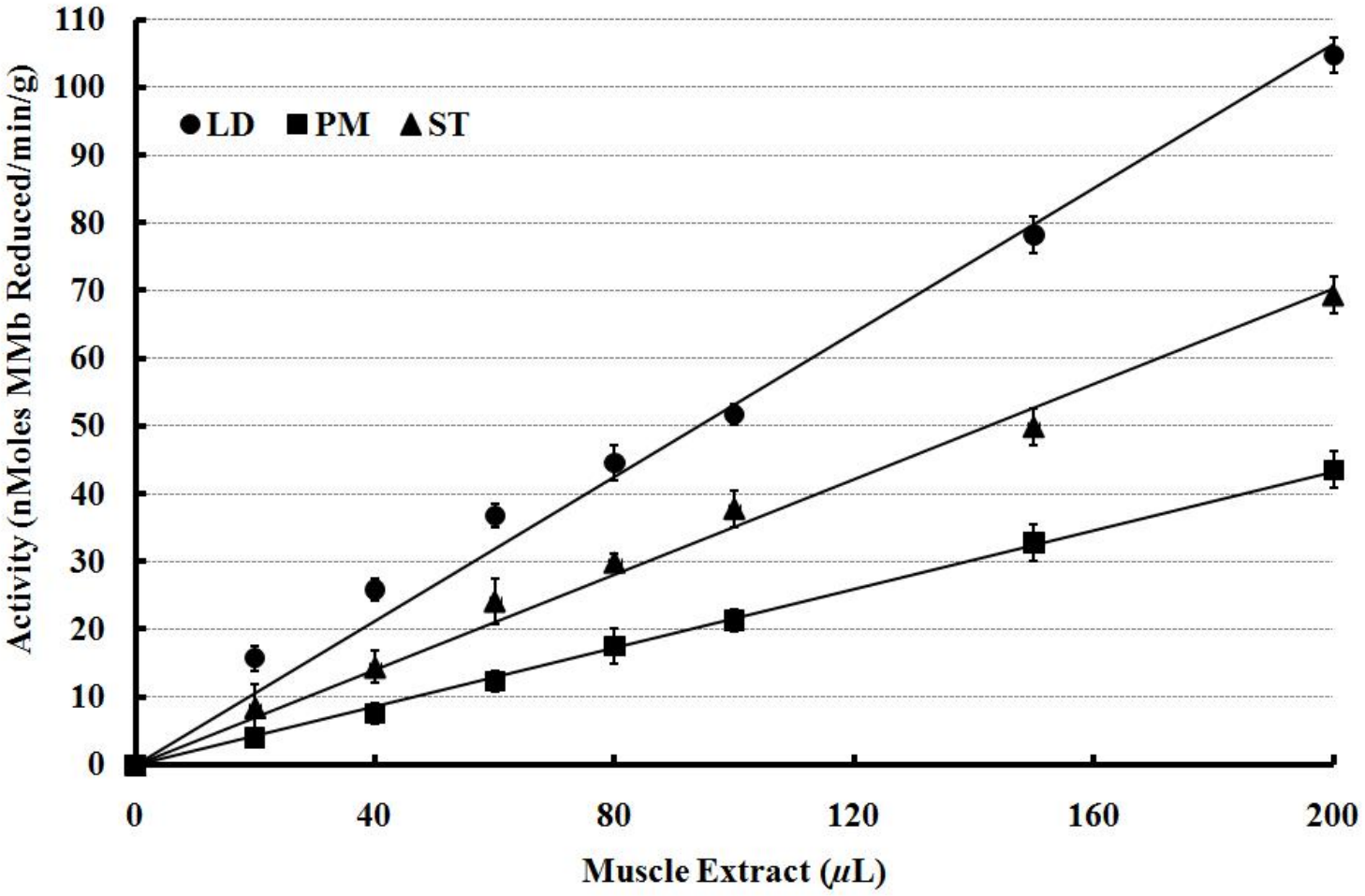

Figure 2.5: *Concentration Effects of Muscle Extracts on Metmyoglobin Reducing Activity.*

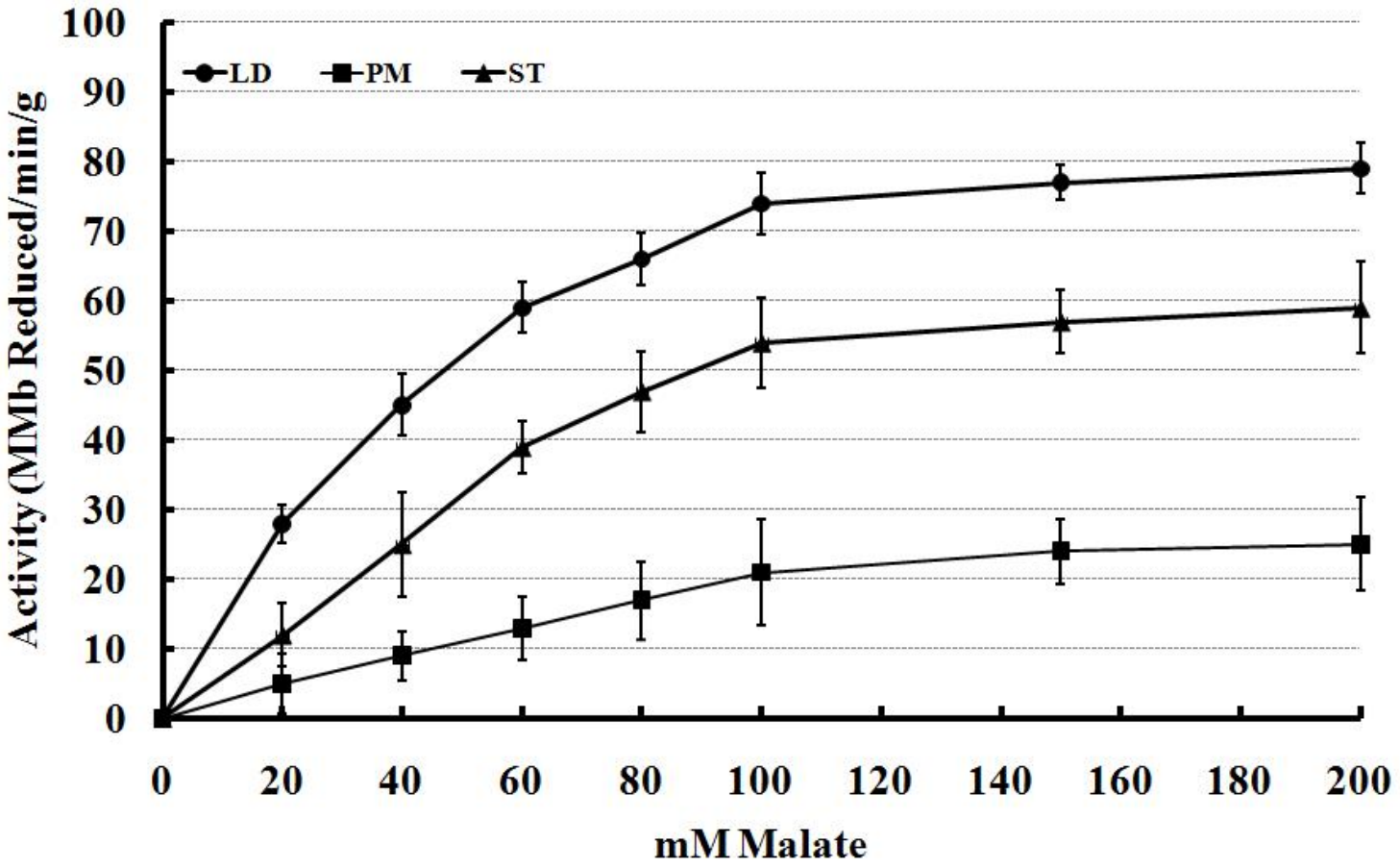

Figure 2.6: *Concentration Effects of Malate on Metmyoglobin Reducing Activity.*

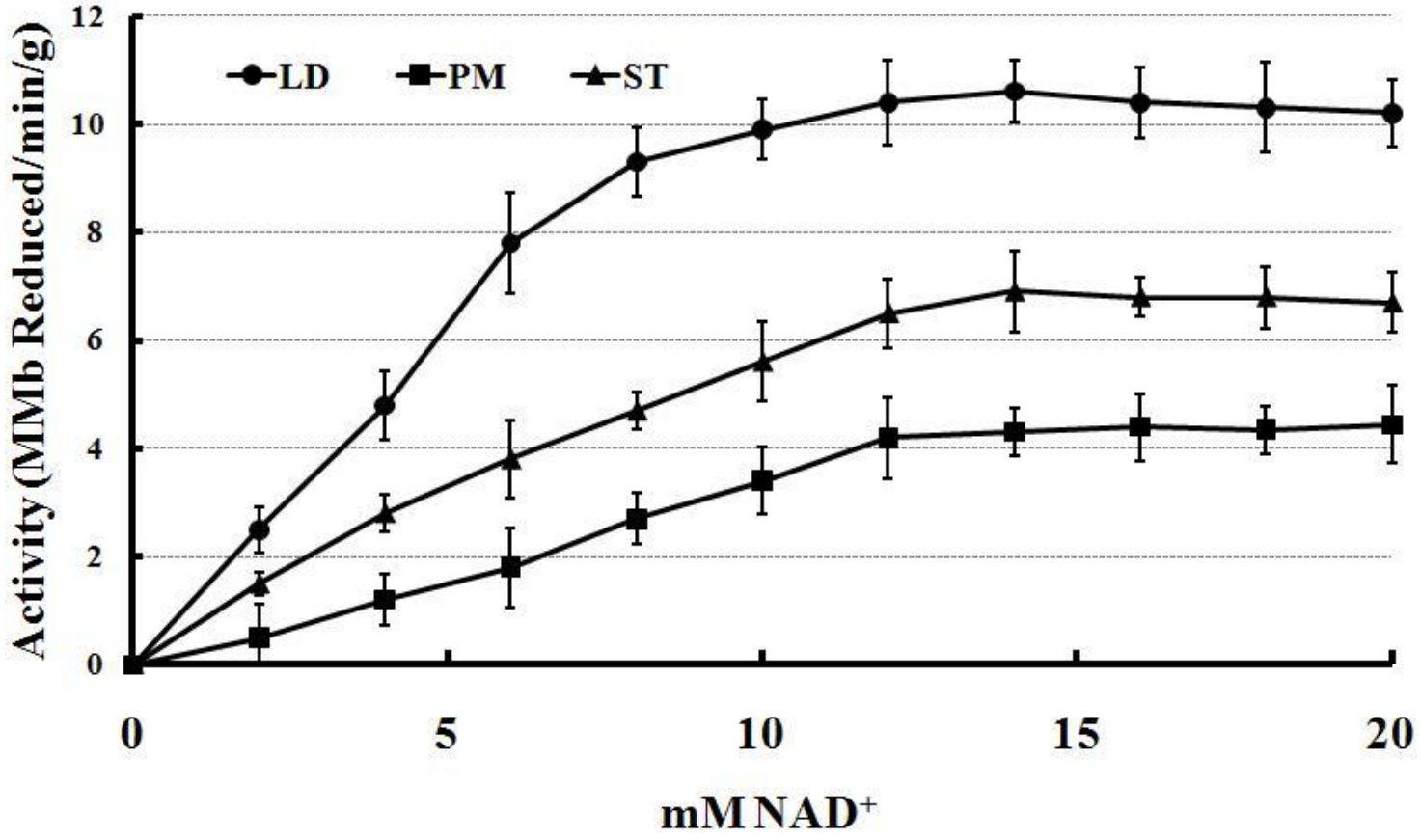

Figure 2.7: *Concentration Effects of NAD on Metmyoglobin Reducing Activity.*

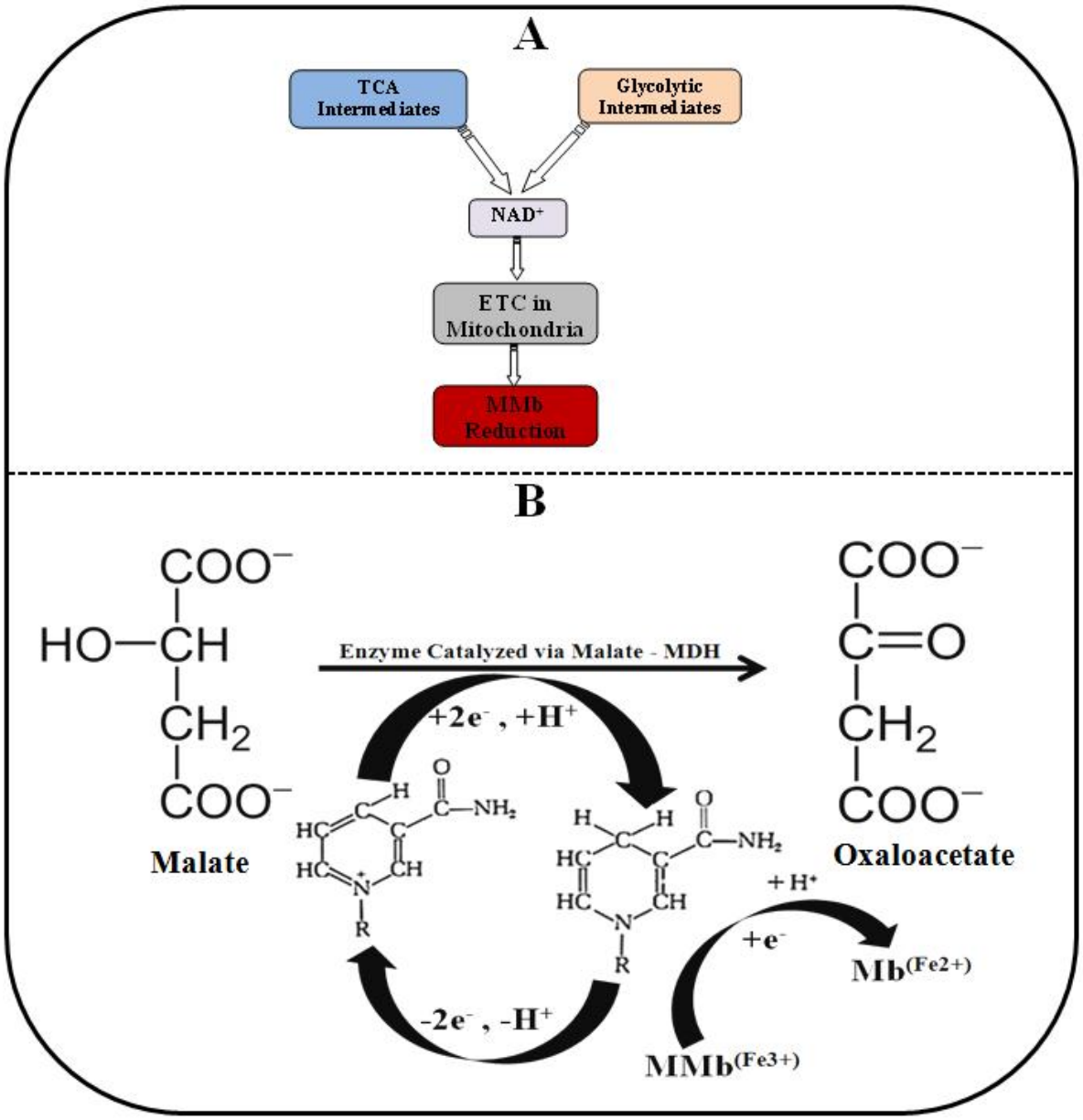

Figure 2.8: *Schematics of proposed pathways of A) nonenzymatic reduction of MMb via malate-MDH system and B) generation of NADH using enzymes of glycolytic and TCA cycles for metmyoglobin-reducing activity (MRA) in meat (Watts et el., 1968).*

Table 2.1: *Nonenzymatic Reduction of Horse Metmyoglobin with Malate-MDH System at 30 °C and pH 7.5 (n = 3)*

				solution components[a]				
FMN 2.0 mM	MB 0.1 mM	NAD^+ 25 mM	L-Malic Acid 100 mM	MDH[b] 0.1 mL	HMA 100 mM	GOT 0.1 mL	Glutamate 100 mM	Activity (nmole/min)
+	+	+	+	+	-	+	+	1.59 ± 0.006
-	+	+	+	+	-	+	+	1.26 ± 0.011
+	-	+	+	+	-	+	+	0.12 ± 0.005
+	+	-	+	+	-	+	+	0.01 ± 0.003
+	+	+	-	+	-	+	+	0.03 ± 0.007
+	+	+	+	-	-	+	+	0.02 ± 0.003
+	+	+	+	+	+	+	+	0.91 ± 0.004
+	+	+	+	+	-	-	+	0.23 ± 0.009
+	+	+	+	+	-	+	-	0.03 ± 0.003

[a] Substances present (+) or absent (-) in mixtures. FMN, flavin mononucleotide; MB, methylene blue; HMA, hydroxy malonic acid: GOT, glutamate-oxaloacetate transaminase; NAD^+, oxidized nicotinamide dinucleuotide. Assays were conducted in an aerobic environment with 0.5 mM equine MMb in 40 mM phosphate buffer. Total assay volume (1.7 mL). Composed of 0.2 mL of equine MMb and combinations of 0.1 mL of FMN, MB, NAD^+, L-malic acid, hydroxymalonic acid, and with deionized distilled water to bring to final volume.

[b] 226.8 units/mL

Chapter 3

Myoglobin Redox Form Stabilization by Compartmentalized Lactate and Malate Dehydrogenases

3.1 Abstract

The purpose of this study was to assess the ability of mitochondrial and cytoplasmic malate dehydrogenase present in postrigor bovine skeletal muscle to utilize malate as fuel for NADH regeneration and MMb reduction via the malate-NAD-MMb system. Furthermore, addition of lactate to beef mitochondrial and cytoplasmic isolates was evaluated to determine if interactions between malate and lactate increased MMb reduction. Addition of malate to isolated beef mitochondrial and cytoplasmic isolates at pH 7.2 increased ($p < 0.05$) MMb reduction. MMb reduction resulting from the addition of malate and lactate was equal or greater than MMb reduction resulting from malate alone. This suggests that a combination of mitochondrial (malate) and cytoplasmic (lactate) factors can be used to regenerate the postmortem pool of NADH resulting in metmyoglobin reduction and meat

color stabilization.

3.2 Introduction

Fresh meat color is determined by the redox status and the relative proportions of myoglobin redox forms, deoxy-, oxy-, and met-myoglobin on and just below the surface of meat. Numerous ingredients are often added to case-ready meats to add value and extend product shelf life. Myoglobin, the protein responsible for red color, interacts with these ingredients through complex biochemical reactions affecting myoglobin redox chemistry and meat color stability. Pathways by which these ingredients interact with myoglobin redox chemistry are unclear and an understanding of the biochemical mechanisms that stabilize myoglobin redox status can offer novel insights into meat color chemistry for the development of new strategies to improve beef color stability.

Mitochondria are important sub-cellular organelles involved in energy metabolism. Reports (Tang, *et al.*, 2005; Seyfert *et al.*, 2006; Ramanathan *et al.*, 2009) indicate that myoglobin redox stability and mitochondrial activity in postmortem skeletal muscles are interrelated. Mitochondria have the potential to affect myoglobin redox stability and meat color by mediation of MMb reduction via tricarboxylic acid (TCA) metabolites by the transfer of reducing equivalents between extra- and intra mitochondrial membranes (Ramanathan *et al.*, 2009). Andrews *et al.*, (1952), Bodwell *et al.*, (1965) and Watt *et al.*, (1966) showed that enzymes involved in glycolysis and the TCA cycle remain active in postmortem muscle, and thus could be possible sources of reducing equivalents. Other evidence (Tang, *et al.*, 2005; Seyfert *et al.*, 2006; Ramanathan *et al.*, 2009) suggests that metabolic activity of mitochondria in postmortem muscles may have a direct role in myoglobin redox form stability.

Arihara *et al.*, (1995) suggested that mitochondria in postmortem skeletal muscles main-

tain metabolic activity muscle for a long period of time and that the enzymes located in mitochondria may reduce MMb. Tang *et al.*, (2005) suggested that the enzymes responsible for MMb reduction are located within the muscle's mitochondria and that the mitochondrial pool of NADH should provide for metmyoglobin-reducing activity (MRA). However, it is unclear, how such enzymes within muscles mitochondria may relate to MRA in the cytoplasm.

Several researchers (Renerre, 1984; Renerre & Labas, 1987; Lanari & Cassens, 1991) indicated that meat discoloration was more indicative of muscle mitochondrial enzyme activity and mitochondria content than the meat's MRA . Muscles with high mitochondrial content were associated with high oxidative metabolism and low color stability (Renerre, 1984). Lanari & Cassens (1991) reported that mitochondrial and sub-mitochondrial particles facilitated the myoglobin redox stability. Giddings (1974) hypothesized that mitochondria may facilitate MMb reduction by supplementing the meat tissue with a postmortem pool of reduced cofactors (NADH) generated by the reversal of electron transport chain. Tang *et al.*, (2005ab) concluded that the addition of succinate to a bovine mitochondria-myoglobin system resulted in both oxygen consumption and MMb reduction. Bodwell *et al.*, (1965) suggested that availability of substrates to TCA enzymes (dehydrogenases) that help regenerate reducing equivalents in postmortem skeletal muscles could be the limiting factor. Collectively, these observations suggest that mitochondria can play an important role in meat color stability.

Furthermore, effects of compartmentalized enzymes between mitochondria and cell sap such as malate dehydrogenase (MDH) and their ability to generate reducing equivalents in postmortem beef skeletal muscles of varied color stability and their influence on myoglobin redox stability has not been reported. Klingenberg & Bucher, (1960) reported a bimodal distribution of several enzymes (including MDH) between mitochondria and cell sap. More specifically, NADH resulting from MDH activity between mitochondrial and cell sap might

play an important role in meat color stability.

MMb may be reduced nonenzymatically (Brown & Snyder, 1969; Mancini & Hunt, 2005; Kim *et al.*, 2006; Mancini & Ramanathan, 2008) or enzymatically (Watts *et al.*, 1966; Saleh & Watts, 1968; Hagler *et al.*, 1979; Mancini & Hunt, 2005; Kim *et al.*, 2006) by enzymes located within muscle mitochondria (Arihara *et al.*, 1995; Mikkelsen et al., 1999) such as cytochome *c* oxidase. Reduction of MMb occurs in the presence of mitochondria, lactate and succinate *in vitro* (Tang *et al.*, 2005ab; Ramanathan *et al.*, 2009). Thus, mitochondria as a source of NADH, a key component of MRA, should provide for MRA (Arihara *et al.*, 1990). It is not known, however, how cytochome *c* oxidase activity within muscles may relate to MRA.

A metabolite-induced regeneration of NADH by compartmentalized enzymes such as MDH and lactate dehydrogenase (LDH) that may mediate MMb reduction and influence myoglobin redox stability has not been reported. More specifically, NADH production resulting from MDH and/or LDH activity between mitochondrial and cell cytoplasm might play an important role in meat color stability. Therefore, we hypothesized that the NADH produced from MDH and/or LDH has the potential to improve myoglobin redox stability. The objectives of this study were to: 1) assess the functional potential of compartmentalized MDH and LDH from mitochondria and cell cytoplasm on MMb reduction and 2) evaluate the relationships among color stabilization mediated by compartmentalized MDH and LDH, metmyoglobin-reducing activity, and cytochrome *c* oxidase (CcOx) activity for three beef muscles differing in color stability.

3.3 Materials and Methods

3.3.1 Raw Materials

Five beef carcass sides representing different animals (USDA Select, A-maturity, with normal color and pH) were selected randomly 2 d postmortem at a commercial abattoir. From each carcass side, the *longissimus lumborum* (LL), *psoas major* (PM), and *semitendinosus* (ST) muscles were obtained and stored in vacuum either 10 or 20 days postmortem at 2 ± 1 °C. Since fiber types vary in these muscles (Hunt & Hedrick, 1977), tissue samples were from the central portion of the LL, the whole cross sectional portion of the PM, and the outer portion of the ST (the small deep red portion of the ST was excluded).

3.3.2 Chemicals

Equine skeletal Mb, magnesium chloride ($MgCl_2$), bovine albumin (BSA), sucrose, Tris[hydroxymethyl]-aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), potassium phosphate monobasic (KH_2PO_4), sodium malate, potassium chloride (KCl), and Nagarse protease (10.5 units/mg) were obtained from Sigma Chemical Co. (St. Louis, MO); sodium succinate was purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were reagent grade.

3.3.3 pH

Samples, visually devoid of fat and connective tissue, were frozen in liquid nitrogen and blended in a Waring table-top blender (Dynamics Corp. of America, New Hartford, CT). To determine pH, 10 g of pulverized sample was combined with 100 mL of deionized water, mixed for 30 s, and the pH value obtained by using an Accumet combination glass electrode connected to an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ).

3.3.4 Proximate Analysis

A 10 g of the pulverized samples were analyzed (n = 5) for protein [LECO Combustion Analysis (AOAC Official Method 990.03; Thiex, 2009] and moisture and fat [CEM SMART and SMART Trac systems (AOAC PVM 1:2003; Keeton *et al.*, 2003].

3.3.5 Mitochondria Isolation

Mitochondria were isolated according to the methods of Bhattacharya *et al.*, (1991) and a slightly modified method of Frezza, Cipolat, & Scorrano (2007). All mitochondrial isolation procedures were performed at 0-4 °C. Ten grams of muscle, devoid of fat and connective tissue, were homogenized in 50 mL of isolation buffer 1 (46mM M KCl, 100 mM Tris HCl, 5 mM $MgCl_2$, 10 mM EDTA, and BSA 0.5%. pH 7.4) by using a homogenizer (Kinematica Polytron benchtop Model PT 3100; Brinkmann, Lucerne, Switzerland). After homogenization, 25 mg of Proteinase K was added and the sample was incubated for 5 min at 0 °C before 100 mL of isolation buffer 1 was added. Further homogenization at 0 °C was accomplished with a Kontes Duall grinder (Vineland, NJ) and then with a Wheaton Potter-Elvehjem grinder (Millville, NJ). All Teflon grinding pestles rotated at 1400 rpm. Each sample was centrifuged at 600 × *g* at 0 °C for 10 min (Sorvall RC-5B, Newtown, CT) and filtered through two layers of cheesecloth. The supernatant was again centrifuged at 14,000 × *g* at 0 °C for 10 min, and the resulting pellet was vortexed with 5 mL of isolation buffer 2 (0.1 M KCl, 50 mM Tris HCl, 1 mM $MgCl_2$, 0.2 mM EDTA, and 0.5% BSA, pH 7.4). Samples were again centrifuged at 7,000 × *g* at 0 °C for 10 min and the resulting pellet was vortexed with isolation buffer 2. Final centrifugation was accomplished at 3500 × *g* at 0 °C for 10 min, and the isolated mitochondria pellet was vortexed with 0.25 M sucrose.

3.3.6 MRA assay with isolated mitochondrial and cytoplasmic protein

Enzyme activity was assessed spectrophotometrically (Hitachi DU-2010, Japan) at 30 °C. Mitochondrial matrix enzyme, malate dehydrogenase (MDH) was assessed in the mitochondrial fractions. Each assay contained varying amounts of mitochondrial and cytoplasmic proteins added to obtain a rate of 0.1 absorbance units/min. Activities are reported as nanomoles of MMb reduced per milligram per minute. MDH activity was measured by following the rate of NADH oxidation at 340 nm (millimolar extinction coefficient = 6.23). Mitochondrial fraction was diluted in 10 mM $KHPO_4$ and subjected to three freeze-thaw cycles. Lactate and malate were used as substrates to assess maximal rates of MMb reduction. Mitochondrial samples were diluted in 10 mM $KHPO_4$, subjected to sonication (Branson Sonifier 250) at 40% full power.

3.3.7 Cytochrome *c* Oxidase Activity

Cytochrome *c* oxidase activity was determined by using a colorimetric-assay kit (Sigma, St. Louis, MO). Samples for CcOx were removed on day 10 and 20 from each of the 3 muscles, frozen in liquid nitrogen, vacuum-packaged, and stored at -80 °C until analysis. Mitochondria were isolated from these samples as described earlier. Determination of CcOx activity was based on a colorimetric assay that quantifies oxidation of ferrocytochrome c to ferricytochrome c via CcOx, a reaction that results in a decrease in absorbance at 550 nm (Sigma CcOx technical bulletin). The decrease in absorbance at 550 nm was monitored by using a spectrophometer (Hitachi DU-2010, Japan). The spectrophotometer was calibrated to zero by using assay buffer (10 mM Tris-HCl and 120 mM KCl, pH 7.0, 25 °C).

In a cuvette, 0.95 mL of assay buffer was combined with 50 μL of enzyme buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.0, 4 °C) and 50 μL of isolated mitochondria. The

reaction was initiated by the addition of 50 μL of ferrocytochrome c (reduced with 0.1 M dithiothreitol), and the decrease in absorbance at 550 nm was measured every second for 1 min by using a kinetics program. Activity was calculated based on the following equation. Units/mL) [(ΔAbs_{550}/ min for the sample - ΔAbs_{550}/min for the blank) $\times$ dilution factor (6) $\times$ total reaction volume (1.1 mL)]/[mitochondria isolate volume (0.05 mL) $\times$ the difference in extinction coefficients between ferro- and ferricytochrome c at 550 nm (21.84)]. One unit oxidized 1 μM reduced cytochrome c per min at pH 7.0 and 30 °C.

3.3.8 Metmyoglobin-Reducing Activity

Metmyoglobin-reducing activity (MRA) was determined by using a 2.54-cm cube from each of the 3 muscles on days 0, 4, and 7, according to a procedure described by Sammel *et al.*, (2002). Samples were submerged for 20 min in a 0.3% solution of sodium nitrite to facilitate nitric oxide metmyoglobin (MMb) formation, then removed, blotted dry, placed in a barrier bag (3-mil, standard-barrier nylon/polyethylene, 0.6 cm3/645.16 cm^2/24 h at 0 °C oxygen transmission rate; Koch Supplies, Inc., Kansas City, MO), and vacuum packaged at $\geq$62.2 cm Hg (Multivac C500, Multivac Inc., Kansas City, MO). On the light-exposed display surface, samples were scanned twice with a HunterLab MiniScan XE Plus Spectrophotometer (D/8-S, 14.3-mm diameter aperture; Hunter Associates Laboratory, Inc., Reston, VA) to obtain 400-700 nm reflectance data. Samples were incubated at 30 °C for 2 h (Thelco model 4; Precision Scientific, Chicago, IL) to induce nitric oxide MMb reduction to deoxymyoglobin (DMb). Upon removal from the incubator, samples were immediately rescanned twice to determine the percentage of remaining surface MMb, using K/S ratios and equations from AMSA (1991). The following equation was used to calculate MRA: (Δ % surface MMb/preincubation% surface MMb) $\times$100.

3.4 Statistical Analysis

The experimental design for determining muscle effects on MDH, LDH, and CcOx activity was a randomized complete block. Type-3 tests of fixed effects for muscle were performed by using the MIXED procedure of SAS (26), and beef carcass sides (n = 5 animals) were considered random blocking effects.

The experimental designs for determining muscle effects (whole plot fixed effect) on metmyoglobin-reducing were split plots. The whole plot consisted of a randomized complete block in which beef carcass sides (n = 5 animals) served as blocks. Time of chemical analysis (subplot treatment) was assigned to one of the three steaks (subplot unit) from each muscle (15 total units; 5 animals × 3 muscle types). Type-3 tests of fixed effects for muscle, muscle types, and their interaction were performed by using the MIXED procedure of SAS. Beef carcass sides (animals) were considered random effects for the randomized complete-block portion of the whole plot, and muscle*animal was used to determine whole-plot error. Least-squares means were separated as described for color analysis. Least-squares means were separated as described for color analysis. Least-squares means for F-tests ($p < 0.05$) were separated and were considered significant at $p < 0.05$

3.5 Results

3.5.1 pH and Proximate Analysis

The PM muscle had the highest pH ($p < 0.05$), but all muscles were between 5.5 and 5.7 (Table 3.1).

3.5.2 Cytochrome *c* Oxidase Activity

Cytochrome c oxidase activities for all muscles were different (Figure 3.1). Cytochrome c oxidase activity for LL and ST were greater than PM ($p < 0.05$) for aging period day 10. No significant differences were observed between ST and PM ($p < 0.05$)for aging period day 20. However, aging period day 20, CcOx activity of LL was greater than PM and ST ($p < 0.05$). Aging from day 10 to day 20 decreased ($p < 0.05$) CcOx activity for all muscle types.

3.5.3 Metmyoglobin Reduction Activity

Metmyoglobin reducing activity for all muscles decreased ($p < 0.05$) for both aging periods (Figure 3.2). Muscle types, LL and ST had greater MRA than PM ($p < 0.05$) for the 10 day aging period. A similar trend was observed for aging period day 20, where PM had the lowest MRA compared with LL and ST. For both aging periods, MRA was lowest for the PM, intermediate for the ST, and highest for the LL. MRA activity decreased with the increase in the aging periods from 10 days to 20 days.

3.5.4 Mitochondrial Protein Content

Figure 3.3 shows the means for mitochondrial concentration (mg mitochondrial protein/g of muscles) for each muscle and for both aging periods. Mitochondrial concentration for PM was greater ($p < 0.05$) than for LL and ST for both aging times. The ST tended to have the lowest mitochondrial concentration; LL intermediate, and highest for PM for both aging times. After aging for day 20, decrease in mitochondrial protein content was greatest for PM compared with LL and ST. Total mitochondrial protein content was lowest for ST, greatest for LL and intermediate for the ST.

3.5.5 Cytoplasmic Protein Concentration

Cytoplasmic protein concentrations (mg cytoplasmic protein/g of muscles) for each muscle and for both aging periods are shown in Figure 3.4. The cytoplasmic protein concentration for PM was greater ($p < 0.05$) than those for LL and ST at 10 days of aging. Cytoplasmic protein concentration was lowest for PM, intermediate for the ST, and highest ($p < 0.05$) for LL for both 20 day. Decreases in cytoplasmic protein content for PM were greatest compared with LL and ST after aging for day 20.

3.5.6 Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Malate Dehydrogenase

Kinetic data (Figure 3.5) showed differences in MMb reduction between muscles aged 10 and 20 days. Increasing concentrations of both the mitochondrial protein and cytoplasmic protein fractions (0.5, 1.0, 1.5, and 2.0 mg Mito Protein/gm) from LL muscle aged for 10 days exhibited greater MMb reduction via the malate-MDH pathways than those from LL muscle aged for 20 days. Reduction of MMb by mitochondrial-MDH (MT-MDH) was greater ($p < 0.05$) than that for cytoplasmic-MDH (CP-MDH) for all three beef muscles. Mitochondrial-MDH from the PM aged for 10 days exhibited greater ($p < 0.05$) MMb reduction via malate-MDH compared to PM muscle aged for 20 days. Reduction of MMb by PM MT-MDH was greater ($p < 0.05$) than CP-MDH. A similar but non-significant trend was observed for MMb reduction by MT-MDH and CP-MDH isolates from beef skeletal muscle ST for both aging periods. MMb reduction by the individual factions of MT-MDH and CP-MDH from the three beef muscles were different. The kinetic data for the MT-MDH-assisted-MMb reduction indicated that PM exhibited greater MMb reduction compared with LL and ST muscles aged for 10 days. However, there were no differences in MT-MDH-assisted-MMb reduction for all three muscles aged for 20 days.

3.5.7 Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Lactate Dehydrogenase

Kinetic data (Figure 3.6) showed that cytoplasmic LDH (c-LDH) exhibited a greater ($p < 0.05$) reduction rates for MMb as compared with mitochondrial LDH (m-LDH). Reduction of MMb by m-LDH was much lower ($p < 0.05$) than c-LDH for all three beef muscles. Increased concentration of mitochondrial protein (0.5, 1.0, 1.5, and 2.0 mg Mito Protein/gm) isolated from the PM showed greater ($p < 0.05$) MMb reduction than did the LL and ST muscle aged for 10 and 20 days. Increasing concentration of cytoplasmic protein isolated from LL muscle aged 10 and 20 days significant difference ($p > 0.05$) in MMb reduction. MMb reduction kinetics using m-LDH from PM muscles aged for 10 days had greater ($p < 0.05$) MMb reduction than the PM muscle aged 20 days. Also, c-LDH activity for MMb reduction by the PM was lower than for the ST and LL. c-LDH activity and MMb reduction was greater for LL compared with PM and ST. Aging of beef muscles from 10 to 20 days tended to have lower c-LDH and m-LDH activities for facilitating MMb reduction.

3.5.8 Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Malate and Lactate Dehydrogenases

Addition of a mixtures of substrates malate and lactate to the beef skeletal muscle mitochondrial and cytoplasmic isolates enhanced MMb reduction compared with addition of lactate or malate alone. Among the three beef muscles, addition of malate and lactate to the mitochondrial isolates from the PM muscle exhibited greater MMb reduction than did the LL and ST muscle aged for 10 days (Figure 3.7). Contrary to mitochondrial isolates, cytoplasmic protein isolated from the PM had minimal MMb reduction as compared with LL and ST. Mitochondrial and cytoplasmic protein isolated from LL and ST showed similar trend for MMb reduction for muscles aged 10 and 20 days. Kinetic data clearly showed that

the rate of MMb reduction was concentration dependent and that increasing concentrations of cytoplasmic and mitochondrial protein increased MMb reduction in muscles aged for and 10 and 20 days as compared with the control.

3.6 Discussion

3.6.1 Reduction of Metmyoglobin by compartmentalized Malate Dehydrogenase

The prime objectives of this experiment were: 1) to investigate the ability of compartmentalized TCA enzyme MDH from postmortem beef skeletal muscle to utilize substrate (sodium malate) supplementation to regenerate reducing equivalents, and 2) to evaluate the effects of compartmentalized MDH in the mediation of MMb reduction and influence myoglobin redox stability via malate-MDH pathways. Our investigations provide substantial evidence indicating that addition of malate to the mitochondrial and cytoplasmic isolates will lead to MMb reduction via regeneration of NADH and malate-MDH pathways.

There are several enzymes in the TCA cycle capable of reducing NAD. However, the position of MT-MDH in the TCA cycle combined with the results of this experiment showing considerable specific activity of MT-MDH for NAD-linked MDH in beef skeletal muscle mitochondria capable of reducing MMb represents a potential mechanism by which substrates of TCA enzymes could be used as a source of NADH postmortem that plays a direct role in myoglobin redox form stability. Although this investigation did not comprehensively evaluate all the compartmentalized enzymes, it appears that, not only do the compartmentalized MDHs differ in their relative abilities to regenerate postmortem pool of NADH, but they also appear to differ in their ability to reduce MMb at different rates in different muscles.

Our results also provide evidence that the enzymatic compositions, specifically of CP-

and MT-MDH from the three beef skeletal muscles differ considerably. Among the three muscles, addition of malate to the mitochondrial isolate from PM exhibited maximal activity for MMb reduction rates, whereas mitochondrial isolates from LL and ST did not show significant differences in their ability to reduce MMb.

Although morphological make-up of mitochondria in muscles of red or white fibers composition may not be different (Seyfert *et al.*, 2004; Tang *et al.*, 2005), it is the concentration and functional ability of the mitochondria postmortem that may lead to greater color stability in some muscles. Of the muscles used in this study, ST has the greatest amount of α-white fibers, the LL has more α-red and α-white, and PM is mostly β-red and α-red fibers (Hunt & Hedrick, 1977). Muscles high in β-red and α-red fibers, such as PM, have greater mitochondrial concentrations, they bloom (oxygenate) more slowly because of increased competition for available oxygen by mitochondria and other cellular organelles vs. myoglobin. Comparatively, muscles with predominantly white fibers have a lower consumption of oxygen (Hoppeler, Hudlicka, & Uhlmann, 1987). Therefore, muscles with more white glycolytic fibers, such as LL and ST, blooms brightly at the initiation of display as oxygen bind to myoglobin without mitochondrial competition. Consequently, LL and ST with predominantly glycolytic muscle fibers should have the greater color stability, and PM, with primarily oxidative fibers, should be more color labile. This study does not support this conclusion directly; however, presence of higher mitochondrial concentration of PM explains the higher oxidative potential of this muscle, and therefore, predicts a rapid discoloration.

Bodwell *et al.*, (1965) and Watts *et al.*, (1966) suggested that availability of substrates to TCA dehydrogenases that help regenerate reducing equivalents in postmortem skeletal muscles could be the limiting factor in meat color stability. Tang *et al.*, (2005ab) reported that the addition of succinate to a bovine mitochondria resulted in both greater oxygen consumption and MMb reduction. Lanari & Cassens (1991) reported that mitochondrial and sub-mitochondrial particles facilitated myoglobin redox stability and suggested that

mitochondria content and postmortem mitochondrial enzyme activity was related with meat discoloration. Renerre (1984) found that beef muscles with high mitochondrial content were associated with high oxidative metabolism and low color stability.

Malic acid, a natural constituent of many fruits and vegetables, is a metabolite of the TCA cycle that is involved in the production of energy in the body under both aerobic and anaerobic conditions through the malate-aspartate redox shuttle (Figure 3.8) via the electron transport chain. As a result of malic acid's role in the malate-aspartate redox shuttle and the TCA cycle, NADH is produced. It is generally accepted that the malate-aspartate shuttle is the principal means for removal of reducing equivalents from the cytoplasm to the mitochondria (LaNoue & Williamson, 1971). Intra-mitochondrially, malate is oxidized to oxaloacetate (OAA) by the TCA cycle regenerating mitochondrial pool of reducing equivalents that ultimately participates in ETC that may reduce MMb near mitochondrial outer membrane as proposed in the model (Figure 3.9).

According to McElroy, Wong, & Williams (1968) model intra-mitochondrial OAA is transaminated and reduced to malate. This process allows the transfer of the electrons from extra-mitochondrial NADH to malate, which reenters the mitochondrion. This model further supports the finding that the energy rich mitochondria should export malate non-energetically (as a source of cytoplasmic NADH) and import malate in the energy-poor state.

Although the role of MT-MDH in the regeneration of mitochondrial pool of NADH via TCA pathways is well documented (Markert & Mdller, 1959), the role of CP-MDH in the generation of reducing equivalents is less clear. The usefulness of having two localized isoenzymes may be a very critical factor when, as in postrigor skeletal muscle, the NADH is the most limiting factor for myoglobin redox stability. Moreover, NADH produced by the activity of lactate dehydrogenase cannot permeate through mitochondria (Purvis & Lowenstein, 1961), which makes malate likely a potential candidate for regeneration and

transport of reducing equivalents to mitochondria by shuttle mechanisms ultimately that ultimately lead to MMb reduction.

However, in postmortem skeletal muscle the role of malate in malate-aspartate shuttle and its permeability to mitochondria is unknown. Future studies should entail the detailed physiochemical properties of the malate, MDH, and the efficacy of these biochemical pathways leading to myoglobin form redox stability.

3.6.2 Reduction of Metmyoglobin by compartmentalized Lactate Dehydrogenase

Another purpose of this experiment was to assess the ability of compartmentalized LDH from isolated mitochondria and cytoplasm from muscles differing in color stability to utilize lactate as a substrate for NADH production and concomitant MMb reduction either directly or via the lactate-LDH system. Addition of lactate to isolated beef mitochondrial and cytoplasmic isolates resulted in MMb reduction via lactate-LDH system. This study provides evidence that, addition of L-lactate to isolated mitochondria and cytoplasmic protein was utilized as substrate for LDH accounting for regeneration of NADH and MMb reduction. The rate of MMb reduction was lower in the mitochondrial fraction as compared with the cytoplasmic fraction. The ability of the m-LDH to convert lactate to pyruvate is more directly related with mitochondrial redox status than LDH isoenzyme pattern (Brooks *et al.*, 1999). To our knowledge, no previous study has evidence for the compartmentalized LDH activity in postmortem muscle with an ability to regenerate NADH and MMb reduction. LDH activity of all muscles followed a different trend compared with m- and CP-MDH. The c-LDH assisted MMb reduction was significantly higher for LL than PM, while ST was not significantly different from LL. Kim *et al.*, (2009) reported that a higher accumulation of MMb and lower MRA was associated with low LDH activity in PM and decreased NADH

concentration postmortem. In conclusion, our results confirm the presence of mitochondrial and cytoplasmic LDH and support a role for MMb reduction via lactate-LDH system .

3.6.3 Influence of Postmortem Aging on Enzyme Activity and Distribution

Enzyme activity (LDH and MDH) and protein content (mitochondrial and cytoplasmic) in postmortem aged meat were examined for relative decreases in enzyme activity. The aging times used in this study (10 or 20 days) represent typical industry practices and meat purchased by consumers.

3.6.4 Cytoplasmic and Mitochondrial Protein Content

Both cytoplasmic and mitochondrial protein of the three muscles indicate important muscle-specific effects that could impact color stability. Aging from 10 to 20 days decreased both the cytoplasmic protein and mitochondrial protein levels. PM had the highest levels of both cytoplasmic and mitochondrial proteins compared with LL and ST muscles; however, aging decreased the cytoplasmic and mitochondrial proteins in all muscles. It is possible that the differences in estimates of mitochondrial and cytoplsmic protein content reported in this study could be due to fiber type (slow and fast twitch) and different metabolic activity of the three beef muscles discussed earlier. Beef skeletal muscles used as sources of muscle mitochondria in this study are comprised of either type I or type IIb fibers . Higher respiratory capacity in red fibers has been attributed to a higher mitochondrial content (Hoppeler, Hudlicka, & Uhlmann, 1987).

3.6.5 Metmyoglobin Reducing Activity Assay

Data for MRA corresponded well with m- and CP-MDH and/or LDH assays. As expected, muscles with greater color stability such as LL and ST had more MRA than the more color unstable muscle, PM during both aging times. Our research documenting muscle differences in their ability to regenerate NADH and concomitant MMb reduction utilizing cytoplasmic and mitochondrial enzymes and their agreement with the specific MRA assay adds a new dimension for color stabilization. Researchers have documented different methods to quantify MRA; however, their findings for relative color stability differences among muscles are similar to those in the present study. Ledward (1985) placed the PM and LL muscles after display into anaerobic environments and measured the extent of MMb reduction by reflectance method. Reddy & Carpenter (1991) reported that muscles with higher reducing activities were the muscles that have been traditionally characterized as the most color stable. Our data clearly show that the linkage between a muscle's ability to regenerate NADH and the subsequent reduction of MMb to be a primary endogenous determinant of relative color stability.

Reddy & Carpenter (1991) used muscle extracts, ferrocyanide, and horse MMb in solution with NADH to show that LL had more MRA than ST and PM muscles. MRA assays used in this study revealed that LL had more MRA than both ST and PM muscles, but the MRA of the ST appeared to be more intermediate that the PM. Sammel *et al.*, (2002ab) used a MRA procedure similar to the current study and reported that superficial portions of the M. *semimembranosus* (SM) had greater MRA and more color stability than SM's deep portions. O'Keeffe & Hood (1982) reported that the MRA of LL, SM, and PM muscles decreased as storage time increased. Madhavi & Carpenter (1993) reported similar findings and suggested that muscle oxygen consumption and its MRA has biphasic affects on color stability during aging. Muscle oxygen consumption was the primary determinant of color stability during the first 7 days postmortem, whereas MRA was most important

after 7 days. Overall, measures of MRA in this study seemed reliable since it showed good agreement with CcOx activity and enzymatic reduction of MMb using cytoplasmic and mitochondrial proteins.

3.6.6 Cytochrome *c* Oxidase Activity

Differences in CcOx activity among the three muscles corresponded well with MRA activity and color stability differences exhibited by these muscles. Furthermore, because mitochondria is considered as the principle site for MMb reduction and a source of NADH, a key component for MRA CcOx activity within different muscles should relate to their reducing ability and the relative color stability differences. Seyfert e al., (2006) assessed CcOx activity of five different bovine skeletal muscles and found that the superficial region (region of muscle adjacent to subcutaneous fat) of the semimembranosus muscle had twice as much CcOx activity as the deep (inner region of muscle closest to the femur) region had.

In summary, our results suggested that the LL had almost twice as much CcOx activity as the PM. Also, beef muscles aged for 10 days had higher CcOx activity than muscles aged for 20 days. A difference in CcOx activity of beef skeletal muscles of varied color stability might explain color stability differences among LL, ST, and PM. Furthermore, the greater differences in CcOx activity of beef skeletal muscles due to aged 10 and 20 days appear to be related with 20 days aging resulting in greater protein and enzymatic denaturation and/or oxidation. It is likely that the conditions of postmortem aging of beef muscles for further 20 days would have caused denaturation of enzymes within mitochondria, particularly CcOx, resulting in a decreased CcOx activity. It is generally believed that reducing activity of individual muscles is the most important factor in determining rate of discoloration. However, relative contribution of mitochondrial enzymes such as, CcOx, and MRA play an important role in regulating myoglobin redox stability and ultimately determine the color stability of muscle.

3.7 Conclusions

The findings demonstrate that mitochondria and cytoplasmic proteins isolated from beef skeletal muscles of different metabolic origin differ substantially in their enzymatic composition. Malate-MDH assisted-MMb reduction using Mitochondrial and cytoplasmic isolates from the three beef skeletal muscles exhibited substantial differences in enzymatic compositions and their ability to reduce MMb *in vitro*. Differences were also observed in the enzymatic characteristics of MDH assisted-MMb among the three beef muscles. This study further confirmed the presence of localized m- and c-LDH and supports a role for MMb reduction via lactate-LDH system. Our data clearly show that the linkage between a muscle's ability to regenerate NADH and the subsequent reduction of MMb to be a primary endogenous determinant of relative color stability. Moreover, finding that an addition of malate and lactate in combination to mitochondrial and cytoplasmic isolates was completely additive in reducing MMB, suggests an important role of glycolytic and mitochondrial TCA metabolites in the enzymatic reduction of reduction of MMb. Relative contributions of other factors such as, CcOx, and MRA may also play an important role in regulating myoglobin redox stability and ultimately determine the color stability of muscle.

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ABBREVIATIONS USED

LL, *longissimus lumborum*; PM, *psoas major*; ST, *semitendinosus*; MRA, metmyoglobin reducing activity; OCR, oxygen consumption rate; MMb, metymyoglobin; DMb, deoxymyoglobin; CcOx, cytochrome *c* oxidase; m-MDA, mitochondrial malate dehydrogenase, c-MDA, cytoplasmic malate dehydrogenase; m-LDH, mitochondrial lactic dehydrogenase; c-LDH, cytoplasmic lactic dehydrogenase

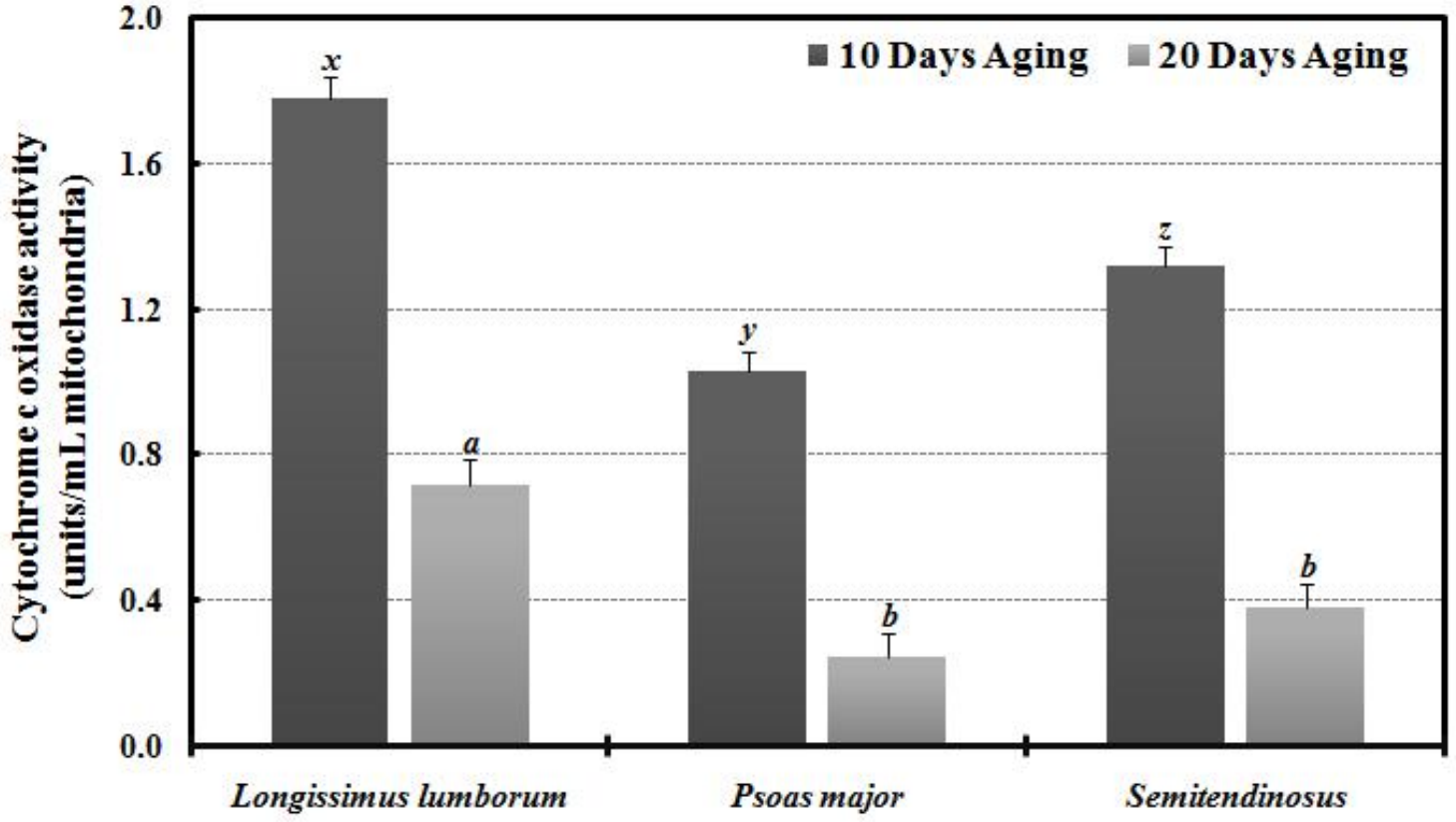

Figure 3.1: *Cytochrome c oxidase activity (CcOx) of three bovine muscles during 10 and 20 days aging times. LL = longissimus lumborum, PM = psoas major, ST = semitendinosus. MRA, or % MMb reduced, was calculated as (Δ % surface MMb/preincubation % surface MMb) × 100. x, y, z = means for different muscle types within 10 days aging time with a different letter differ ($p < 0.05$). a, b, c = means for different muscle types within 20 days aging time with a different letter differ ($p < 0.05$).*

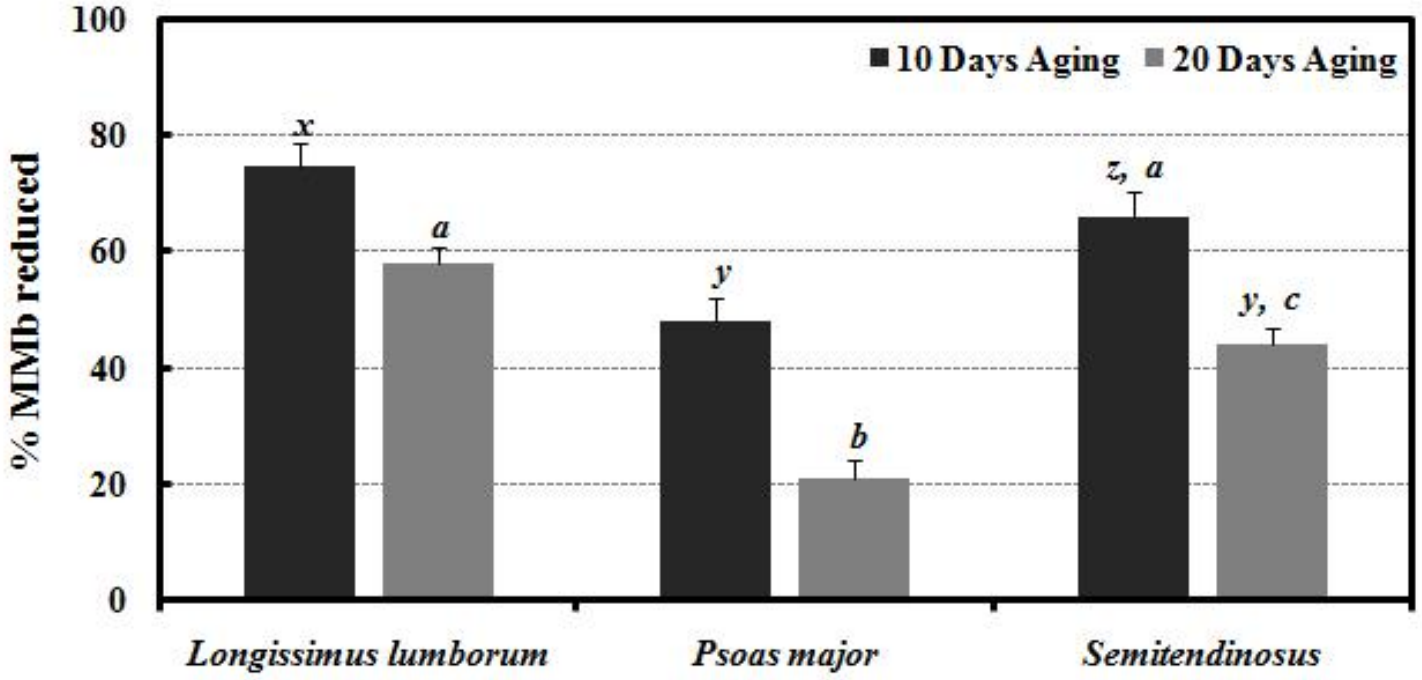

Figure 3.2: *Metmyoglobin-reducing activity (MRA) of three bovine muscles during 10 and 20 days aging times. LL = longissimus lumborum, PM = psoas major, ST = semitendinosus. MRA, or % MMb reduced, was calculated as (Δ % surface MMb/preincubation % surface MMb) × 100. x, y, z = means for different muscle types within 10 days aging time with a different letter differ ($p < 0.05$). a, b, c = means for different muscle types within 20 days aging time with a different letter differ ($p < 0.05$).*

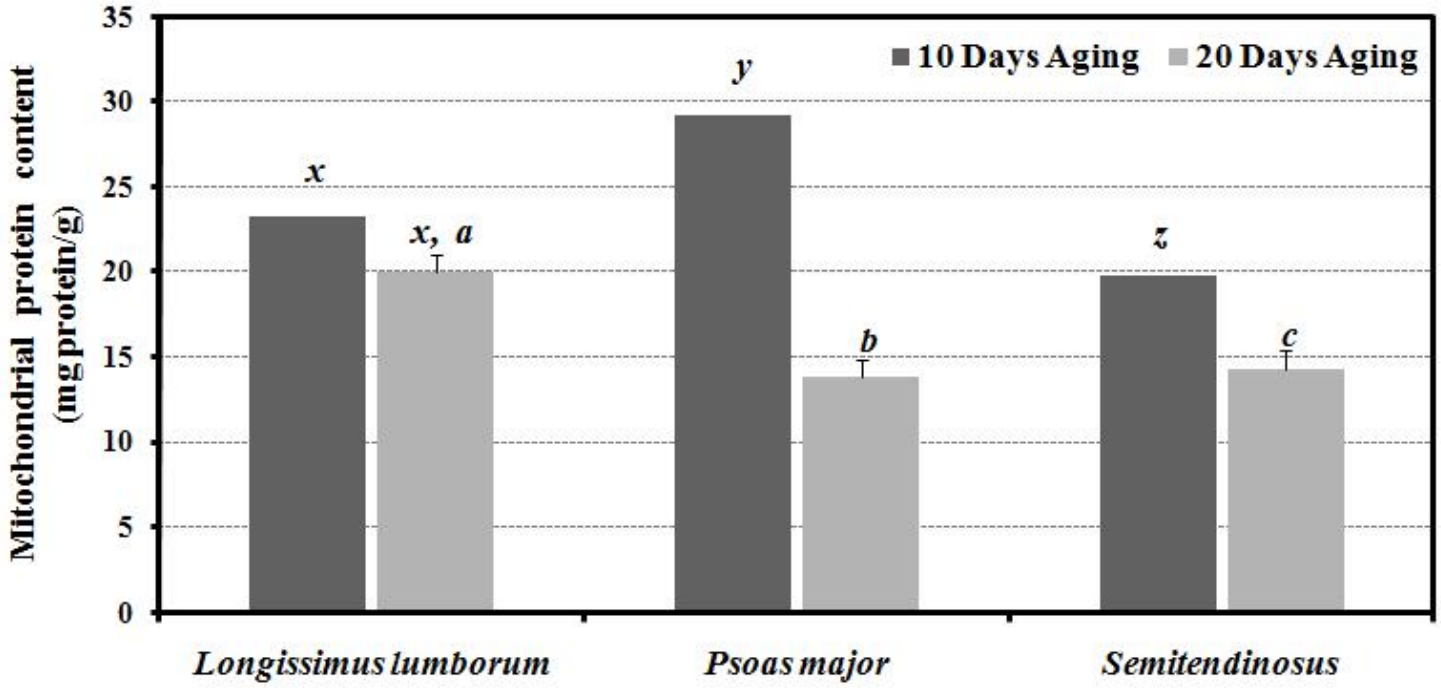

Figure 3.3: *Cytoplasmic protein content for Longissimus lumborum (LL), Psoas major (PM), and Semitendinosus (ST) of beef skeletal muscle aged for 10 and 20 days.*

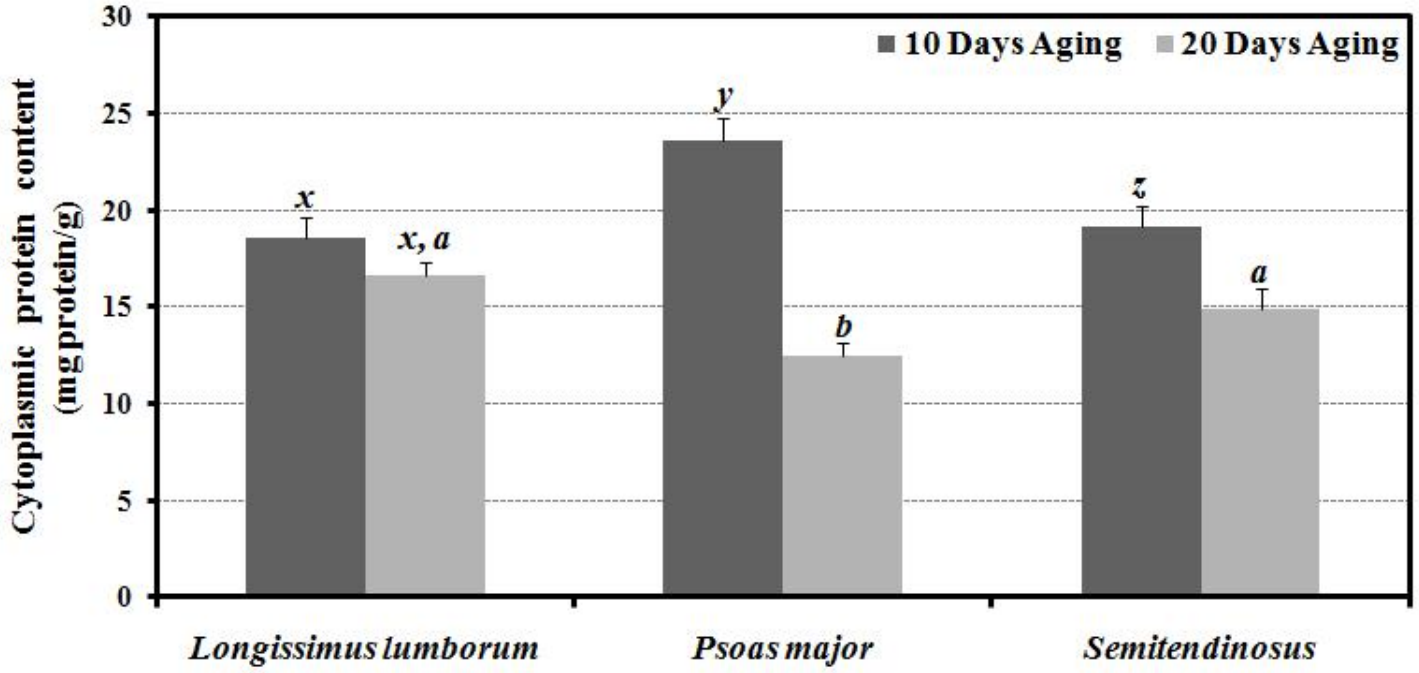

Figure 3.4: *Mitochondrial protein content for Longissimus dorsi (LL), Psoas major (PM), and Semitendinosus (ST) of beef skeletal muscle aged for 10 and 20 days.*

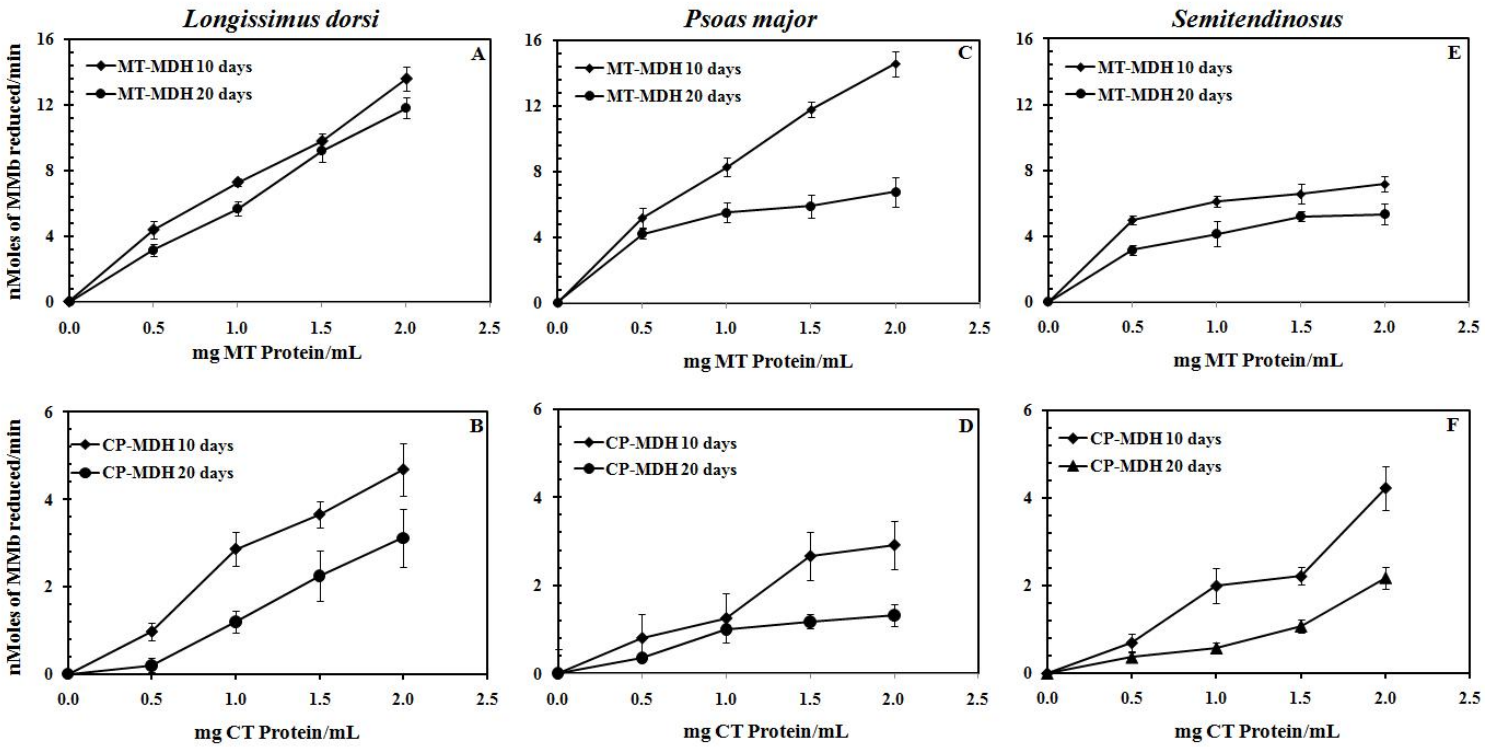

Figure 3.5: *Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic malate dehydrogenase (CP-MDH & MT-MDH). Concentration effects of the amount of the individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (Longissimus lumborum [LL], Psoas major [PM] and Semitendinosus [ST]), on the MRA of beef skeletal muscle's mitochondrial and cytoplasmic isolates.*

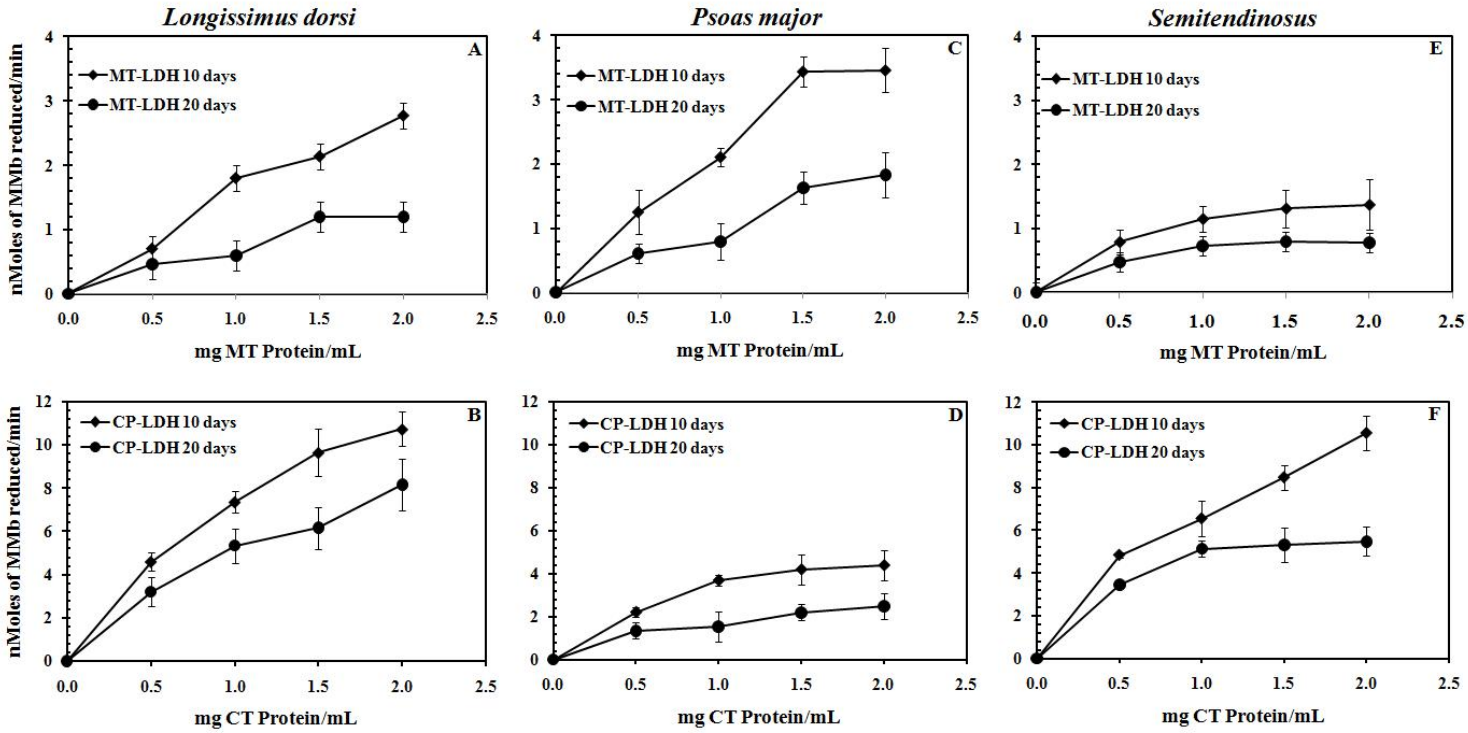

Figure 3.6: *Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic lactate dehydrogenase (c-LDH & m-LDH). Concentration effects of the amount of the individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (Longissimus lumborum [LL], Psoas major [PM] and Semitendinosus [ST]), on the MRA of beef skeletal muscle's mitochondrial and cytoplasmic isolates.*

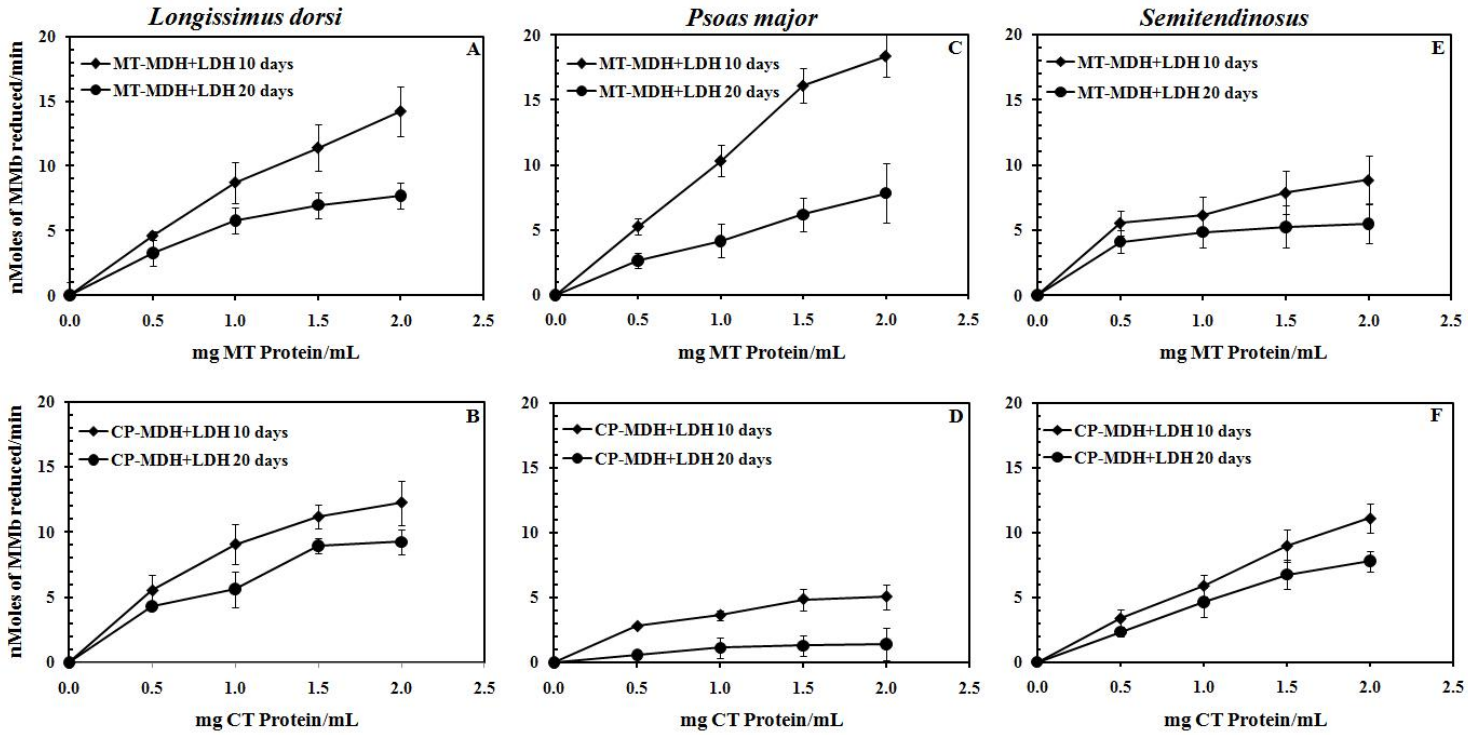

Figure 3.7: *Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic lactate and malate dehydrogenases (CP-LDH, CP-MDH & MT-LDH, MT-MDH). Concentration effects of the amount of the individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (Longissimus lumborum [LL], Psoas major [PM] and Semitendinosus [ST]), on the MRA of beef skeletal muscle's mitochondrial and cytoplasmic isolates*

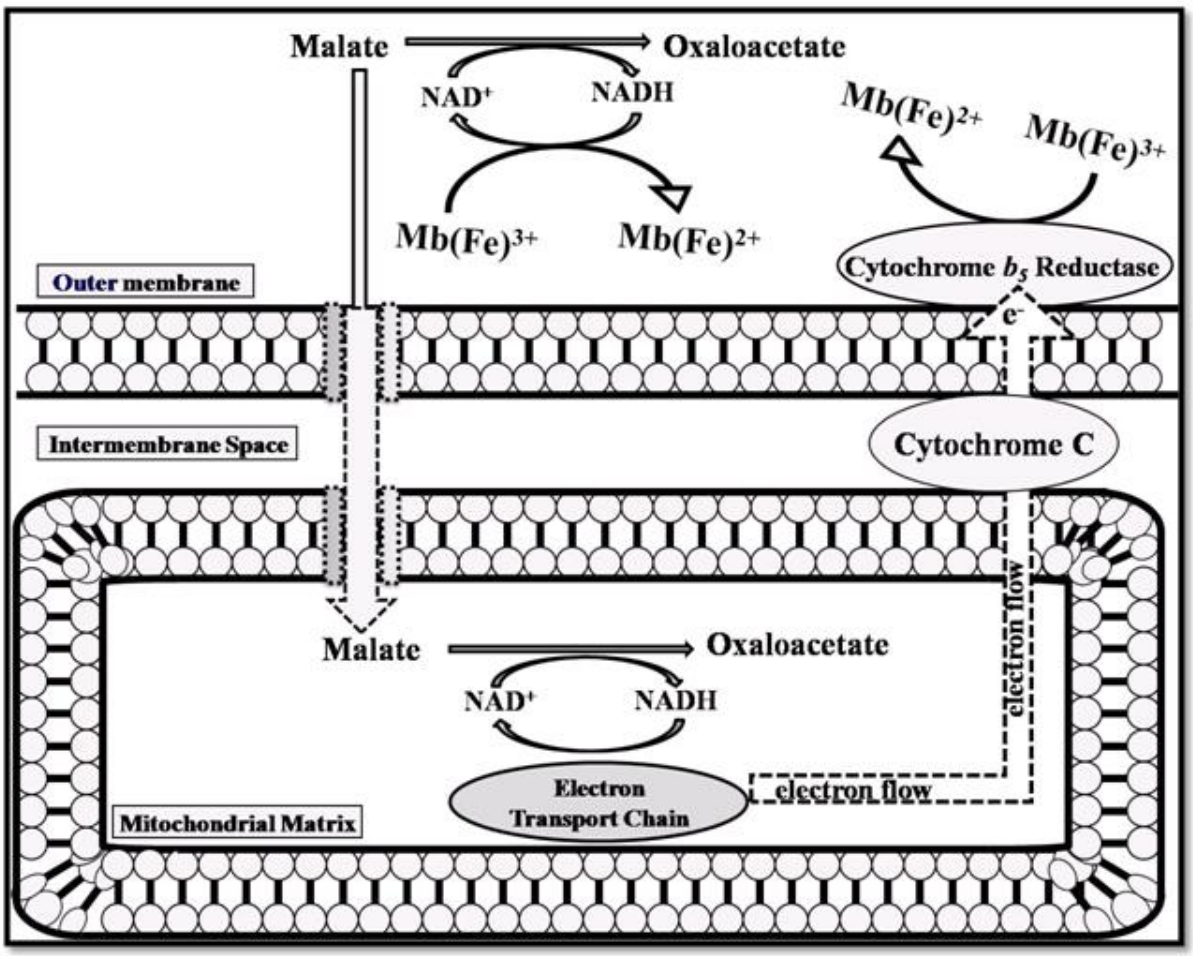

Figure 3.8: *Proposed model and biochemical pathways for enzymatic and nonenzymatic reduction of metmyoglobin and depiction of the NADH regeneration via cytoplasmic and mitochondrial malate-MDH activity. The entry of malate into the mitochondrial matrix is facilitated by malate-aspartate shuttle. Mitochondrial MDH (MT-MDH), oxidizes malate to oxaloacetate regenerating postmortem pool of NADH and further oxidation of NADH via electron transport chain (ETC) ultimately reducing metmyoglobin localized near mitochondrial outer membrane (a site of low oxygen partial pressure causing formation of metmyoglobin).*

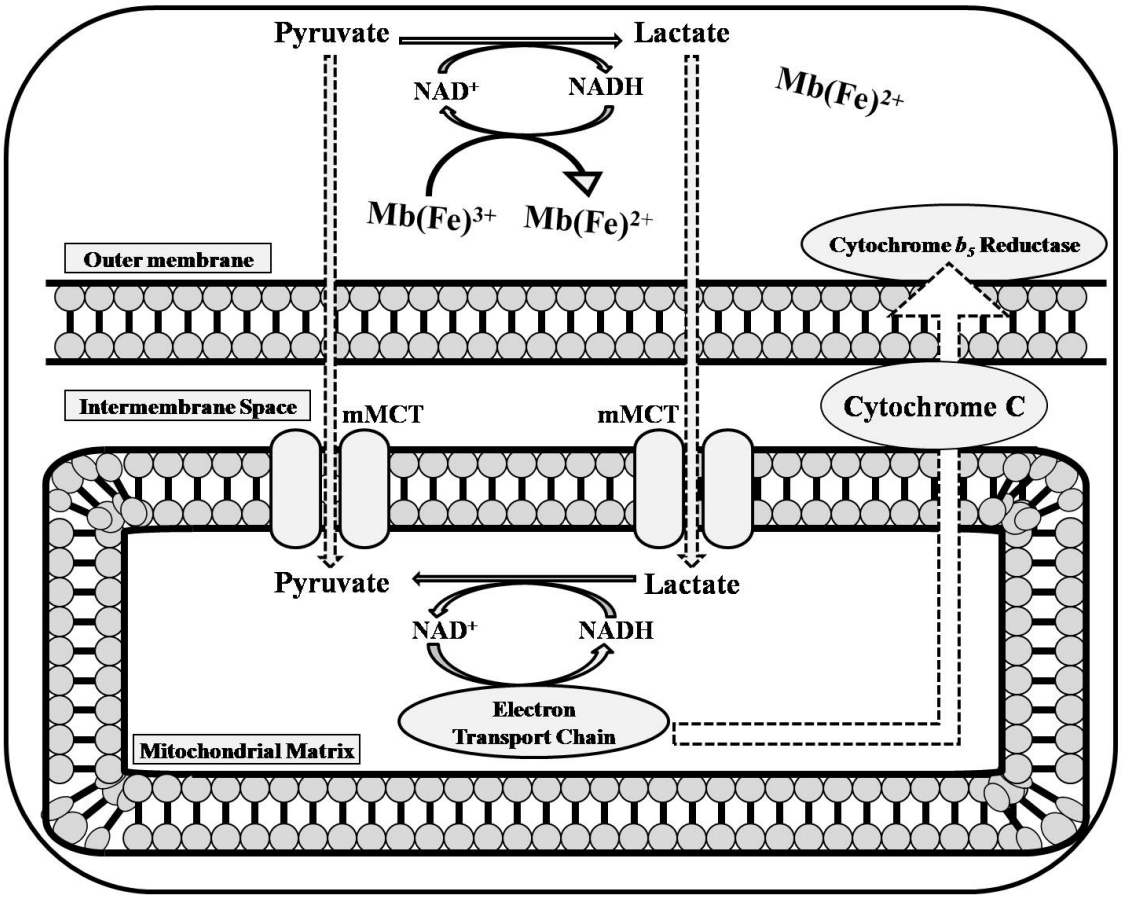

Figure 3.9: *Proposed model and biochemical pathways for enzymatic and nonenzymatic reduction of metmyoglobin and depiction of the NADH production between cytoplasmic and mitochondrial LDH. The entry of lactate and pyruvate into the mitochondrial matrix is facilitated by mMCT. There, lactate is oxidized to pyruvate via mitochondrial LDH (MT-LDH) regenerating postmortem pool of NADH with changes in mitochondrial redox potential, and the produced NADH is further oxidized via electron transport chain (ETC) ultimately reducing metmyoglobin localized near mitochondrial outer membrane (a site of low oxygen partial pressure causing formation of metmyoglobin).*

Table 3.1: *Least-squares means*[b] *for pH, moisture, protein, and fat of three beef muscles*

Trait	muscles[a]			
	LL	PM	ST	SE[c]
pH	5.8^{x}	5.6^{x}	5.7^{x}	0.04
Moisture(%)	71.3^{x}	71.2^{x}	74.1^{y}	0.12-0.47
Crude protein(%)	23.6^{z}	21.9^{x}	22.4^{y}	
Fat(%)	4.2^{y}	1.4^{x}	1.5^{x}	
Ash(%)	5.1^{x}	5.5^{x}	2.0^{y}	

[a] LL = *longissimus thoracis*, PM = *psoas major*, ST = *semitendinosus*

[b] ^{xz}Means in a row with a different letter differ ($p < 0.05$).

[c] Standard error.

Chapter 4

Effects of Lactate, Malate, and Pyruvate on Myoglobin Redox Stability of Three Bovine Muscles

4.1 Abstract

We investigated the effects of three glycolytic and tricarboxylic acid cycle metabolites on myoglobin redox forms and their influence on meat color stability. Eighteen combinations of malate (M), lactate (L), and pyruvate (P) were added to beef *Longissimus lumborum*, *Psoas major*, and *Semitendinosus* muscle homogenates to study their effect on metmyoglobin formation during incubation at 25 °C. Changes in surface color at 0, 2, 4, 8, and 12 hrs were evaluated using reflecto-spectrophotometry [both $L^*a^*b^*$ and wavelengths specific for metmyoglobin (MMb)]. Addition of M, L, and P either alone or in combinations stabilized ($p < 0.05$) $L^*a^*b^*$ values and myoglobin redox forms in muscle homogenates; however there was a trend for P to be least effective. At the 2% concentrations for the individual metabolites, the most effective metabolite at retarding MMb formation was L > M > P in

the ST, and M > L > P in the PM and LL muscles. MMb was reduced most effectively with combination of metabolites where M+L > M+P > L+P. Enhancement of meat with these metabolites can effectively extend color life of post-rigor meat apparently by providing more reducing conditions for myoglobin, thus increasing myoglobin redox form stability.

Key words: Meat color; Myoglobin redox forms; Malate, Pyruvate, Lactate

4.2 Introduction

Acceptability of meat by consumers is frequently judged by its visual appearance, with a bright cherry-red color being preferred. Once meat discolors, the product losses retail value and the retailers have to either discount the price or discard the product. MacKinney *et al.*, (1966) suggested that the visual appearance of meat products determines whether a consumer will make a purchase or not. It is generally accepted that color is one of the most important visual attributes in the selection of meat products, and the single greatest factor determining the purchase of meat at retail (Hedrick *et al.*, 1994; Kropf, 1980).

The increased demand for central packaging of case-ready meat products has led to the application of new processing protocols that enhance the quality of meat products and extends shelf life (Kropf, 1980; Madhavi & Carpenter, 1993; Ramanathan *et al.*, 2009). However, the effects of non-meat ingredients in enhancement fresh meat color stability are not fully understood. Mancini & Ramanathan (2009) suggested that addition of certain glycolytic and tricarboxylic acid cycle (TCA) metabolites (as non-meat ingredients) may involve postmortem processes that increase myoglobin redox stability.

Kim *et al.*, (2006) reported that beef *longissimus* steaks enhanced with 2.5% lactate exhibited higher lactate dehydrogenase (LDH) and metmyoglobin reducing activity (MRA) and contained significantly more NADH than treatments without lactate. These researchers concluded that lactate-mediated LDH activity likely contributed to the increased NADH and

MRA. Ramanathan *et al.*, (2009) reported that combining lactate, LDH, and NAD with bovine mitochondria increased oxygen consumption and suggested that lactate addition to beef cardiac mitochondria can generate NADH via LDH activity, and that the NADH can be utilized to reduce metmyoglobin. Saleh & Watts (1968) suggested that addition of certain glycolytic and TCA to meat may be associated with regeneration of NADH and an improved MRA. Some early work noted that enzymes of glycolysis and the TCA cycle remain active in postmortem muscle, and could be used as a possible source for replenishment of the postmortem pool of reducing equivalents for metmyoglobin reduction (Andrews *et al.*, 1952; Bodwell *et al.*, 1965). Jerez *et al.*, (2003) noted that NAD-NADH metabolism and color stability could be related to glycolytic components such as glyceraldehyde 3-phosphate oxidation, pyruvate reduction, and enolase activity.

Although, it is generally accepted that fresh meat color life is associated with the maintenance of a bright-cherry red color, the effectiveness of combinations of glycolytic and TCA metabolites for mediation of meat color during both storage and display needs more focused research. Thus, our objectives were to assess the effects of lactate, pyruvate, and malate alone or in combination on myoglobin redox stability during an accelerated display and storage.

4.3 Materials and Methods

4.3.1 Chemicals.

Sodium malate and sodium pyruvate was obtained from Sigma Chem. Co. (St. Louis, MO). Potassium lactate was obtained from PURAC America, Inc., (PURASAL HiPure P, 60% potassium lactate/40% water) Lincolnshire, IL.

4.3.2 Raw Materials.

Five beef carcass sides representing different animals (USDA Select, A-maturity, with normal color and pH (5.61 to 5.77) and no obvious quality defects) were selected randomly 2 d postmortem at a commercial abattoir. From each carcass side, the *longissimus lumborum* (LL), *psoas major* (PM), and *semitendinosus* (ST) muscles were obtained and stored in vacuum packages in the dark for 10 days at 2 ± 1 °C.

4.3.3 Preparation of Skeletal Muscle Homogenates

Twenty-five grams (devoid of fat and connective tissue) of minced bovine skeletal muscle from each muscle (n= 5) were homogenized as one of eighteen treatments, each at 50 mL containing phosphate buffer, malate, lactate, pyruvate, malate + lactate, malate + pyruvate, and lactate + pyruvate. Samples were homogenized using a variable speed PowerGen Homogenizer (PowerGen Homogenizer, Model 35, Fisher Scientific, Park Lane Drive, Pittsburgh, PA) for 25 s.

4.3.4 Color and pH Measurement

Homogenates were poured into sterilize polystyrene petri dishes (60mm + 15mm) and covered with a clear, snug fitting lid. Instrumental color measurements were measured immediately after samples were poured into the petri dishes. Immediately after the samples were poured into the petri dishes, CIE L^*, a^*, and b^* values and wavelengths from 400 to 700 nm values were collected using a HunterLab MiniScan XE Plus Spectrophotometer (D/8-S, 45/0 LAV, 14.3 mm diameter aperture, 10 ° standard observer, Illuminant A; Hunter Associates Laboratory, Inc., Reston, VA, USA) stabilized by a stand that allowed the dishes to sit on the inverted spectrophotometer with the aperture upward. A black tile (Hunter Associates Laboratory, Inc., Reston, VA) was placed over the dishes and scanned at 2 hr intervals

during 12 hr of incubation at 25 °C. Estimation of MMb was computed using reflectance data from 400-700 nm converted to K/S values and ratios of wavelengths at 525 and 572 were inserted into equations with values for the samples and 0% and 100% of the pigment forms necessary for MMb quantification (Hunt *et al.*, 1991).Sample pH was recorded using an Accumet combination glass electrode connected to an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ). The pH of each treatment solution added to the minced muscle also was measured.

4.4 Statistical Analysis

The experimental design was a randomized complete block with repeated measurements and each experiment was replicated 5 times. The eighteen treatments within the randomized complete block included a control and one of eighteen treatments. Each of the eighteen treatments was assigned once within a replicate. Color measurements were taken repeatedly on the same experimental unit during the incubation period. Duplicate color measurements taken on the same experimental unit were averaged for statistical analysis. Fixed effects included treatment, incubation time, and their interaction. Type-3 tests of fixed effects for changes in L^*, a^*, and b^* during incubation were evaluated using the Mixed procedure of SAS. Least square means were generated for significant F-tests ($p < 0.05$) and separated using least significant differences.

4.5 Results

The individual metabolites, combinations of metabolites, and the levels of the metabolites are shown in Table 4.1. Because of the number of treatments (n=18), superscripts are not shown in the figures and tables; however the standard errors are listed in the captions or

footnotes can be used to rationalize significant differences. Detailed analysis of the data indicated that the least magnitude for a significant difference for the spectrophotometric traits and MMb percentages was generally 1.5 of the standard errors.

4.5.1 Instrumental color effects of homogenates containing individual metabolites

Lightness, L^* values

In the LL muscle homogenates (Figure 4.1A) that contained M and P at either concentration (1 or 2 %) and 1L, there were no significant differences in the L^*-values during 12 hr of incubation. However, L^*-values for 2L decreased (became darker) at 2 hr, but then held constant, whereas the controls progressively decreased after 4 hr of incubation. The PM muscle had the smallest (darkest) L^*-values of the three muscles (Figure 4.1D). L^* values for homogenates containing all metabolites except 1P were not different ($p > 0.05$) during the 12 hr incubation, but the 1P homogenates became lighter after4 hrs. Control homogenates were lightest in color initially but became darker after 4 hrs. L^* values for the ST muscle (Figure 4.1G) were more variable and generally higher than those for the LL and PM. Control homogenates for the ST were the lightest initially, but they became progressively darker as incubation time increased. Homogenates containing 1L and 1 and 2M were the most stable over time. Those with 2L and 1 and 2 P decreased significantly compared with the control and other metabolite compositions for 6 hr and then were reasonably stable.

Redness, a^* values

LL Homogenates (Figure 4.1B) containing 2M were the most red and those with 2L were intermediate for all metabolites up to 8 hr of incubation. After 12 hr of incubation, the 2m and 2L were most red, 2P was intermediate, and those with 1% M, L or P were the least

red. Control homogenates were less red compared with all metabolite homogenates at all times except 0 hr. For PM homogenates (Figure 4.1E), a^* values decreased ($p < 0.05$) for all treatments from 0 to 2 hr. At 4hr, homogenates with 1L and the control were slightly less red. At 6 hr of incubation, the control, 1L and 1P were less red than all other metabolite homogenates. At the end of incubation, homogenates with 1 and 2% M and 2L were the reddest, those with 1L and 2P were intermediate, and the control, and 1P were least red. The a^* values of ST homogenates with 1 and 2% M were the reddest, those with 1 and 2% L were intermediate, and the control and both P homogenates were least red (Figure 4.1H). Among all three beef muscles, the a^* values was highest initially in the PM, but the homogenate color declined the faster compared with the LL and ST. However, the a^*-values for ST treated with 2M were more stable as compared with LL and PM during the incubation period of 0 to 12 hrs.

Yellowness, b^* values

Overall, b^* values were the highest for the LL, intermediate for the ST, and slightly lower for the PM. The most stable b^* values occurred in the LL, whereas the PM and ST values declined more during incubation. In all muscles, b^* values for the control homogenates declined ($p < 0.05$) at a faster rate and to a greater extent than all other homogenates.

4.5.2 Instrumental color effects of homogenates containing combinations of metabolites

Lightness, L^* values

Among the three muscles, the L^* values for the controls were the lightest in color (although not always significant) compared with the combination treatments. If addition, L^* values for metabolite combinations (Figure 4.2A and D and Figure 4.3A, D, and G) were fairly

consistent in magnitude over incubation time. However, the ST muscle (Figure 4.2G) was more variable across metabolite combinations where the 1M+1L and 2M+2L were the most stable in lightness; the 1L+1P was intermediate, and the least stable in lightness were the 1M+1P, 2M+2P, and 2L+2P combinations. As expected, the PM homogenates were the darkest ($p < 0.05$) and the LL and ST were lighter. Addition of any of the metabolites seemed to lower L^* values regardless of the muscle and when the L^* values decreased, the extend of change was greatest from 0 to 4 hr of incubation.

Redness, a^* values

Initial a^* values were higher ($p < 0.05$) for the PM than either the LL or ST muscles. The greatest and most consistent declines in a^* values occurred in the PM, whereas the LL and ST were similar in extent of loss of redness. Metabolite combinations that maintained a^* values the most were 2M+2L (LL), 2M+2P and 2M+2L (PM), and 2M+2L (which increased redness from 6 to 12 hr) and 1M+1L (ST). This trend agreed with that of single metabolites (Figure 4.1B, E, and H) where M and L at either 1 or 2% were most effective in stabilizing a^* values.

Yellowness, b^* values

Initial values for b^* were similar for all three muscles (Figures 4.2 and 4.3, panels C, F and I). The declined in b^* values were greatest for the PM and the LL and ST were similar over the 12 hr incubation. Metabolite combinations that stabilized PM b^* values were 2M+2L, 2M+2P, 1M+1P, and 1M+1L, which agreed with a^* stabilization in the PM. However, metabolite combinations that stabilized b^* values most in the LL and ST were 1M+1P, 1L+1P and 1M1L (LD and ST) generally were opposite to those that were most stabilizing for a^* values in the LL and ST muscles.

4.5.3 Effects of individual metabolites on metmyoglobin formation

The percentages of metmyoglobin formation in the three muscles during 12 hr of incubation with metabolites are in Tables 4.1 (LL), 4.2 (PM), and 4.3 (ST). The initial percentages for MMb in control homogenates of the three muscles ranged from 14.6 to 21.6. The averages of the six individual metabolite samples at 0 hr were 17.6 for the LL, 18.4 for the PM, and 15.4 for the ST; none of these averages were significantly different from their respective controls and the only individual metabolite sample that was significantly higher at 0 hr was the % Malate treatment from the ST muscle. Thus, it appears that the control and individual treatment samples started at an equal level of MMb initially.

As incubation time increased, MMb levels increased for the control and individual metabolite samples (Tables 4.1 (LL), 4.2 (PM), and 4.3 (ST)). The rate of MMb formation was the fastest and most extensive for the PM muscle where MMb was over 92% at 8 hr of incubation compared with 79% for the LL and 63% for the ST. Control samples after 12 hr of incubation for the three muscles were over 82% MMb. At 2% inclusing, M, L, and P were more effective ($p < 0.05$) in delaying MMb accumulation over time of all three muscles compared to the individual metabolites at 1%, which had 1.5 to 3 times greater MMb than the 2% levels. At the 2% concentrations for the individual metabolites, the most effective metabolite at retarding MMb formation was L > M > P in the ST, and M > L > P in the PM and LL muscles.

4.5.4 Effects of combinations of metabolites on metmyoglobin formation

Combinations of M, L, and P (Tables 4.1, 4.2, and 4.3) followed the same pattern as the individual homogenate compositions in that MMb increased as incubation time increased

albeit at different rates and extents. When the treatments concentrations were 2%, the M+L slowed MMb accumulation the most ($p < 0.05$), M+P was intermediate ($p < 0.05$), and L+P was least repressive ($p < 0.05$) for MMb; however, all of these compositions retarded MMb formation greater ($p < 0.05$) than all other treatment combinations at 1% levels for both metabolites, at 1%+2% combinations, and 2%+1% levels. Treatment compositions containing 1 or 2% P generally had the greatest levels of MMb formation.

4.6 Discussion

Jerez *et al.*, (2003) suggested that inhibition of postmortem glycolysis could alter NADH content and therefore, effect color stability. In their study, beef muscles treated with citrate tended to have more NADH than control samples. Saleh & Watts (1968) indicated the possible involvement of malate and glutamate in metmyoglobin reduction via their participation in pathways that regenerate NADH. Kropf, (1980) indicated that during the retail display of meat, reduction of MMb requires NADH for the metmyoglobin reducing activity that utilizes NADH. Andrews *et al.*, (1952) noted that enzymes involved in glycolysis, the tricarboxylic acid cycle, and the electron transport chain, remain active in postmortem muscle capable of producing reducing equivalents. Saleh & Watts (1968) suggested a reductive pathway through which intermediates of the glycolytic and tricarboxylic acid pathway can be used for the production of NADH that is eventually used for MMb reduction. The interest in using endogenous enzyme systems that utilize numerous ingredients as a metabolite for NADH regeneration has potential for adding value, extending product shelf-life, and stability of meat color.

Messer, Jackman & Willis, (2004) suggested that malate accounts for the majority of the carbon in tricarboxylic acid cycle intermediates. They further reported that malate and pyruvate are the most limiting substrates in skeletal muscles and provided evidence that

the addition of malate and pyruvate were able to support oxidative energy production in isolated skeletal muscle mitochondria (Baldwin, Hooker, & Herrick, 1978). In this study, combination of malate and lactate were more effective in maintain the redness of both the *Longissimus* and *Psoas* muscles homogenates during the accelerated incubation and display (Figure 4.2). Differences in color stability between the *Longissimus*, *Semitendinosus*, and *Psoas major* have been attributed to their intrinsic muscle-to-muscle variation in MMb reducing activity and endogenous enzyme activity (Ledward, 1985; McKenna *et al.*, 2005). As a result, the effects of non-meat metabolites on myoglobin redox stability could be muscle-specific (Claus *et al.*, 2005). In the current project, muscle effects on color stability were dependent on packaging type (Table 4.1).

The main focus of this investigation was to develop an understanding of how addition of metabolic intermediates such as malate, lactate, and pyruvate affect myoglobin redox stability. Reflectance color measurements and estimates of MMb formation during the incubation of muscle tissue homogenates from the LL, ST, and PM muscled clearly indicated that addition of malate alone and in combination in with lactate were capable of significant improvements in stabilization of myoglobin redox forms. Our data for MMb formed in homogenates clearly show that individual and combinations of M, L and P were effective inhibitors of MMb. Individual metabolites at 1% were not as effective as the 2% level. Moreover, combination ingredients, especially at 2% levels for both metabolites, were better in stabilizing color as compared with other combinations at 1%+2% or 2%+1% levels.

This study clearly supports the suggestion by Mancini & Ramanathan (2009) that the interrelationship between non-meat metabolites and myoglobin redox stability merits further investigation. M, L and P are glycolytic and/or mitochondrial metabolites and these and perhaps others in these two biochemical pathways appear to have potential to improved color stability of meat during retail display. Tang *et al.*, (2005ab) reported that succinate added to bovine mitochondria can be used by Complex II within the electron transport

chain; producing electrons that can be donated to metmyoglobin via cytochrome c. Thus, metabolites such as M, L and P have the potential to produce NADH postmortem which should improve myoglobin redox stability.

Research by Kim *et al.* (2006) reported that nonenzymatic reduction of MMb occurred in a model system containing L, LDH, and NAD and the deletion of one of the components from the system limited MMb reduction. Several studies show that color stabilizing effects due to enhancement of meat with NaL or KL. (Kim *et al.*, 2009a; Mancini & Ramanathan, 2008ab). Additionally, Kim *et al.*, (2009b) assessed effects of enhancement solutions containing containing phosphate and/or calcium lactate on beef strip loins packaged in high-oxygen (80% O_2) modified-atmosphere packaging, and/or irradiated at 2.4 kGy. These authors reported that beef strip loins enhanced with calcium lactate and phosphate maintained the most stable red color, demonstrated increased NADH concentration, and were the least oxidised. Among irradiated steaks, calcium lactate with phosphate treatment significantly minimized lipid oxidation, and had a higher a^* value. Their study concluded that lactate inclusion improves color stability of fresh beef by providing superior antioxidant capacity and increased reducing activity of myoglobin by elevating NADH concentration.

4.7 Conclusion

Postmortem enhancement of beef subprimal cuts containing the LL, PM, and ST muscles with biochemical intermediates as substrates for enhancing NADH formation will lead to increased myoglobin color stability. Malate and/or L at 2% level will be more effective than combinations including pyruvate in improving the color stability during retail storage and display. Our data for MMb formed in homogenates clearly show that individual and combinations of M, L and P will be effective inhibitors of MMb. Individual metabolites at 1% will not as effective as at 2%. Moreover, combination ingredients, especially at 2% levels

for both metabolites, will stabilize color better than combinations at 1%+2% or 2%+1% levels of L and P when combined with M. Furthermore, using 2% each of two metabolites will be better than only using 1% or combinations with other intermediates. Enhancement of meat with these metabolites can effectively extend color life of post-rigor meat apparently by providing more reducing conditions for myoglobin, thus increasing myoglobin redox form stability.

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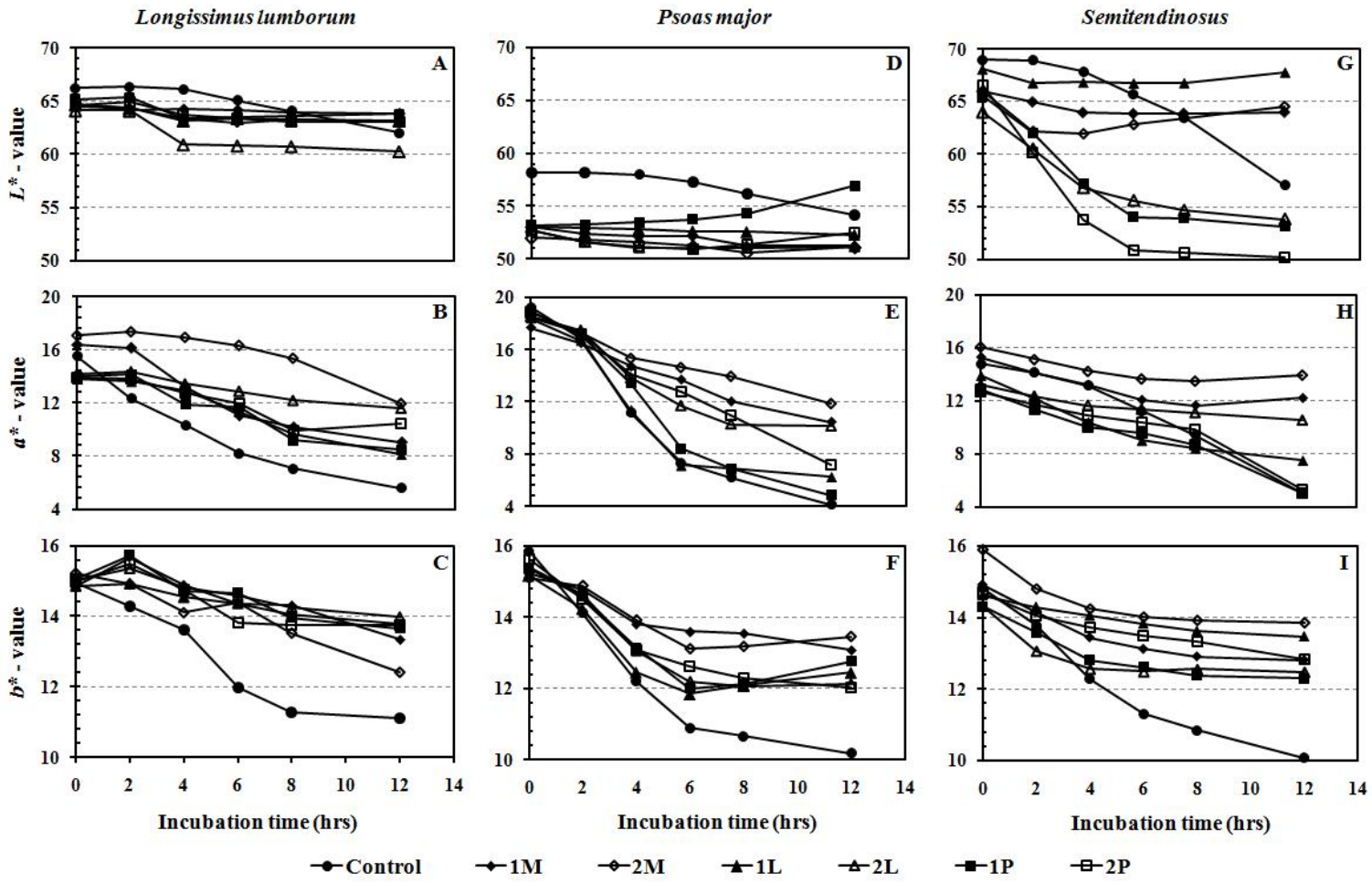

Figure 4.1: *Muscle type × display effects of different levels of individual metabolites[a] on color properties (L^*, a^*, and b^*) of bovine Longissimus lumborum (A, B, and C), Psoas major (D, E, and F), and Semitendinosus (G, H, and I) muscles during a 12 hr incubation at 25 °C. Larger values for L^*, a^*, and b^* indicate a lighter, redder, or more yellow color, respectively. Standard error for differences of Least-squares means = 1.8.* [a] *1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+2L = 1% malate and 2% lactate*

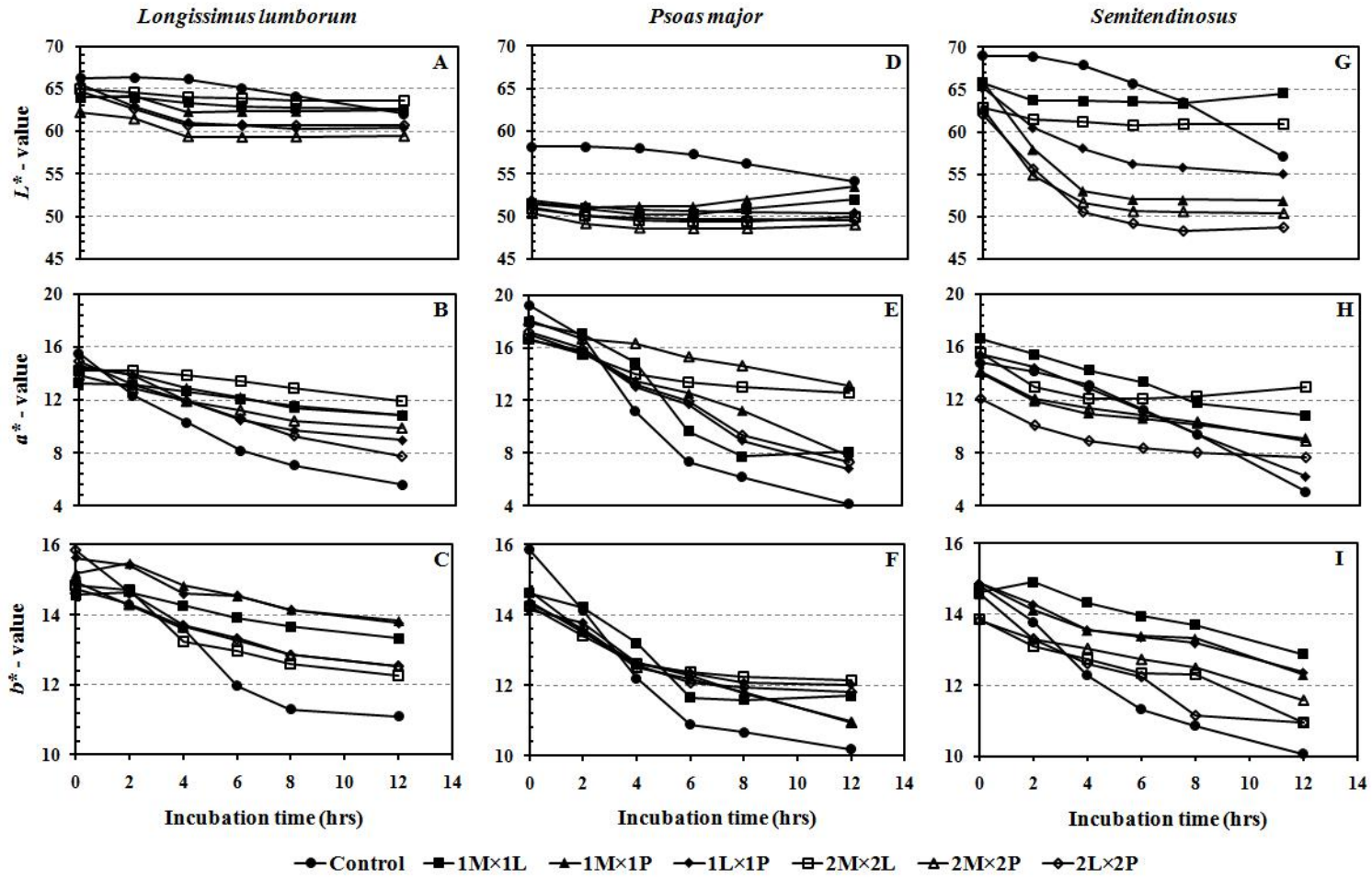

Figure 4.2: *Muscle type × display effects of different metabolite combinations[a] on color properties (L^*, a^*, and b^*) of bovine Longissimus lumborum (A, B, and C), Psoas major (D, E, and F), and Semitendinosus (G, H, and I) muscles during a 12 hr incubation at 25 °C. Larger values for L^*, a^*, and b^* indicate a lighter, redder, or more yellow color, respectively. Standard error for differences of Least-squares means = 2.1. [a]1M + 1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L + 1P = 1% lactate and 1% pyruvate; 2M +2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate*

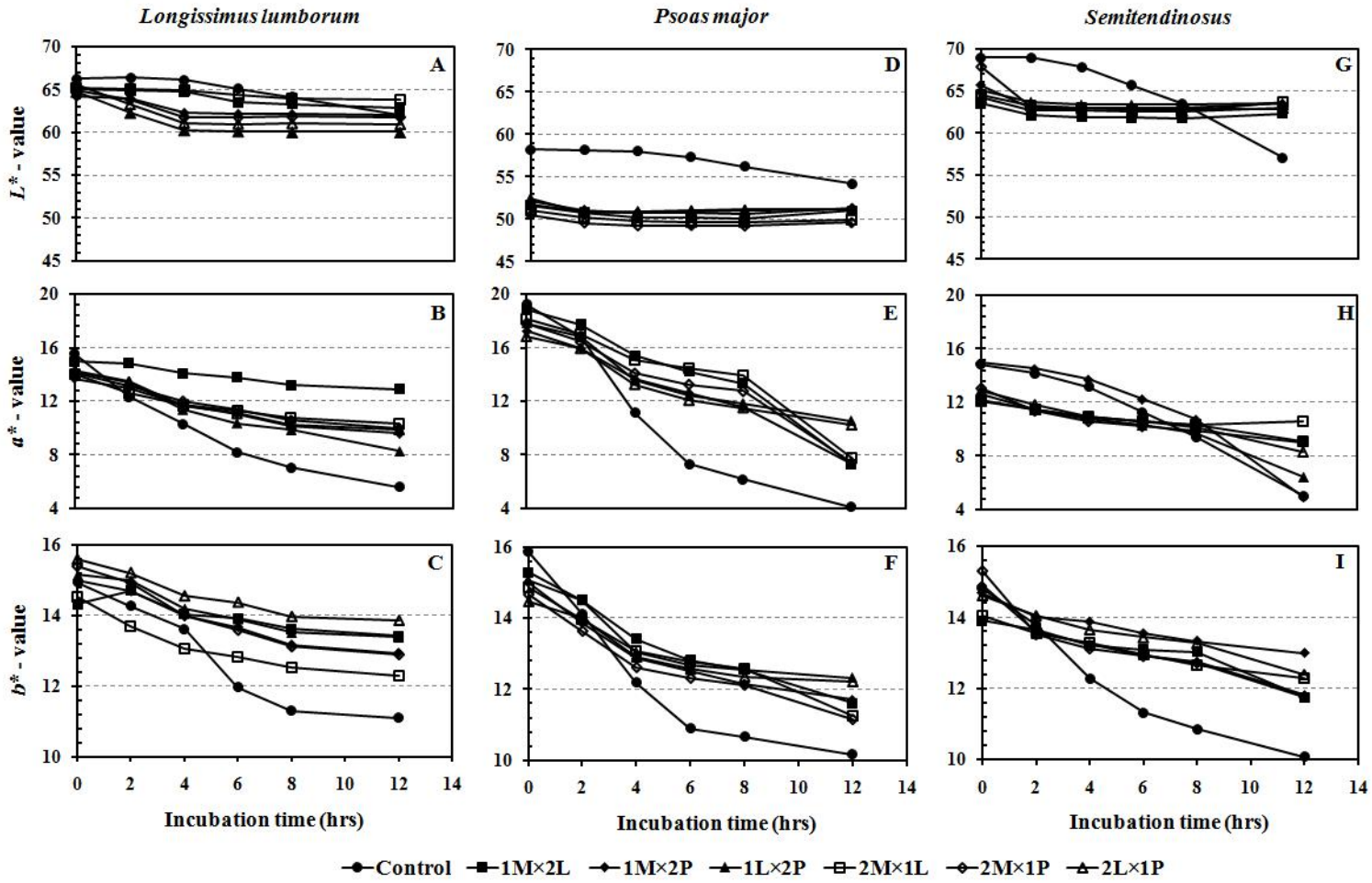

Figure 4.3: *Muscle type × display effects of different metabolite combinations[a] on color properties (L^*, a^*, and b^*) of bovine Longissimus lumborum (A, B, and C), Psoas major (D, E, and F), and Semitendinosus (G, H, and I) muscles during a 12 hr incubation at 25 °C. Larger values for L^*, a^*, and b^* indicate a lighter, redder, or more yellow color, respectively. Standard error for differences of Least Square Means = 1.4. [a] 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.*

Table 4.1: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Longissimus lumborum muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	21.6	45.4	58.3	79.4	83.4	85.4
Malate	1M	15.2	19.9	24.2	38.3	52.3	66.3
Lactate	1L	19.0	22.6	26.5	44.8	61.8	68.8
Pyruvate	1P	19.6	23.4	33.3	48.4	63.4	76.4
Malate	2M	18.6	27.4	34.7	31.2	35.2	42.2
Lactate	2L	17.7	22.7	22.3	29.7	41.2	47.8
Pyruvate	2P	15.3	21.9	28.2	39.1	47.9	54.9
Malate + Lactate	1M+1L	15.2	19.9	26.9	38.3	48.3	57.3
Malate + Pyruvate	1M+1P	19.0	21.6	28.5	45.6	55.7	78.8
Lactate+ Pyruvate	1L+1P	19.6	23.4	33.3	48.4	63.4	76.4
Malate + Lactate	2M+2L	8.6	11.4	12.1	13.2	17.2	24.2
Malate + Pyruvate	2M+2P	17.7	22.7	22.3	27.2	29.2	33.2
Lactate+ Pyruvate	2L+2P	15.3	25.9	28.2	32.4	40.2	47.3
Malate + Lactate	1M+2L	29.2	29.9	31.9	37.3	45.3	51.3
Malate + Pyruvate	1M+2P	17.5	24.2	28.9	41.8	54.8	63.8
Lactate + Pyruvate	1L+2P	29.6	31.4	33.3	48.4	51.4	63.4
Malate + Lactate	2M+1L	28.6	26.4	28.5	33.2	39.4	42.2
Malate + Pyruvate	2M+1P	27.7	26.2	22.3	31.8	37.2	41.2
Lactate+ Pyruvate	2L+1P	15.3	21.9	23.2	26.9	33.9	36.9

[a] Standard error for muscle type + incubation and display of muscle = 1.34 - 2.17.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.

Table 4.2: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Psoas major muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	17.6	39.4	64.3	92.4	94.4	96.4
Malate	1M	20.2	19.9	44.9	72.3	72.3	72.3
Lactate	1L	19.0	27.6	46.5	74.8	74.8	74.8
Pyruvate	1P	19.6	43.4	53.3	78.4	83.4	86.4
Malate	2M	18.6	29.4	34.9	33.2	39.2	42.7
Lactate	2L	17.7	26.7	35.3	47.2	51.6	53.2
Pyruvate	2P	15.3	26.3	38.2	51.9	58.9	63.9
Malate + Lactate	1M+1L	15.2	33.9	43.2	57.3	63.3	67.3
Malate + Pyruvate	1M+1P	16.6	31.6	48.5	65.6	75.7	78.8
Lactate+ Pyruvate	1L+1P	19.6	38.4	47.3	77.4	83.4	87.4
Malate + Lactate	2M+2L	28.6	21.4	16.1	13.2	15.2	22.2
Malate + Pyruvate	2M+2P	11.7	22.7	22.3	26.2	39.2	47.2
Lactate+ Pyruvate	2L+2P	15.3	35.9	38.2	47.4	55.8	58.3
Malate + Lactate	1M+2L	18.2	39.9	44.9	52.3	62.3	72.3
Malate + Pyruvate	1M+2P	15.0	37.6	53.5	64.8	67.8	71.3
Lactate + Pyruvate	1L+2P	14.3	33.4	49.7	58.4	63.4	76.4
Malate + Lactate	2M+1L	18.6	23.4	31.9	33.2	36.2	42.7
Malate + Pyruvate	2M+1P	12.7	16.7	25.3	37.2	41.6	53.2
Lactate+ Pyruvate	2L+1P	10.3	16.3	28.2	41.9	48.9	63.9

[a] Standard error for muscle type + incubation and display of muscle = 1.54 - 2.79.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.

Table 4.3: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Semitendinosus muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	14.6	32.8	46.7	63.4	71.7	82.2
Malate	1M	25.2	29.1	34.7	48.1	56.4	66.3
Lactate	1L	14.0	25.8	36.5	54.7	65.2	71.5
Pyruvate	1P	12.8	30.4	41.8	49.8	58.7	66.7
Malate	2M	18.6	21.1	20.4	25.2	33.2	41.8
Lactate	2L	10.4	12.7	14.7	17.7	24.2	28.1
Pyruvate	2P	11.1	17.3	28.2	38.3	42.9	47.9
Malate + Lactate	1M+1L	18.5	19.9	22.4	35.7	52.5	64.4
Malate + Pyruvate	1M+1P	12.8	21.6	28.5	45.6	55.7	78.8
Lactate + Pyruvate	1L+1P	19.6	28.4	38.3	55.4	63.4	76.4
Malate + Lactate	2M+2L	23.5	17.3	16.4	20.7	22.3	29.8
Malate + Pyruvate	2M+2P	17.7	22.7	22.3	27.2	39.2	48.2
Lactate + Pyruvate	2L+2P	16.7	23.4	36.9	46.6	48.3	52.7
Malate + Lactate	1M+2L	14.4	15.5	18.6	22.7	36.4	44.4
Malate + Pyruvate	1M+2P	12.6	26.6	35.5	48.6	55.7	61.4
Lactate + Pyruvate	1L+2P	19.6	28.4	38.3	55.4	63.4	76.4
Malate + Lactate	2M+1L	13.5	24.3	26.6	29.1	31.3	34.4
Malate + Pyruvate	2M+1P	27.7	22.7	24.3	27.2	36.1	38.2
Lactate + Pyruvate	2L+1P	16.7	29.8	39.9	52.6	63.3	67.7

[a] Standard error for muscle type + incubation and display of muscle = 2.14 - 2.88.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.

Chapter 5

Effects of Fiber Orientation, Myoglobin Redox Form, and Postmortem Storage on NIR Tissue Oximeter Measurements of Beef *Longissimus* Muscle

5.1 Abstract

To determine near infrared tissue oximeter responses to muscle fiber orientation, display time, and surface color differences of beef *longissimus lumborum* steaks, beef loins were cut into steaks either perpendicular or parallel to the muscle fiber orientation. Surface color differences were created by packaging steaks in vacuum (VAC), 80% O_2 and 20% CO_2 modified atmosphere packaging (HiOx MAP), polyvinyl chloride film overwrap (PVC), and HiOx MAP converted to PVC (HiOx-PVC) after 2 d. Changes in surface color and

subsurface pigments during display (0, 2, 4, 10 and 15 days at 10 °C) were characterized by using a reflecto-spectrophotometer and a near-infrared tissue oximeter, respectively. Fibre orientation, storage, and packaging affected ($p < 0.05$) color, total pigment, deoxymyoglobin, and oxymyoglobin content. Tissue oximetry measurements appear to have potential for real-time monitoring of myoglobin redox forms and oxygen status of packaged meat, but fiber orientation needs to be controlled.

5.2 Introduction

Meat color perceived by consumers is a valuable assessment of overall quality and wholesomeness of meat (Faustman & Cassens, 1990; Kropf, 1993). Among all sensory attributes of meat, color is considered one of the most important physical traits because once color is deemed unacceptable, all other sensory attributes lose their significance to consumers (Bekhit, Simmons, & Faustman, 2005; Mancini, & Hunt, 2005) and their purchasing decisions are negatively influenced (Arnold, Scheller, Williams, & Schaefer, 1992; McKenna, Mies, Baird, Pfeiffer, Ellebracht, & Savell, 2005). Myoglobin is the primary pigment associated with meat color. The ability of fresh meat to oxygenate myoglobin and retain a bright cherry-red color of "bloomed" meat during refrigerated storage or display differs among muscles (Atkinson & Follett 1973; Hood, 1980; McKenna *et al.*, 2005; O'Keeffe & Hood, 1982; Renerre & Labas, 1987; Urbin & Wilson 1961). The bloomed color of the meat's surface is influenced by oxygen consumption of skeletal muscle cells, oxygen partial pressure, and depth of myoglobin oxygenation (Lanari & Cassens, 1991; O'Keeffe & Hood, 1982).

Visual appraisal permits inferences to be made about human perception of meat discoloration, but it is subjective, time consuming, and depends upon the skill and preferences of the observer (Harrison, Kropf, Allen, Hunt, & Kastner, 1980; Kropf, 1993; Ockerman &

Cahill 1969). Objective procedures utilizing reflectance colorimetry and spectrophotometry are convenient, rapid, and nondestructive, but their qualitative and quantitative relationship with human perceptions of color can be variable (Hunt, 1980), and they only measure surface color because of limited penetration of the surface. However, near infrared (NIR) tissue oximeter measurements can penetrate several centimeters below the surface (Cui, Kumar & Chance, 1991; Rea, Crowe, Wickramasinghe, & Rolfe, 1985) and have been used to determine the oxygen-dependent absorption of myoglobin and hemoglobin (Marcinek, Amara, Matz, Conley, & Schenkman, 2007; Ferreira, Hueber, & Barstow, 2007) in medical diagnostics and exercise physiology (Quaresima, Lepanto, & Ferrari, 2003). Wavelengths of NIR light were differentially absorbed by the oxygenated and deoxygenated forms of myoglobin (Bowen, 1949). Thus, tissue oximetry seems to have potential for continuous real-time measurement of changes in myoglobin oxygenation leading to information on tissue oxygenation and hemodynamics (Ferreira *et al.*, 2005, 2007).

Binzoni *et al.*, (2006) reported that the propagation of photons in the human *biceps brachii* muscle was anisotropic and suggested that the probability of a photon traveling along the fiber direction was higher than if traveling perpendicular to the fibers. Physical, chemical, and anatomical differences in muscles cause variations in color from cut to cut, within a cut, and in cuts made perpendicular (PD) or parallel (PL) to the muscle fiber (Hunt & Hedrick 1977; Smith, Belk, Sofos, Tatum, & Williams, 2000). It appears that these tissue structures affect variations in meat color and color stability. Muscles cut PL to the meat fiber direction had faster water and heat transfer (Godsalve, Davis, & Gordon, 1977), were more tender (Bouton, Ford, Harris, & Ratcliff, 1975; Marks, Stadelman, Linton, Schmieder, & Adams, 1998; Munro, 1983), and had higher cooking yields (Boles & Shand, 2008). However, variations in myoglobin redox dynamics, oxygen penetration, meat color life, and color stability due to orientation of the muscle fibers (PL or PD) are not well documented.

This study was undertaken to determine how NIR tissue oximeter measurements of post-rigor beef skeletal muscle relate with the more established methods of quantifying myoglobin redox states. Our specific objectives were to (1) determine the amount of oxymyoglobin (OMb) and deoxymyoglobin (DMb) in beef *longissimus* muscle in several packaging formats by using NIR tissue oximetry and reflecto-spectrophotometry, (2) evaluate the effects of muscle fiber orientation (PL vs. PD) on NIR tissue oximeter responses, and (3) determine whether tissue fiber orientation affects myoglobin redox forms in beef muscle packaged in several packaging formats.

5.3 Materials and Methods

5.3.1 Raw materials

The *longissimus lumborum* (LL) from six beef loins (USDA Select, A-maturity) were fabricated at 10 d postmortem into portions about 5 cm 8 cm 10 cm. Fiber orientation of three of the loins was either PD or PL to a designated muscle surface. The portions were cut thick (5 cm) to ensure that NIR light did not escape from the tissue.

5.3.2 Packaging and display

The muscle portions were assigned to four packaging treatments: (1) VAC (62.2 cm Hg vacuum; Multivac C500, Multivac Inc., Kansas City, MO); (2) HiOx MAP (80% O_2, 20% CO_2; Air Gas certified gas, Mid South, Inc., Tulsa, OK); (3) PVC (MAPAC L, 21,700 cc O_2/m2/24 h; Borden Packaging and Industrial Products, North Andover, MA) on foam trays (17S; McCune Paper Company, Salina, KS) with a Dri-Loc soaker pad (AC-50, Sealed Air Corp, Duncan, SC); and (4) HiOx-PVC. Steaks packaged in HiOx MAP (Ross Jr. S-3180, Ross, Midland, VA) were placed in 4.32-cm-deep rigid plastic trays (CS977, Cryovac

Sealed Air Corp., Duncan, SC) and covered with oxygen-barrier film (Lid 550; 1.0 mil; less than 20.0 oxygen transmission cc/24 h/m2 at 4.4 °C with 100% relative humidity; and moisture vapor transmission less than 0.1 g/24 h/645.2 cm2 at 4.4 °C and 100% relative humidity; Cryovac Sealed Air Corp., Duncan, SC) with a soaker pad, flushed with one of two certified gas blends (80% O_2, 20% CO_2), and sealed with shrinkable-barrier film. Steaks were displayed at 2 °C for 10 d under 2150 ± 50 lux of continuous fluorescent lighting (bulb F32T8/ADV830, 3000 K, CRI = 86; Phillips, Bloomfield, NJ) in open-top display cases (DMF8; Tyler Refrigeration Corp., Niles, MI). Packages were rotated daily to minimize case location effects.

5.3.3 Proximate analyses and pH

Meat remaining after packaging preparation was used for proximate analysis of the meat. Random samples were analyzed in triplicate for moisture, fat, and protein. The pH of steaks was measured on d 10 postmortem by inserting the tip of pH probe (MPI pH probe, glass electrode, Meat Probes Inc., Topeka, KS) into the steaks

5.3.4 Color analysis

Steaks were scanned from 400 to 700 nm in triplicate on days 0, 2, 4, 10, and 15 for instrumental color (HunterLab MiniScan XE Plus Spectrophotometer 45/0 LAV, 2.54-cm diameter aperture, 10 standard observer; Hunter Associates Laboratory, Inc., Reston, VA). Values for CIE L^*, a^*, and b^* (Illuminant A); hue angle ($\tan^{-1} b^*/a^*$); and saturation index $[(a^*)^2 + (b^*)^2]$ were calculated for days 0, 2, 4, 10, and 15 of display (AMSA, 1991) DMb and OMb were calculated from reflectance data for myoglobin isobestic wavelengths (AMSA, 1991).

To obtain reference values for 100% of DMb and OMb, steaks not used in the experiment

were either vacuum packaged and allowed to deoxygenate overnight at 10 °C to obtain 100% DMb or placed in a bag containing 100% oxygen.

5.3.5 NIR tissue oximetry

Muscle portions were evaluated for myoglobin redox form by using a NIR tissue oximeter (OxiplexTS model 96208, ISS, Inc., Champaign, IL), which is based on the relative tissue transparency for light in the NIR region and on the oxygen-dependent absorption changes of myoglobin (van Beek & Westerhof, 1996). Using a frequency-domain NIR spectrophotometer that generates modulated light at 110MHz, it was possible to separate between optical absorption and scattering. Because of their nearly identical spectral characteristics, it was not possible to distinguish between hemoglobin and myoglobin, but because most of the pigment in meat is myoglobin (Groot, Zuurbier, & van Beek, 1999), it was assumed that the data were primarily from myoglobin. Absorption changes at the discrete wavelengths are converted into concentration changes of DMb and OMb. Data were sampled in real time and stored on disk for off-line analysis.

NIR measurements were made by using a probe consisting of eight laser diodes operating at two different wavelengths (690 and 830 nm, four at each wavelength) and a photomultiplier tube. The diodes were arranged in two parallel rows of emitter fibers and one detector fiber bundle such that the source-detector separations were 2.0, 2.5, 3.0, and 3.5 cm for each wavelength. Measurements were made by placing the probe longitudinally to the long axis of packaged meat sections. The NIR spectrometer was calibrated on each test day following the manufacturer's recommendations. By using light at 630 and 830 nm, it was possible to differentiate between DMb and OMb. The sum of [OMb] and [DMb] concentrations equaled the total myoglobin [TMb].

5.3.6 Statistical analysis

The experimental design for the complete experiment was a randomized complete block with split-plot design. The experimental design within the whole plot was a randomized complete block with carcass serving as a random effect (a block) and muscle serving as the treatment. Within the subplot, packaging type served as the treatment assigned to steaks (subplot experimental units). Data were analyzed separately for each of the three variables (OMb, DMb, and TMb]. Multiple NIR tissue oximetry scans on each steak (three per steak) were averaged for statistical analysis. Thus, for each variable, analysis consisted of 120 total observations (three animals two orientations four packaging 5 days). Type-3 tests of fixed effects for muscle, muscle packaging, muscle day, and muscle packaging day were evaluated by using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). F-test denominator degrees of freedom were estimated by using the Satterthwaite adjustment. Least squares means for significant F-tests were separated by using least significant differences.

5.4 Results

5.4.1 pH and proximate analyses

There were no differences ($p < 0.05$) in meat pH, moisture, fat, or protein for samples in Table 5.1

5.4.2 NIR tissue oximetry: Oxymyoglobin and deoxymyoglobin

There was a fiber orientation × packaging × day interaction ($p < 0.05$) for NIR tissue oximeter responses for OMb and DMb (Table 5.2). The LL steaks cut PD to the fiber orientation and packaged in HiOx MAP had 27% OMb on day 0, whereas steaks cut PL had 23% OMb. On display day 2, the OMb level of PD steaks increased ($p < 0.05$) to 77%

in HiOx MAP and remained at that level to day 4. By day 10, there was a slight decline in OMb, and by day 15, the decline was significantly less (53%). Levels of OMb of steaks cut PL followed a similar oxygenation pattern as steaks cut PD, but levels of OMb were about 10% lower. Steaks cut PL declined in OMb to 55% ($p < 0.05$) on day 10 and to 42% ($p < 0.05$) on day 15.

Steaks with PD fibers packaged in HiOx-PVC MAP had significantly higher levels of OMb (30%) than steaks with a PL orientation (23% OMb) on display day 0. On display day 2, OMb levels of PD steaks increased ($p < 0.05$) to a greater extent than those of PL cut steaks (78% vs. 68%) in HiOx-PVC MAP, and these levels were maintained through display day 4. On days 10 and 15, OMb levels in steaks cut PD and PL were lower (p ¡ 0.05) than the previous levels.

As expected, oxygenation of myoglobin of the LL muscle cut either PD or PL was less ($p < 0.05$) when steaks were packaged in PVC and was considerably less ($p < 0.05$) compared with either of the HiOx MAP systems. There was an increase ($p < 0.05$) in OMb level for both PD and PL steaks in PVC from days 0 to 10 of display. However, compared with steaks cut PD, PL cut steaks packaged in PVC systems had significantly lower percentages of myoglobin oxygenation throughout display. The LL muscle cut either PD or PL and packaged in vacuum did not show any changes ($p > 0.05$) in OMb percentages throughout display.

NIR tissue oximeter response for DMb exhibited a similar but opposite pattern for fiber orientation effects (Table 5.2). Steaks with PD orientation packaged in HiOx MAP had 73% DMb on day 0 of display, which decreased ($p < 0.05$) to 22% by day 2 and remained at that level throughout the display period. The HiOx-PVC packaging system containing PD steaks had relatively the same level of DMb as the HiOx MAP system throughout display. However, PD steaks packaged in PVC had 92% DMb on day 0, which decreased ($p < 0.05$) to 56% on day 2 of display, whereas PD steaks in VAC contained $\leq$ 95% DMb and, as

expected, maintained predominately DMb throughout display. Steaks with PL orientation did not differ ($p > 0.05$) in DMb levels from PD steaks in HiOx MAP, HiOx-PVC, PVC, or VAC systems (Table 5.2).

Oximeter measurements of TMb (3.71mg/g) did not differ ($p > 0.05$) because of fiber orientation except in steaks packaged in HiOx-PVC (Figure 5.1), in which TMb was higher ($p < 0.05$) in steaks cut PL.

5.4.3 Reflecto-spectrophotometry: Oxymyoglobin and deoxymyoglobin

Percentages for OMb and DMb were essentially the same for HiOx MAP and HiOx-PVC during display until days 10 and 15, when color deteriorated and amount of OMb declined but DMb levels were maintained in the HiOx-PVC packages (Table 5.3). Steaks cut both PD and PL and packaged in PVC had less OMb and more DMb than comparable steaks in the HiOx MAP packaging. The VAC steaks had, as expected, the greatest DMb and only traces ($< 2\%$) of OMb during display. In general, PD steaks exhibited a higher percentages of OMb compared with PL steaks, whereas DMb percentages were greater for steaks cut PL than for those cut PD.

5.4.4 Reflecto-spectrophotometric color

For all color parameters shown in Figures 5.2 and 5.3, the level of color was depressed for steaks cut PL compared with those cut PD in the HiOx MAP and HiOx-PVC packaging systems. Differences in color parameters were smaller for steaks in PVC and were not different for steaks in VAC.

Steaks cut PD and PL and packaged in HiOx MAP, HiOx-PVC, or PVC became darker, less red, yellow, and saturated in color (lower L^*, a^*, b^* and chroma values; $p < 0.05$) as

display time advanced from days 0 to 15, and hue angles generally increased after day 10 (Figures 2 and 3). On days 2, 4 and 10, PD steaks packaged in HiOx MAP and HiOx-PVC had greater redness intensity (higher a^* and chroma values; $p < 0.05$) than comparable PL steaks (Figures 2B vs. 2E and 3A vs. 3C). From days 0 to 15, a^* values for PL steaks packaged in HiOx MAP, HiOx-PVC, and PVC systems were not different ($p > 0.05$), whereas PD steaks in PVC had lower ($p < 0.05$) a^* values than those in HiOx MAP and HiOx-PVC (Figure 2B vs. 2E). Furthermore, a^* values decreased ($p < 0.05$) during display more sharply (from days 4 to 15) for PD steaks in HiOx-PVC than for PL steaks. Instrumental b^* values also decreased more slowly for PD steaks compared with PL steaks packaged in HiOx MAP, HiOx-PVC (Figure 2C vs. 2F). By day 15, steaks with OMb were discolored regardless of packaging system and fiber orientation. There were no significant differences in color between steaks cut PD and PL and packaged in PVC and VAC.

5.5 Discussion

NIR tissue oximetry has been used extensively to determine the oxygen-dependent absorption of myoglobin and hemoglobin (Marcinek, Amara, Matz, Conley, & Schenkman, 2007) in medical diagnostics and exercise physiology. The focus of this investigation was to develop an understanding of (1) how quantitative measurements obtained from an NIR tissue oximeter of beef skeletal muscle relate to more established methods of quantifying muscle pigments and (2) effects of muscle fiber orientation of meat cuts on NIR and reflecto-spectrophotometric measures of meat color parameters and color stability. NIR tissue oximetry was used to quantify the myoglobin redox forms on and below the surface of cuts, whereas reflecto-spectrophotometric data were used to characterize meat surface myoglobin redox forms, color, and discoloration.

This research revealed three principle findings: (1) NIR oximetry can quantitate OMb

and DMb in and on meat that are logical for the packaging systems used and which compared favorably with spectrophotometric estimates of myoglobin redox forms and surface color measures of surface meat color, (2) Both NIR and spectrophotometric measurements are affected by muscle fiber orientation, and (3) Tissue fiber orientation interacts with the extent of oxygenation of myoglobin and its resulting color stability.

Because there were no differences ($p > 0.05$) in pH, chemical composition, and total pigment between steaks cut PD and PL to fibers, the significant effects observed because of fiber orientation, packaging format, and display time appear to have more substance. The packaging systems used were successful in creating differences in myoglobin redox forms and in the dynamic changes in the levels of OMb and DMb during display.

Previous research has documented color differences within beef cuts (McKeith *et al.*, 1985; Johnson, Chen, Muller, Costello, Romans, & Jones, 1988; Sammel, Hunt, Kropf, Hachmeister, Kastner, & Johnson, 2002); however, we found no data supporting the effect of fiber orientation on myoglobin redox status and meat color variation within a muscle. Farouk, Zhang, & Cummings (2005) reported that steaks cut PD to the fiber orientation had a higher proportion of myofibrils and potentially higher pigment concentration compared with steaks cut PL to the fiber orientation. Gou, Comaposada, & Arnau (2002) reported that muscle fiber orientation was related to biophysical properties of postmortem muscle, and they stated that fiber could possibly affect myoglobin redox form and thus meat color. Marquez, Wang, Lin, Schwartz, & Thomsen (1998) reported that the muscle fiber orientation of chicken skeletal muscle affected absorption and reduced the scattering coefficient of NIR measurements and that these changes were related to the structural anisotropy of the alignment of muscle fibers. In the current study, surface reflectance measurements of redness intensity indicated that steaks cut PD and packaged in HiOx MAP and HiOx-PVC were more likely to maintain their redness longer during display than steaks in PVC, which had a shorter color life. Fiber orientation affects on myoglobin forms and color were much

less pronounced for steaks in VAC.

NIR tissue oximetry was a noninvasive measure of muscle oxygenation and provided more detailed information related to meat color stability. Reflectance measurements were limited to differences of surface myoglobin redox forms and did not provide any insight into pigment dynamics in the depths of the meat. Behrends (2004) reported that bovine skeletal muscle oxygen penetration depth and oxygen consumption were indicators of lean color and potential discoloration and that the decrease in the oxygen penetration depth of muscle during display can be used as a tool to assess meat color stability. Madhavi & Carpenter (1993) stated that oxygen consumption alters penetration of oxygen into the muscle; when there is deep penetration of oxygen into the muscle, there is a slower conversion of oxymyoglobin to metmyoglobin, and when there is shallow oxygen penetration into the muscle, there is a rapid deterioration of meat color. In addition, the ability of meat to keep the metmyoglobin layer from advancing to the meat's surface is slowed with better metmyoglobin reducing capacity (Behrends, 2004; Mancini & Hunt, 2005; Seyfert, Hunt, Mancini, Tang, & Faustman, 2006).

It would have been advantageous if the oximeter also measured metmyoglobin, but the biomedical applications do not need this capability. Thus, combined use of tissue oximetry and reflectance measurements may be a good way to further investigate the dynamic changes in myoglobin redox forms as meat discolors. Our NIR data suggest that steaks cut PD to fibers will attain a greater level of oxygenated pigment that is more stable in aerobic packaging systems than steaks cut PL to fibers. Thus, physical structure of the meat, pigment concentration, chemical state of pigments, and oxygen uptake and metmyoglobin reducing activity in the meat are major factors that contribute to color and color stability of fresh meat (DeVore & Solberg, 1974; Hunt & Hedrick, 1977; Mancini & Hunt, 2005; Seyfert, Hunt, Mancini, Tang, & Faustman, 2006). Within the parameters of this study, muscle fiber orientation in relation to myoglobin redox forms affecting meat color and color stability is

very important from the consumer perspective. Since the color and discoloration of steaks were affected by fiber orientation and because many cuts cannot be fabricated without some fibers being 100% PL or PD, to obtain the most uniform appearance, meat should be cut to minimize differences in muscle fiber orientation.

5.6 Summary

Fiber orientation and display days affected ($p < 0.05$) OMb, DMb, and instrumental color measurements in all packaging formats. Steaks with PD fiber orientation had more OMb and greater color stability than steaks cut PL to the fiber orientation. Packaging format did not affect TMb; however, OMb increased and DMb decreased as exposure to oxygen increased. As postmortem storage advanced, OMb levels decreased.

5.7 Implications

NIR tissue oximeter measurements have potential for use in real-time monitoring of the myoglobin redox forms and oxygen status of meat packaged in a variety of packaging formats. To obtain repeatable NIR tissue oximetry measurements on post-rigor muscle, fiber orientation, tissue oxygen exposure, and storage time must be controlled.

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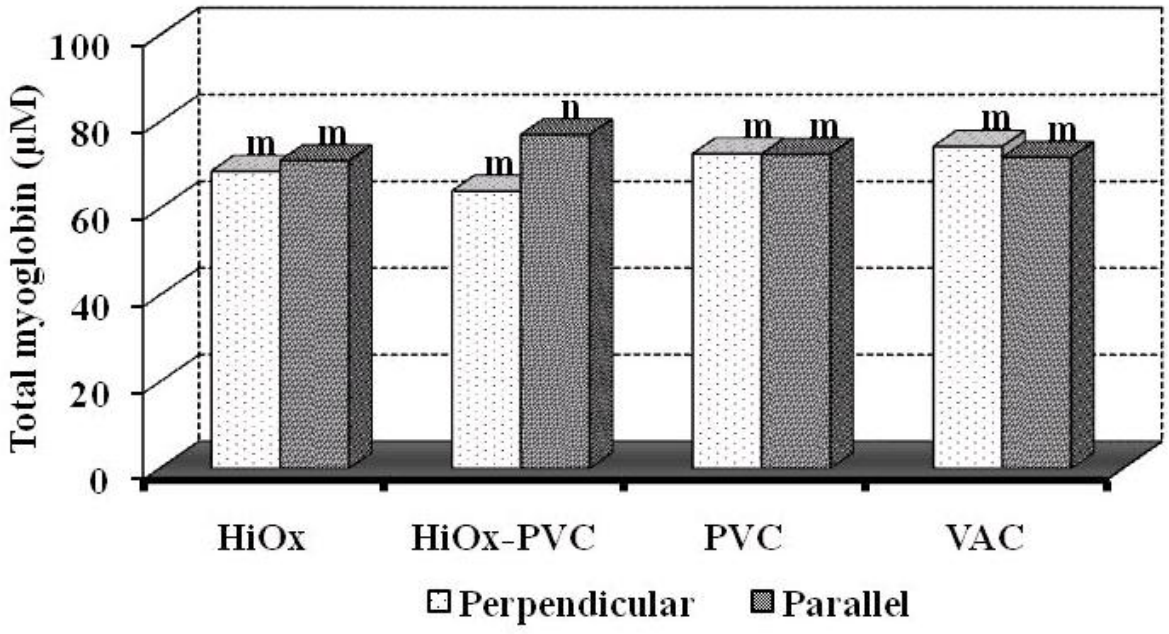

Figure 5.1: *Effects of fiber orientation, and packaging on total myoglobin content of bovine longissimus muscle. HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 hours and then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging.* ^{m,n} *Means with a different letter differ* ($p < 0.05$).

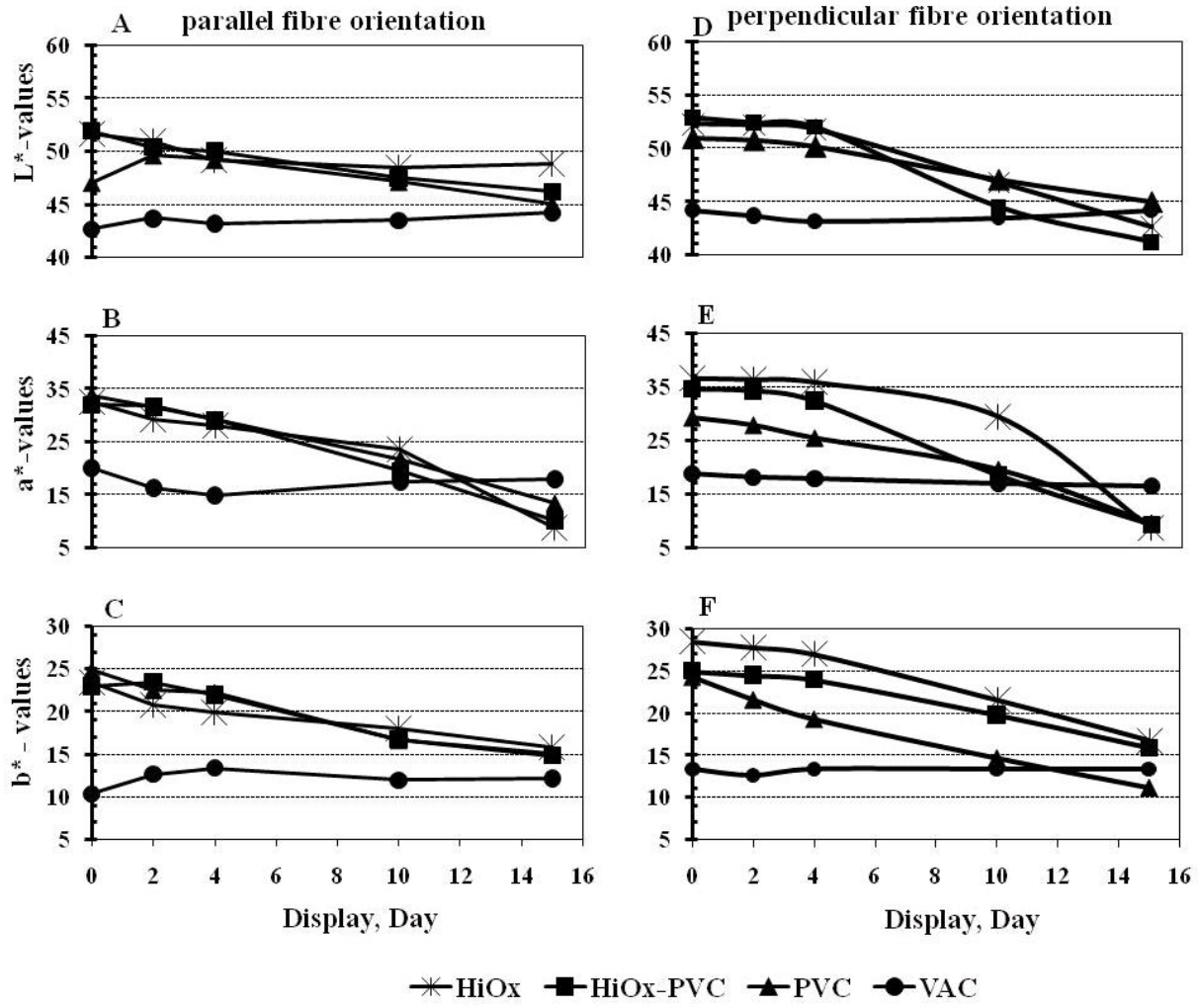

Figure 5.2: *Effects of fiber orientation, packaging, and display time on percentage of oxymyoglobin forms of bovine longissimus muscle by reflecto-spectrophotometer HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 hours and then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging. A, B, and C = parallel fiber orientation; D, E, and F = perpendicular fiber orientation. Larger values for L^*, a^* and b^* indicate a lighter, redder, or more yellow color, respectively.*

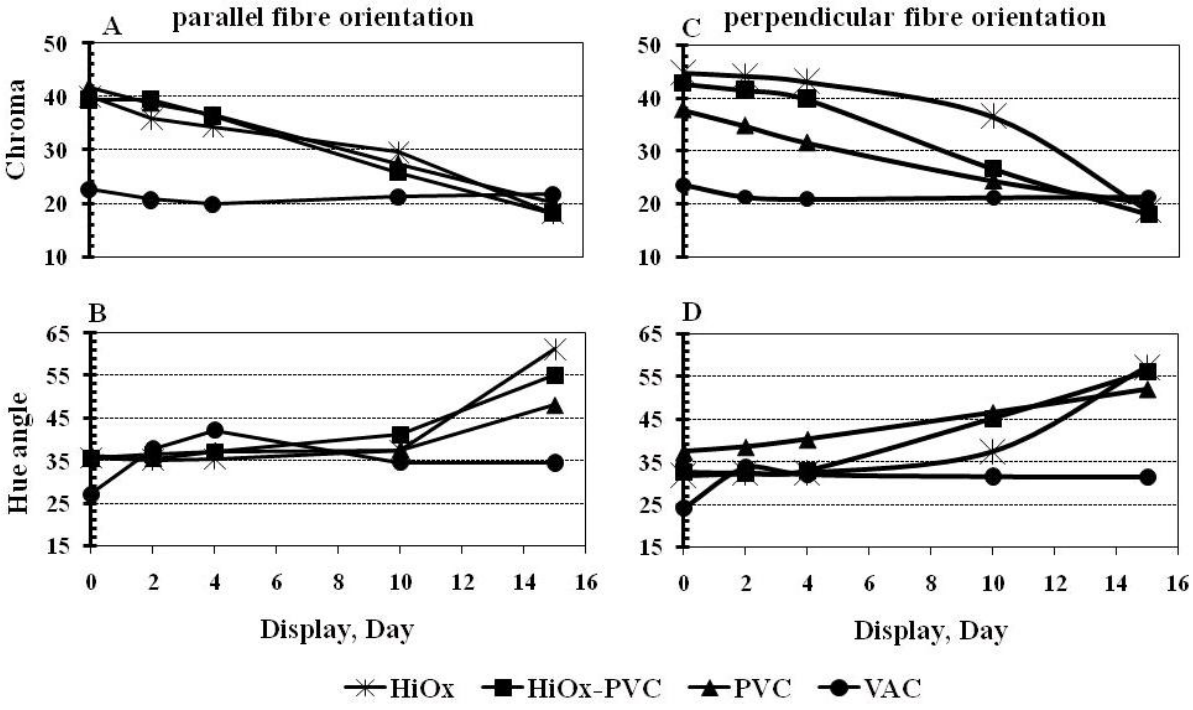

Figure 5.3: *Effects of fiber orientation, packaging, and display time on percentage of deoxymyoglobin forms of bovine longissimus muscle by reflecto-spectrophotometer HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 hours and then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging. A and B = parallel fiber orientation; C and D = perpendicular fiber orientation. Larger values for chroma and hue angle are a more saturated red or more yellow/ brown, respectively.*

Table 5.1: *Effect of muscle fiber orientation on pH and proximate composition of beef LL muscle*

Fiber Orientation	pH	Moisture(%)	Protein(%)	Fat(%)
Perpendicular	5.5	71.3	23.6	4.2
Parallel	5.5	72.3	23.4	3.7

Table 5.2: *Oxymyoglobin[a] and deoxymyoglobin[b] percentages of the beef LL muscle measured by using a NIR tissue oximeter during 15 days of retail display in different packaging formats[c] (Means[e-i],Means[v-y])*

Pigment form	Fiber orientation	Package type	Display time, day				
			0	2	4	10	15
Oxymyoglobin	Perpendicular	HiOx	27.1^{ghv}	77.6^{ix}	77.7^{ix}	71.1^{ix}	53.0^{gw}
		HiOx-PVC	30.3^{gv}	78.3^{iy}	75.6^{iy}	65.7^{ix}	45.5^{fw}
		PVC	7.3ev	47.5^{gw}	48.2^{gw}	48.3^{gw}	10.8^{ev}
		VAC	4.5^{ev}	4.8^{ev}	2.5^{ev}	4.1^{ev}	13.1^{ew}
	Parallel	HiOx	23.2^{fhv}	67.8^{hy}	67.6^{hy}	55.4^{hx}	42.1^{fw}
		HiOx-PVC	23.3^{fhv}	68.6^{hy}	65.2^{hy}	53.8^{hx}	45.6^{fw}
		PVC	8.7^{ev}	34.7^{fw}	38.8^{fw}	32.5^{fw}	8.8^{ev}
		VAC	5.2^{ev}	7.7^{ev}	8.1^{ev}	8.1^{ev}	11.1^{ev}
Deoxymyoglobin	Perpendicular	HiOx	73.1^{ew}	22.5^{ev}	22.3^{ev}	23.2^{ev}	27.3^{fv}
		HiOx-PVC	69.8^{ex}	21.9^{e}	24.3^{ev}	24.4^{ew}	25.9^{fv}
		PVC	92.5^{fw}	56.1^{gw}	55.9^{gw}	53.9^{fv}	51.3^{gw}
		VAC	95.8^{fv}	95.5^{iv}	97.5^{iv}	96.2^{hv}	88.9^{iw}
	Parallel	HiOx	76.2^{ey}	32.1^{fx}	32.3^{fx}	24.4^{ew}	17.3^{ev}
		HiOx-PVC	75.7^{ey}	31.6^{fx}	33.3^{fx}	21.4^{ew}	14.9^{ev}
		PVC	90.1^{fw}	65.3^{hv}	64.5^{hv}	63.9^{gw}	59.2^{hv}
		VAC	94.8^{fv}	95.6^{iv}	95.4^{iv}	94.4^{hv}	92.9^{iv}

[a] Standard error = 3.57 - 4.13.

[b] Standard error = 3.21 - 4.29.

[c] HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 hours and then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packagin

[e-i] Means within a pigment form within a column with a different letter differ ($p < 0.05$).

[v-y] Means within a row with a different letter differ ($p < 0.05$).

Table 5.3: *Oxymyoglobin[a] and deoxymyoglobin[b] percentages of beef LL muscle measured using a reflectance spectrophotometry during 15 days of retail display in different packaging formats[c](Means[e-h],Means[v-y])*

Pigment form	Fiber orientation	Package type	Display time, day				
			0	2	4	10	15
Oxymyoglobin	Perpendicular	HiOx	68.0^{gx}	68.4^{gx}	68.3^{gx}	48.9^{iw}	25.6^{iv}
		HiOx-PVC	68.9^{gx}	66.7^{gx}	63.0^{gw}	17.7^{gv}	17.8^{hv}
		PVC	61.6^{fx}	56.5^{fw}	55.7^{fw}	18.1^{gv}	17.2^{hv}
		VAC	0.1^{ev}	0.8^{ev}	0.3^{ev}	1.6^{ev}	0.3^{ev}
	Parallel	HiOx	66.0^{fgy}	56.4^{fx}	54.4^{fx}	18.1^{gw}	9.4^{gv}
		HiOx-PVC	62.9^{fx}	57.3^{fw}	54.9^{fw}	6.8^{fv}	5.0^{fv}
		PVC	60.2^{fy}	55.0^{fx}	53.0^{fx}	24.3^{hw}	10.9^{gv}
		VAC	0.3^{ev}	0.7^{ev}	1.0^{ev}	2.1^{ev}	0.9^{ev}
Deoxymyoglobin	Perpendicular	HiOx	13.7^{ex}	10.3^{ewx}	10.7^{ewx}	9.8^{ew}	4.4^{ev}
		HiOx-PVC	13.4^{ew}	10.6^{ew}	11.7^{ew}	10.1^{ew}	5.4^{ev}
		PVC	23.9^{gv}	21.6^{fv}	21.1^{gv}	21.4^{gv}	24.4^{fv}
		VAC	85.6^{iw}	80.9^{gv}	79.2^{hv}	84.0^{iw}	91.7^{gx}
	Parallel	HiOx	18.5^{fx}	18.2^{fx}	$18.7\mathrm{f}^{gx}$	$13.4\mathrm{e}^{fw}$	4.1^{ev}
		HiOx-PVC	17.7^{fw}	17.2^{fw}	$15.9\mathrm{e}^{fw}$	14.6^{fw}	7.8^{ev}
		PVC	29.2^{hw}	18.4^{fv}	20.5^{gv}	19.4^{gv}	20.7^{fv}
		VAC	86.7^{iw}	85.3^{gw}	84.7^{hw}	78.7^{hv}	89.4^{gw}

[a] Standard error = 0.89.

[b] Standard error = 0.77.

[c] HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 hours and then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging.

[e-i] Means within a pigment form within a column with a different letter differ ($p < 0.05$).

[v-y] Means within a row with a different letter differ ($p < 0.05$).

Chapter 6

Near-Infrared Oximetry of Three Post-rigor Skeletal Muscles for Following Myoglobin Redox Forms

6.1 Abstract

We investigated the response of frequency-domain multidistance (FDMD) near-infrared (NIR) tissue oximetry for detecting absolute amounts of myoglobin (Mb) redox forms and their relationship to meat color stability. Four packaging formats were used to create different blends of Mb redox forms and meat colors during display. Changes in surface color and subsurface pigment forms during simulated display (0, 2, 4, and 10 d at 2 °C) were evaluated using surface reflecto-spectrophotometry (both $L^*a^*b^*$ and specific wavelength analyses) and FDMD NIR tissue oximetry. Data for both methods of direct measurement of oxymyoglobin and deoxymyoglobin were strongly related and accounted for 86 to 94% of the display variation in meat color. Indirect estimates of metmyoglobin ranged from r^2 = 59 to 85%. It appears that NIR tissue oximetry has potential as a noninvasive, rapid

method for the assessment of meat color traits and may help improve our understanding of meat color chemistry in post-rigor skeletal muscle.

KEYWORDS: Near-infrared tissue oximeter; reflectance spectroscopy; modified atmosphere packaging; meat color; myoglobin redox forms

6.2 Introduction

Meat color is the most important factor that influences consumer meat purchasing decisions (Kropf, 1993). The inability to control factors affecting meat color and to assess the potential of meat to have good color life contributes to millions of dollars lost per year at retail because of discoloration (McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005). Muscle oxygen uptake, its subsequent utilization by enzymes and other cellular compartments, and the ability to reduce metmyoglobin (MMb) determines the color life of meat (Mancini & Hunt, 2005).

In living skeletal muscle, myoglobin (Mb) undergoes rapid oxygenation and deoxygenation in response to fluctuations in oxygen supply and tissue demand (Millikan, 1939), whereas post-rigor muscles respond more slowly and differently, resulting in variable degrees of "worst point" discoloration on meat surfaces (Mancini & Hunt, 2005). Maintenance of a bright red color of fresh meat is a delicate interaction between retail cold chain management and the inherent chemistry of the meat, which results in various Mb redox forms on the exposed meat surface and a few millimeters below the surface. DeVore and Solberg (1975) found that muscle tissue respiration accounts for 80% of the oxygen uptake in the first 10 h of display. Reducing oxygen tensions within muscle tissue promote the autoxidation of deoxymyoglobin (DMb) and oxymyoglobin (OMb), causing surface discoloration (Atkinson & Follett, 1973). Bendall & Taylor (1972) reported the nicotinamide adenine dinucleotide (NADH) linked oxidation of mitochondrial tricarboxylic cycle metabolites pyruvate-malate

and muscle oxygen consumption rates in pre-rigor beef muscle. Tang *et al.*, (2005) reported that Mb serves as the oxygen reservoir and transporter for mitochondria in meat animal tissue.

Meat scientists have used several methods to determine muscle oxygen uptake and consumption (OC) including the Warburg flask (Urbin & Wilson, 1961), differential respirometry (DeVore & Solberg, 1975), Clark oxygen electrodes (Lanari & Cassens, 1991), reflecto-spectrophotometry (Madhavi & Carpenter, 1993), and headspace oxygen analyzers (Sammel, Hunt, Kropf, Hachmeister, Kastner & Johnson, 2002). None of these methods provide real-time, noninvasive monitoring of meat color and thus have limited practical application in the meat industry.

Interactions between light and muscle pigment in meat offer an opportunity to develop methodology for detecting the redox dynamics of Mb using near-infrared (NIR; 700-1000 nm) technology. Recently, frequency-domain multidistance (FDMD) NIR tissue oximetry has been used in exercise physiology and biomedical applications to provide a direct measure of absolute concentrations of hemoglobin (Hb) and Mb oxygen saturation in skeletal muscle and brain tissue (Chance, Dait, Zhang, Hamaoka & Hagerman, 1992). Because Hb and Mb have similar NIR absorption and because there are minimal amounts of Hb in well-bled animal tissue (Hunt & Hedrick, 1977a), it is possible to distinguish DMb, OMb, and MMb in post-rigor skeletal muscle. This approach has been used successfully to assess the dynamics of Mb oxygenation and deoxygenation during step changes in cardiac workload (de Groot, Zuurbier & van Beek, 1999). Mohan *et al.*, (2010) reported that fiber orientation and Mb redox form affected NIR tissue oximetry (FDMD) data in post-rigor muscle.

We are not aware of research using FDMD-based NIR tissue oximetry to evaluate color and color stability of beef skeletal muscle. Thus, the objectives of this project were to investigate (1) NIR tissue oximeter response from three bovine post-rigor muscles varying in pigment concentration, Mb redox state, and packaging, (2) NIR tissue oximeter response

for redox dynamics of Mb in post-rigor muscle over time, and (3) relationships of NIR tissue oximetric and spectrophotometric data for color and redox forms during retail display.

6.3 Materials and Methods

6.3.1 Raw Materials

Six *longissimus lumborum* (LL; color stable), *semitendinosus* (ST; intermediate color stability), and *psoas major* (PM; color unstable) muscles were obtained from USDA Select, A-maturity carcasses at a commercial plant and were fabricated at 10-d postmortem into 5.0-cm thick portions with the fiber orientation perpendicular to the muscles' cut surface. The thick portions ensured that NIR light did not escape from the muscle tissue.

6.3.2 Packaging and Display

Cut portions of each muscle were assigned randomly to the following packaging treatments: (1) vacuum packaging (VAC; 62.2 cm Hg, Multivac C500, Multivac Inc., Kansas City, MO), (2) modified atmosphere packaging (MAP) with high oxygen (HiOx; 80% O_2, 20% CO_2, AirGas certified gas, MidSouth, Inc., Tulsa, OK), (3) overwrapped with polyvinyl chloride film (PVC; MAPAC L, 21,700 cc O_2/m_2/24 h, Borden Packaging and Industrial Products, North Andover, MA) on foam trays (17S; McCune Paper Company, Salina, KS) with a Dri-Loc soaker pad (AC-50; Sealed Air Corp, Duncan, SC), and (4) HiOx-MAP converted to PVC after 2 d and stored as PVC (HiOx-PVC). Steaks packaged in MAP (Ross Jr. S-3180, Ross, Midland, VA) were placed in 4.32-cm deep rigid plastic trays (CS977, Cryovac Sealed Air Corp., Duncan, SC), covered with oxygen barrier film (Lid 550; 1.0 mils; less than 20.0 oxygen transmission cc/24 h/m^2 at 4.4 °C with 100% relative humidity, and moisture vapor transmission less than 0.1 g/24 h/645.2 cm^2 at 4.4 °C and 100% relative humidity; Cryovac

Sealed Air Corp., Duncan, SC) with a soaker pad, flushed with (80% O_2 and 20% N_2) gas blends, and sealed with shrinkable barrier film. Steaks were displayed at 2 °C for 10 d under 2150 ± 50 lux of continuous fluorescent lighting (bulb F32T8/ADV830, 3000 K, CRI = 86; Phillips, Bloomfield, NJ) in open-top display cases (DMF8; Tyler Refrigeration Corp., Niles, MI). Packages were rotated daily to minimize case location effects.

6.3.3 Proximate and pH Determinations

The pH of steaks was measured on d 14 postmortem by inserting the tip of a pH probe (MPI pH probe, glass electrode, Meat Probes Inc., Topeka, KS) into the steaks. A composite sample of tissue remaining after packaging from each of the 18 muscles was used for proximate analysis. Samples were analyzed in triplicate for protein [LECO Combustion Analysis (AOAC Official Method 990.03; 18)] and moisture and fat [CEM SMART and SMART Trac systems (AOAC PVM 1:2003; 19)].

6.3.4 Color Analysis

Steaks were scanned in triplicate on display d 0, 2, 4, 10, and 14 for instrumental color (HunterLab MiniScan^{TM} XE Plus Spectrophotometer 45/0 LAV, 2.54-cm-diameter aperture, 10 ° standard observer; Hunter Associates Laboratory, Inc., Reston, VA). Values for CIE L^*, a^*, and b^* (Illuminant A) were collected, and hue angle ($tan^{-1}b^*/a^*$) and saturation index ($(a^{*2} + b^{*2})^{1/2}$) were calculated (20) from instrumental measures. Estimation of DMb and OMb was computed using reflectance data from 400-700 nm with a HunterLab MiniScan XE Plus Spectrophotometer (D/8-S, 14.3-mm-diameter aperture; Hunter Associates Laboratory, Inc., Reston, VA). Wavelengths isobestic for DMb and OMb were measured and converted to K/S values, and ratios of wavelengths were inserted into equations with values for 0% and 100% of the pigment form being calculated (20).

6.3.5 Application of NIR Tissue Oximeter on Beef Skeletal Muscles

Myoglobin redox forms present in the muscle portions on display d 0, 2, 4, and 10 were evaluated using a NIR tissue oximeter (OxiplexTS model 96208, ISS, Champaign, IL), which is based on the relative tissue transparency for light in the NIR region and on the O_2-dependent absorption changes of Mb. The OxiplexTS is a FDMD spectrophotometer that permits calculation of absolute (μM) concentrations of OMb and DMb using dynamic calculation of the reduced scattering coefficient. It was not possible to distinguish between Hb and Mb because of their nearly identical spectral characteristics; however, there is usually less than 5 to 8% Hb in post-rigor muscle. The absorption changes at the discrete wavelengths were converted into concentrations of OMb and DMb. The concentration of MMb was estimated as 100 - ([OMb] + [DMb]).

The NIR tissue oximeter was calibrated each test day according to the manufacturer's recommendations. Simultaneous NIR oximetry measurements were made using a single probe consisting of eight laser diodes operating at two different wavelengths (692 and 834 nm, four at each wavelength) and a photomultiplier tube (21). The laser diodes and photomultiplier tube were connected by optical fibers consisting of two parallel rows of emitter fibers and one detector fiber bundle arranged in four source-detector separations of 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. Measurements were obtained by placing the probe longitudinally on the top of the steak with packaging material intact (Figure 6.1).

6.3.6 Statistical Analysis

The experimental design for the experiment was a randomized complete block with a split-plot design, and within the whole plot, carcass served as a random effect (a block) and muscles served as the treatments. Within the subplot, packaging format served as the

treatment assigned to steaks (subplot experimental units). Data were analyzed separately for each variable (OMb, DMb, and MMb). Multiple oximetry scans on each steak (three per steak) were averaged for statistical analysis. Thus, for each variable, the analysis consisted of 288 total observations [six animals (replications) × three muscles per animal × four packaging formats per muscle × four display days per packaging format]. Type-3 tests of fixed effects for muscle, muscle × packaging, muscle × day, and muscle × packaging × day were evaluated using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). F-test denominator degrees of freedom were estimated using the Satterthwaite adjustment. Least squares means for significant F-tests were separated using least significant differences.

To assess the strength of relationships between reflecto-spectrophotometric and NIR tissue oximeter measurements, simple correlation coefficients were computed using the CORR procedure of SAS. Correlation graphics of fitted data with 95% prediction limit and 95% confidence limits were generated using the ODS function of SAS.

6.4 Results

6.4.1 pH, Moisture, Protein, Fat, and Myoglobin

All muscles had a pH between 5.5 and 5.7 (Table N.1). The ST had the most moisture (74.4%; $p < 0.05$), least fat (1.5%), and intermediate protein (22.4%; $p < 0.05$) of the three muscles. The LL had the highest protein (23.6%) and intermediate fat (4.2%; $p < 0.05$). The PM had the most fat (5.4%), least protein (21.9%), and intermediate moisture (71.2%; $p < 0.05$). Myoglobin concentrations were greatest in the LL, intermediate in the PM, and least in the ST ($p < 0.05$). The overall means for oximeter reduced scattering coefficients varied by muscle at 692 nm but not at 834.

6.4.2 NIR Tissue Oximetry of DMb and OMb

A muscle × packaging × day interaction ($p < 0.05$) occurred for NIR tissue oximetry response for OMb and DMb (Table N.2). On d 0, the LL, PM, and ST portions packaged in HiOx and HiOx-PVC MAP had 25-32% OMb, but samples in PVC and VAC had levels from 2.6 to < 6%. By d 2, the HiOx and HiOx-PVC packages contained 50 to 78% OMb, whereas OMb levels in PVC were 35 to 42% and in VAC were < 5%. On d 4, OMb decreased significantly in the HiOx and HiOx-PVC samples for the LL and PM muscles, but OMb in the ST was equal to or higher than d 2 quantities. By d 10, OMb levels in the HIOX and HiOx-PVC further decreased ($p < 0.05$) but were still higher than on d 0. Oxygenated myoglobin in portions from the three muscles packaged in PVC changed ($p < 0.05$) from 5 to 12% on d 0 to 35 to 44% on d 2 and 4. By d 10, PVC-packaged portions had significantly less OMb. Portions packaged in VAC had the lowest (generally 3 to 5%) OMb throughout display.

The LL and PM portions packaged in HiOx-PVC MAP had significantly higher levels of OMb (30%) than the ST on display d 0, likely because of their greater Mb content. On d 2 of display, Mb oxygenation of LL portions increased ($p < 0.05$) to a greater extent than in PM and ST (77% vs. 62% in PM and 52% in ST) and continued to retain the same level through d 4 of display. The most rapid decrease ($p < 0.05$) in OMb level occurred in PM and ST muscles packaged in HiOx-PVC MAP after d 4. Oxygenation of Mb was considerably less ($p < 0.05$) for all three muscles ($p < 0.05$) packaged in PVC than for either of the HiOx MAP systems (Table N.2). At the end of display, the LL muscle had the most stable red color, the PM retained the least OMb, and the ST was intermediate. Portions of all three muscles in VAC contained ≤ 5% OMb.

The NIR tissue oximetry response of all three muscles for DMb exhibited a similar but opposite pattern (Table N.2). All samples initially had relatively high amounts of DMb because they were measured shortly after placement in the packages. Samples in the HiOx

and HiOx-PVC MAP packaging systems contained significantly less DMb (66-73 %) on d 0 than the PVC and VAC packages (85-94%). Furthermore, the DMb decreased ($p < 0.05$) by d 2 to 20% in LL, 29% in PM, and 39% in the ST. The LL, PM, and ST portions in HiOx packages contained relatively the same level of DMb from d 4 to 10. Samples in HiOx-PVC decreased in DMb on d 2 and 4, which accompanied their removal from the HiOx environment. However, LL portions packaged in PVC had 92.5% DMb on d 0, which decreased ($p < 0.05$) to 49.4% on d 2 of display, and maintained predominately DMb throughout display. All the three muscles in VAC contained 93-97% DMb and, as expected, maintained predominately DMb throughout display.

6.4.3 Reflecto-spectrophotometry of DMb and OMb

A muscle × packaging × day interaction ($p < 0.05$) also occurred for reflectance measurements of OMb and DMb (Table N.3) on the meat's surface. Percentages of OMb were higher ($p < 0.05$) for LL and ST muscles than for the PM in all three aerobic packaging systems throughout display, and, as expected, OMb decreased as time postmortem increased. The LL samples packaged in HiOx, HiOx-PVC, and PVC contained the most ($p < 0.05$) OMb from display d 2 to 10, whereas OMb in the ST and PM samples with comparable packaging was intermediate and lowest, respectively. The OMb of all three muscles packaged in VAC was essentially the same and did not change ($p > 0.05$) during display.

The DMb levels followed essentially the same pattern for the three aerobic packaging systems during display until d 10, when less reduced redox forms occurred as color deteriorated (Table N.3). During display, the amount of OMb declined but DMb levels were maintained in the HiOx-PVC and PVC MAP packages. The VAC packages, compared with HiOx, HiOx-PVC, and PVC for all three muscles had, as expected, the greatest ($p < 0.05$) DMb at the beginning of display; DMb in the VAC packages increased ($p < 0.05$) on d 2 and thereafter remained constant throughout the display period. Reflecto-spectrophotometric

measurements demonstrated that in general, LL portions in oxygen-containing packaging had the highest percentages of OMb, PM portions had the least, and the ST portions were intermediate. Percentages of DMb were greater for PM and ST muscles than for the LL.

6.4.4 NIR Tissue Oximetry and Reflecto-spectrophotometry of MMb

Because FDMD NIR tissue oximeter directly measured only DMb and OMb, estimates of MMb were calculated and are presented in Table 6.4. At d 0, MMb estimates were $< 3.8\%$. On d 2, LL portions in HiOx and all muscles in VAC had low estimates of MMb, whereas LL muscle in PVC and the PM and ST muscles in all non-VAC packaging had increased MMb. At d 4, MMb estimates further increased for all non-VAC samples, and the largest estimated values of MMb occurred by d 10.

Levels of MMb measured on the meat's surface by reflectance (Table 4) followed a pattern of change similar to MMb estimated from NIR data, but the absolute values were different. Estimates of MMb by reflectance increased as display time increased, especially between d 4 and 10. Packages containing oxygen had more MMb than samples in VAC. At the end of display, the PM had the most MMb, the ST was intermediate, and the LL had the least.

6.4.5 Instrumental Meat Color Measurements

Values for L^* (Figure 6.2 A, D, and G) during display for all muscles in all packaging formats were more stable than values for a^* (Figure 6.2 B, E, and H) and b^* (Figure 6.2 C. F. and I). Products in VAC were consistently darker (lower L^* values). Each muscle experienced a loss ($p < 0.05$) of redness (smaller a^* values) during display in aerobic packaging systems (HiOx, HiOx-PVC, and PVC). The a^* values for PM declined ($p < 0.05$) more rapidly during display than those for the LL and ST (Figure 6.2 B, E, and H). Overall, the LL was the most red

(greater a^*), the ST was intermediate, and the PM was least red throughout display. Values for a^* for all muscles were most stable in VAC. Values for b^* were most stable for products in VAC. In general, b^* values declined ($p < 0.05$) similar to a^* values throughout display in all three aerobic packaging systems (Figure 6.2 C, F, and I) but to a lesser magnitude. All three muscles packaged in VAC had little or no change in b^* throughout display.

Chroma (vivid redness calculated from a^* and b^* values) decreased ($p < 0.05$) for all muscles during display in all three aerobic packaging systems (Figure 6.3 A, B, and C). Chroma values of LL and ST were stable and declined gradually ($p > 0.05$) during display; PM chroma values declined rapidly by d 4 with only a slight further decrease at d 10. At d 10, chroma values of all three muscles were at their lowest values; the LL, ST, and PM had the highest, intermediate, and lowest values, respectively. Chroma values in the VAC packages changed little during display. Hue angle, a measure of the loss of surface redness of meat, increased ($p < 0.05$) most for the PM; ST was intermediate, and LL had least change in hue (Figure 6.3 D, E, and F).

6.4.6 Correlation of Reflecto-spectrophotometric and NIR Tissue Oximetry Measurements

To graphically show the strength of the relationship between reflectance and NIR tissue oximeter measurements, data were plotted with a fitted line and confidence lines in Figure 6.4. Correlations between reflectance and NIR oximetry data were the lowest (r = 0.19, $p < 0.27$ to 0.69, $p < 0.0001$) on d 0 regardless of the Mb redox form (Figure 6.4 A, E, and I). For OMb and DMb, significant correlations of ≥ 0.93 were obtained between the two measurements on d 2, 4, and 10 for OMb (Figure 6.4 B, C, and D) and DMb (Figure 6.4 F, G, and H), which was located principally below the meat's surface. Correlations for MMb at d 2, 4, and 10 were significant but lower than those for OMb and DMb.

Overall, the reflecto-spectrophotometic data for Mb redox forms and colorimetric values ($L^*a^*b^*$) were highly correlated after d 0 and accounted for 86 to 94% of the variation between the two variables for OMb (located mainly on the surface) and DMb (located mainly subsurface). Correlations of the two measures for MMb (located both below and on the meat's surface) accounted for 59 to 84.6% of the variation.

6.5 Discussion

The muscles used in this study had a pH, proximate composition, myoglobin concentration, and initial visual color typically expected for those cuts; therefore, the data collected should be a reasonable test for the methodologies applied. The main focus of this study was to A) investigate the potential use of FDMD-based NIR tissue oximetry as a noninvasive, continuous, direct method for determining the oxygenation and hemodynamics of post-rigor beef skeletal muscle and B) compare NIR tissue oximetry data with reflectance measures of meat color and Mb redox forms created by various packaging systems and display using muscles inherently different in Mb content and color stability. The principal new finding of our study is that the quantitative determination of absolute levels of OMb and DMb and the indirect estimate of MMb is possible using NIR tissue oximetry.

6.5.1 NIR Tissue Oximetry and Reflectance of Mb Redox Forms

Previous research (Mancini & Hunt, 2005; McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005; O'Keefe & Hood, 1982; Renerre & Labas, 1987; Seyfert, Mancini, Hunt, Tang, Faustman & Garcia, 2006) has shown that the color stability of the three muscles in the present study would be ranked as LL > ST > PM. Our NIR tissue oximetry and reflectance data confirmed that muscle ranking for meat displayed in aerobic packaging systems. O'Keeffe & Hood (1982) reported that the LL had > 4 d more color stability than the

PM. This seems related to the greater population of white-type muscle fibers in the LL vs. the PM, which is a muscle with predominately red-type fibers. Most of the ST is whiter than the LL, but the ST has fewer intermediate type fibers that seem to contribute positively to color life (Hunt & Hedrick, 1977a). Consequently, the LL and ST, with predominantly glycolytic fibers, are more color stable, and the PM, a muscle with more mitochondria and aerobic metabolism is least stable in maintaining oxygenated Mb during display.

In living animals, different muscles have unique physiological roles and differ in their micro-anatomy, biochemical activity, and Mb concentration (Hunt & Hedrick, 1977a; Hunt & Hedrick, 1977b; Lanari & Cassens, 1991). Muscles with greater oxidative activity show greater oxygen consumption (Hunt & Hedrick, 1977a; Madhavi & Carpenter, 1993; Mancini & Hunt, 2005; Mohan, Hunt, Barstow, Houser, Bopp & Hueber, 2010; Seyfert, Mancini, Hunt, Tang, Faustman & Garcia, 2006) and are more color labile than those with predominantly glycolytic activity (O'Keefe & Hood, 1982; Madhavi & Carpenter, 1993;, Mancini & Hunt, 2005; McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005). Post-rigor skeletal muscles differ in fiber types and metabolic function on the basis of their specific physiological role (DeVore & Solberg, 1975; Hunt & Hedrick, 1977b). Thus, each muscle experiences different levels of oxygen demand and consumption when exposed to atmospheric oxygen (DeVore & Solberg, 1975; Lanari & Cassens, 1991; Renerre & Labas, 1987; Seyfert, Mancini, Hunt, Tang, Faustman & Garcia, 2006).

Colour stability is primarily an interaction between a muscle's OC and its ability to keep metmyoglobin reduced (Mancini, Hunt & Kropf, 2003; Mancini & Hunt, 2005). Generally, muscles with greater OC are less color stable during display than those with less OC (Kropf, 1993; Lanari & Cassens, 1991; Tang, Faustman, Hoagland, Mancini, Seyfert & Hunt, 2005). Other researchers have found that MMb reduction is also critically related to color stability (Kropf, 1993; Mancini & Hunt, 2005; McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005; Mohan, Hunt, Barstow, Houser, Bopp & Hueber, 2010; Seyfert, Mancini, Hunt, Tang,

Faustman & Garcia, 2006). Disparities in the importance of OC vs. MMb reduction are likely related to the unknown nature of the chemical and structural interactions for the two processes in post-rigor meat (Mancini & Hunt, 2005).

Tang *et al.*, (2005) reported that there was a direct relationship between the postmortem muscle OC and meat color stability in LL from Holsteins. Colour-labile muscles, such as the PM, have a higher OC rate than color-stable muscles (Mancini & Hunt, 2005; McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005). Tang *et al.*, (2005) reported that, in general, the PM tended to have the greatest OC and the LL had the least. Greater OC also decreases surface oxygen penetration, resulting in a shallow OMb layer and a thinner MMb layer below the surface that moves to the surface faster (Bendall & Taylor, 1972; McKenna, Mies, Baird, Pfeiffer, Ellebracht & Savell, 2005). Because DMb is more prone to oxidation and less stable than OMb (O'Keefe & Hood, 1982), a higher proportion of DMb in muscles with greater OC would discolor faster because the subsurface layer of MMb would appear more rapidly compared with muscles that have less OC. Currently available meat color measurement techniques such as reflectance colorimetry and spectrophotometric methods are limited to surface measurement of meat color because of the inability of the visible light to penetrate the meat surface. In contrast, the FDMD NIR tissue oximeter penetrates several centimeters below the meat surface, and its ability to determine the oxygen-dependent absorption of Mb and Hb has been demonstrated (Hueber *et al.*, 2001; Marcinek, Amara, Matz, Conley & Schenkman, 2007). Bowen (1949) demonstrated that wavelengths of the NIR lights are differentially absorbed by the oxygenated and deoxygenated forms of Hb.

Hueber *et al.*, (2001) used FDMD NIR tissue oximeter for monitoring tissue hemoglobin concentration and oxygenation in the brain of newborn piglets during periods of hypo- and hyperoxia. In their study, a strong linear correlation ($r^2 = 0.98$) was observed between tissue oxygen saturation and the average of arterial and venous saturation. Because Mb and Hb absorb at the same wavelength in the NIR region, a similar approach was undertaken in

this study to investigate the potential use of FDMD NIR tissue oximeter for determining Mb oxygen saturation and its redox stability under conditions simulating retail packaging and display to predict the color stability of beef skeletal muscles.

6.5.2 NIR Tissue Oximetry vs. Reflectance Spectroscopy

A continuous challenge in the meat industry is obtaining a reliable technique that can provide important information on meat quality traits throughout production and retail display. Numerous methods have been developed, but most are either invasive or difficult to implement as a reliable, online, continuous measurement of meat quality. The techniques used for the assessment of meat quality serve as diagnostic tools to optimize and control quality. Reflecto-spectrophotometry is an established method for the assessment of meat discoloration and MMb accumulation on meat surfaces. Quantification of Mb redox forms using reflectance data often produces values that are either less or more than 100%, which causes problems with accuracy. Moreover, reflecto-spectrophotometry provides only an indirect estimate of Mb redox forms on meat surfaces, and there was no clear trend observed for the beef muscles of varying color stability and Mb oxygen status. Reflectance measures may be excellent for describing appearance of meat color, but they offer very little information about Mb redox chemistry at subsurface levels.

Although qualitative and quantitative reflectance data have been very useful in meat color chemistry and for providing insight into meat color problems, these data are not particularly useful for predicting or relating changes in Mb redox status, muscle oxygen status, and muscle-color stability. For the characterization of the surface discoloration, decreases in a^* values (decrease in red color) and chroma and/or hue angles are generally accepted monitoring meat color changes. However, a^* values obtained from reflecto-spectrophotometric measurements seem to be useful only for the qualitative characterization of the surface color of meats. The use of an NIR tissue oximeter on meat provided complimentary information

such as Mb redox status, muscle oxygen status, and potential information about muscle color stability during a simulated retail display. Moreover, the instrumental color data obtained for a^* values corresponded well to the NIR tissue oximeter measurements of Mb redox forms, and the data seem to be adaptable to color stabilization of postmortem beef skeletal muscles in several packaging formats during display. Also, NIR tissue oximetry results seemed to be able to differentiate among high, intermediate, and low color stable beef skeletal muscles on the basis of Mb oxygen status and redox dynamics during retail display. Beef skeletal muscles with greater color stability (LL and ST) exhibited greater total muscle oxygen saturation combined with a lower OC resulting in a higher OMb concentration than the color-labile muscle (PM). The results presented in this paper clearly demonstrate the advantages of using a FDMD-based NIR tissue oximeter to understand the chemistry associated with the color change in postmortem beef skeletal muscles during retail display. The correlation data presented in Figure 6.4 are interesting where the NIR tissue oximeter measurements show a high, positive correlation with reflecto-spectrophotometric data, indicating that information provided by the NIR tissue oximeter is reliable. The rate at which meat discolors during retail display is considered one of the most important traits of meat retailing. The ability of the NIR tissue oximeter to relate with the meat discoloration rate and the relative change in Mb redox forms with a high degree of accuracy highlights the potential of using an NIR tissue oximeter to predict several quality traits important to meat color chemistry in a rapid, noninvasive manner. Overall, NIR tissue oximetry data appear to be reliable for determining the color differences and color stability of beef skeletal muscles, and agreement of the NIR tissue oximeter measurements with instrumental color parameters suggests that NIR tissue oximetry has potential for predicting several traits important to meat color chemistry.

It is easier to conduct repeated color measurements of meat packaged in PVC overwrap than in MAP trays with a headspace between the film and the meat. Color measurements

of MAP packaged meat can be taken by inverting the package so that the meat contacts the film, but repeated measurements taken in this manner result in film stains, fat smearing, and unwanted light scatter. Other researchers open packages before instrumental color measurement, allowing direct contact between the meat surface and the instrument's aperture, but other packages must be available for later storage periods. The NIR tissue oximetry measurements of meat color do not circumvent these packaging constraints, but they do expand the data from surface characterization to internal data that relate to factors believed to be related to color stability such as Mb oxygen status, NADH pools, and MMb reduction mechanisms (Mancini & Hunt, 2005; Millikan, 1939; Seyfert, Mancini, Hunt, Tang, Faustman & Garcia, 2006; Urbin & Wilson, 1961).

In this study, we demonstrated for the first time that FDMD NIR tissue oximetry was able to detect and quantitatively measure the dynamic changes in surface and subsurface levels of Mb redox forms of post-rigor muscle packaged and displayed under simulated industry conditions. Additionally, the effects of packaging, postmortem storage, and display conditions on Mb redox stability were highly correlated (r values > 0.9) between the NIR tissue oximetry and reflectance methods. Our data clearly show the potential for using a FDMD NIR tissue oximeter for measurement of OMb and DMb of post-rigor muscle and the indirect estimation of MMb. Furthermore, the NIR tissue oximeter detected a heterogeneous blend of Mb redox forms on (or in) meat during packaged storage and display.

6.6 Conclusion

In this study, we demonstrated for the first time that FDMD NIR tissue oximetry was able to detect and quantitatively measure the dynamic changes in surface and subsurface levels of Mb redox forms of post-rigor muscle packaged and displayed under simulated industry conditions. Additionally, the effects of packaging, postmortem storage, and display condi-

tions on Mb redox stability were highly correlated (r values > 0.9) between the NIR tissue oximetry and reflectance methods. Our data clearly show the potential for using a FDMD NIR tissue oximeter for measurement of OMb and DMb of post-rigor muscle and the indirect estimation of MMb. Furthermore, the NIR tissue oximeter detected a heterogeneous blend of Mb redox forms on (or in) meat during packaged storage and display.

Abbreviations used

LL, *longissimus lumborum*; PM, *psoas major*; ST, *semitendinosus*, DMb, deoxymyoglobin; MMb, metmyoglobin; OMb, oxymyoglobin; FDMD, frequency-domain multidistance; NIR, near infrared; Hb, hemoglobin; Mb, myoglobin, OC, oxygen consumption; NADH, nicotinamide adenine dinucleotide; HiOx-MAP, high oxygen modified atmosphere; PVC, polyvinyl chloride; VAC, vacuum packaging.

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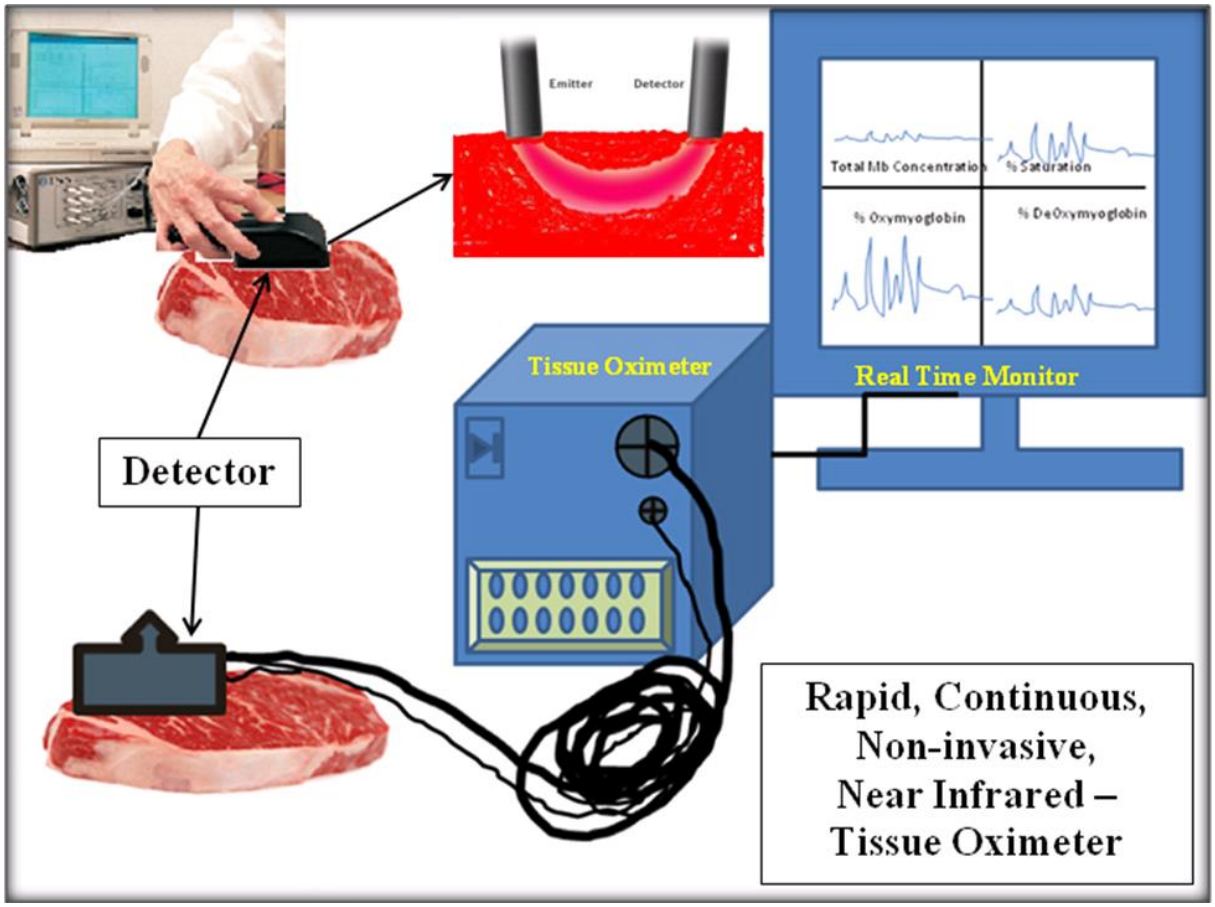

Figure 6.1: *A diagrammatic representation of measuring meat color properties and myoglobin redox with a near-infrared tissue oximeter.*

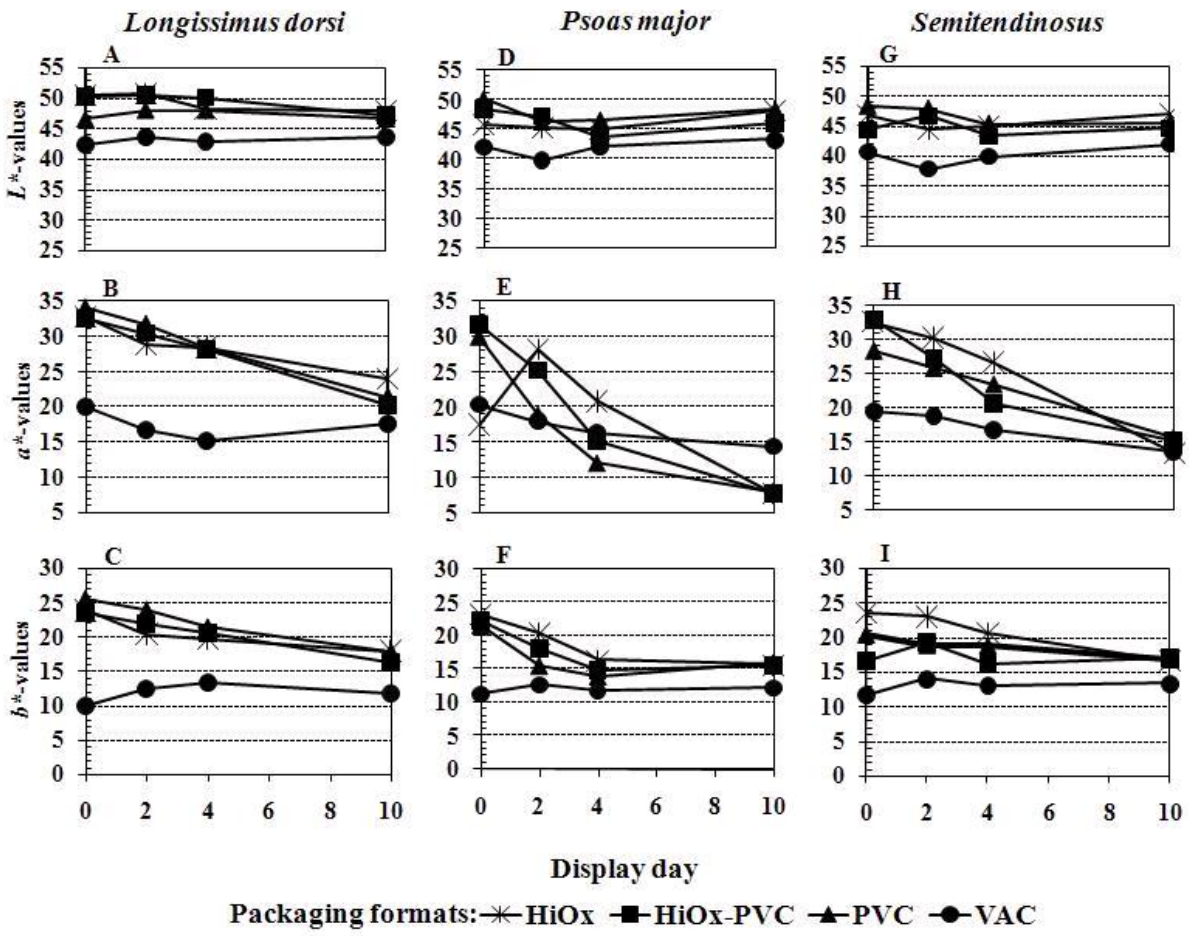

Figure 6.2: *Muscle type × packaging × display day effects on color properties (L^*, a^*, and b^*) of bovine Longissimus lumborum (A, B, and C), Psoas major (D, E, and F), and Semitendinosus (G, H, and I) muscles. HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 h and was then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging. Larger values for L^*, a^* and b^* indicate a lighter, redder, or more yellow color, respectively.*

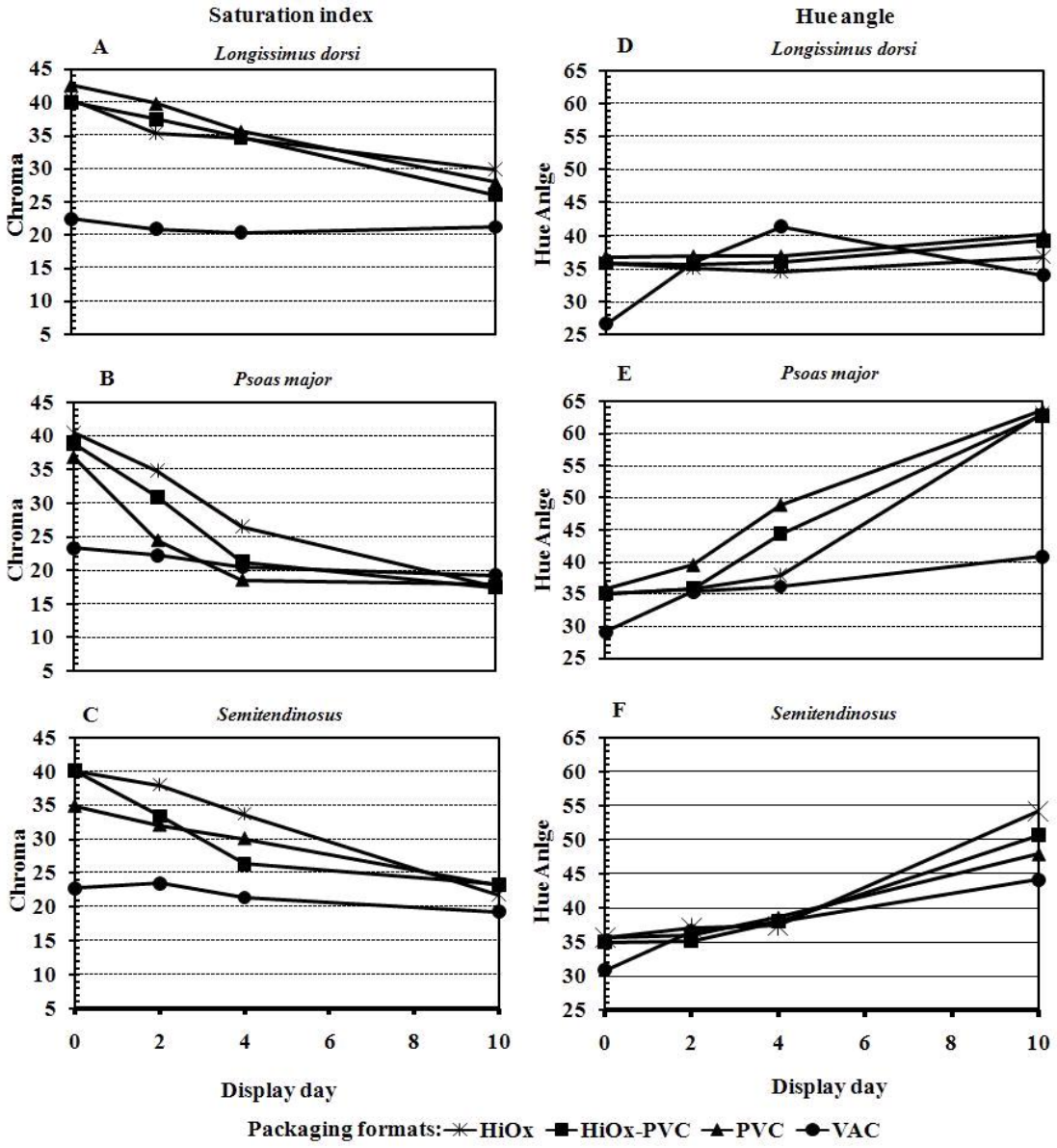

Figure 6.3: *Muscle type × packaging × display day for saturation index (chroma; **A**, **B**, and **C**) and hue angle (**D**, **E**, and **F**) of bovine longissimus lumborum, semitendinosus, and psoas major muscles. HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 h and was then converted to PVC; PVC = Polyvinyl chloride; VAC = Vacuum packaging.*

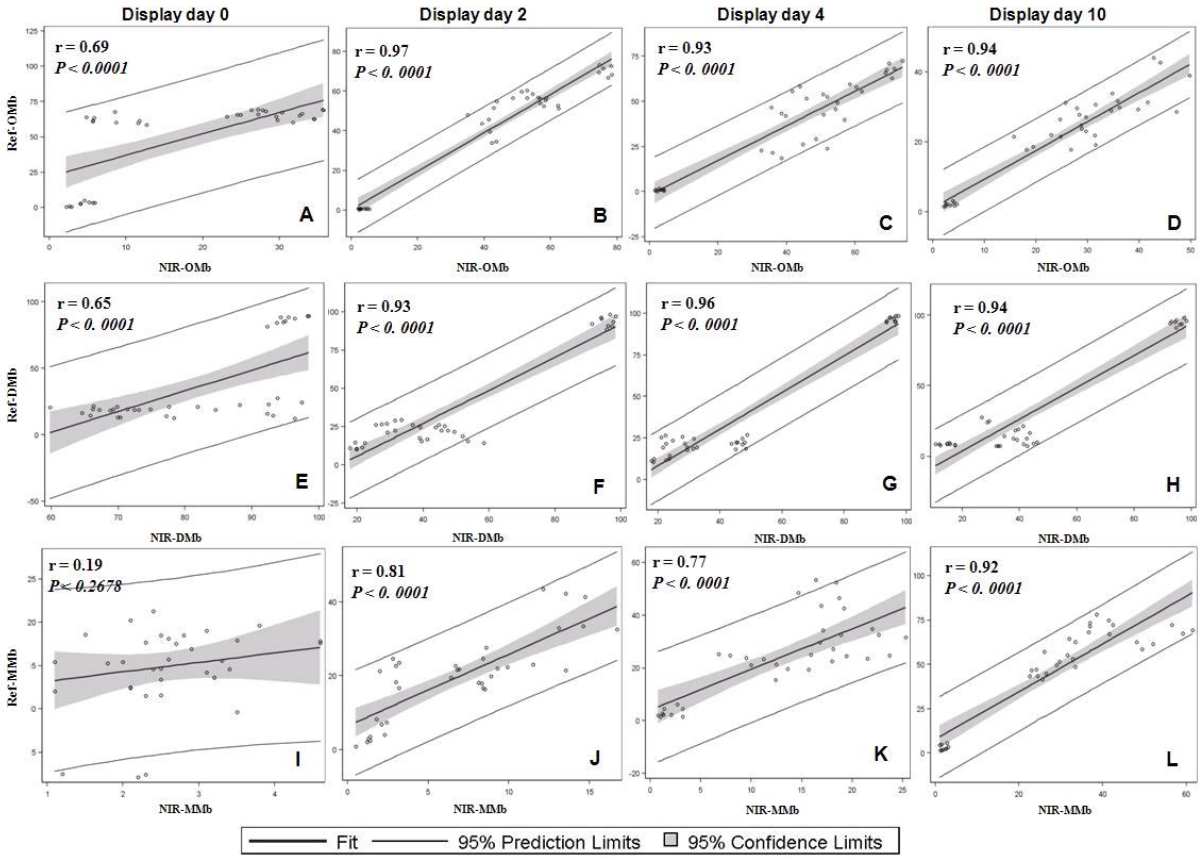

Figure 6.4: *Scatter plot matrix of correlation between reflecto-spectrophotometric and near-infrared (NIR) tissue oximetry data. Ref-OMb = OMb measured by reflecto-spectrophometric method. NIR-OMb = OMb measured by NIR tissue oximetry. Ref-DMb = DMb measured by reflecto-spectrophometric method. NIR-DMb = DMb measured by NIR tissue oximetry. Ref-MMb = MMb measured by reflecto-spectrophometric method. NIR-MMb = MMb measured by NIR tissue oximetry.*

Table 6.1: *Least Squares Means*[a] *for pH, Proximate Analysis, and Reduced Scattering Coefficients*[b] *of Three Beef Muscles*[c]

	muscles			
trait	LL	PM	ST	SE[d]
pH	5.6^x	5.5^x	5.7^x	0.04
moisture(%)	71.3^x	71.2^x	74.4^y	0.36
protein(%)	23.6^z	21.9^x	22.4^y	0.27
fat(%)	4.2^y	5.4^z	1.5^x	0.12
ash(%)	0.8^x	1.4^y	1.6^y	0.19
myoglobin (mg/g)	6.9^z	5.1^y	4.2^x	0.07
μ_s' (λ = 692 nm)	6.4	2.1	4.6	
μ_s' (λ= 834 nm)	5.2	5.1	5.2	

[a] xyz = Within a row, means with a different letter differ ($p < 0.05$).

[b] μ_s' = Overall mean (summed over all treatments) for the reduced scattering coefficients for tissue oximeter data.

[c] LL = *Longissimus lumborum*, PM = *Psoas major*, ST = *Semitendinosus*

[d] Standard error.

Table 6.2: *Least Squares Means*[a] *for Percentages of Oxymyoglobin and Deoxymyoglobin in Three Beef Muscles*[b] *in Four Packaging Formats*[c] *Evaluated by NIR Tissue Oximetry During 10 d of Display*

		Mb[d] redox form							
		OMb[e] display day				DMb[f] display day			
muscle	packaging format	0	2	4	10	0	2	4	10
LL	HiOx	28.2^{fw}	78.3^{jz}	71.4^{ky}	49.8^{jx}	70.3^{fz}	19.7^{efw}	18.6^{ew}	17.4^{ew}
	HiOx-PVC	27.8^{fw}	77.2^{jz}	70.6^{ky}	47.2^{jx}	69.9^{fy}	19.8^{ew}	22.6^{ew}	28.7^{fx}
	PVC	5.7^{ew}	43.7^{gy}	41.6^{gy}	35.8^{ix}	92.5^{hy}	49.4^{hx}	47.2^{gx}	41.2^{gw}
	VAC	2.6^{ew}	2.1^{ew}	2.2^{ew}	2.4^{ew}	94.9^{hw}	96.1^{iw}	96.4^{iw}	96.5^{jw}
PM	HiOx	31.7^{gw}	62.2^{iy}	56.9^{ix}	31.5^{gw}	66.2^{ez}	28.9^{fy}	21.1^{ex}	11.9^{fw}
	HiOx-PVC	29.7^{gx}	62.3^{iz}	51.8^{hy}	26.7^{gw}	67.2^{ex}	29.1^{gw}	29.5^{fw}	31.8^{jw}
	PVC	11.7^{fw}	42.3^{gz}	38.5^{fy}	18.1^{fx}	84.5^{fx}	45.6^{gw}	45.1^{hw}	44.9^{iw}
	VAC	5.2^{ew}	4.8^{ew}	4.2^{ew}	4.2^{ew}	93.7^{hw}	93.9^{iw}	94.8^{iw}	94.6^{jw}
ST	HiOx	24.5^{fw}	52.8^{hy}	60.3^{jz}	34.5^{ix}	72.4^{fy}	39.1^{gx}	21.7^{ew}	17.4^{fw}
	HiOx-PVC	24.9^{fw}	51.2^{hz}	50.6^{hy}	28.9^{hx}	73.1^{fy}	38.9^{gx}	31.3^{gw}	39.1^{hx}
	PVC	5.7^{ew}	35.0^{fy}	35.5^{fy}	27.9^{hx}	93.1^{gy}	51.9^{gx}	48.4^{hwx}	46.1^{hw}
	VAC	4.1^{ew}	2.9^{ew}	3.3^{ew}	4.7^{ew}	94.7^{hw}	96.6^{iw}	95.9^{iw}	92.9^{iw}

[a] efghijk = Within a column across muscles, means with a different letter differ ($p < 0.05$). wxyz = Within a row and myoglobin redox form, means with a different letter differ ($p < 0.05$). Standard error for packaging × display day or muscle = 3.06 - 3.74.

[b] LL = *Longissimus lumborum* = color stable, ST = *Semitendinosus* = intermediate color stability, PM = *Psoas major* = color labile.

[c] HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 h and was then converted to PVC; PVC = Polyvinyl chloride overwrap; VAC = Vacuum packaging.

[d] Myoglobin

[e] Oxymyoglobin

[f] Deoxymyoglobin

Table 6.3: *Least Squares Means*[a] *for Percentages of Oxymyoglobin and Deoxymyoglobin in Three Beef Muscles*[b] *in Four Packaging Formats*[c] *Evaluated by Reflectance Spectrophotometry During 10 d of Display*

		Mb[d] redox form							
		OMb[e] display day				DMb[f] display day			
muscle	packaging format	0	2	4	10	0	2	4	10
LL	HiOx	68.0^{gx}	68.3^{jx}	68.3^{mx}	39.0^{iw}	13.4^{ew}	10.3^{ew}	10.7^{ew}	7.8^{ew}
	HiOx-PVC	68.9^{gy}	66.7^{jy}	63.0^{lx}	28.7^{hw}	13.4^{ew}	10.6^{ew}	11.7^{ew}	24.1^{fx}
	PVC	61.6^{fy}	56.5^{hx}	55.7^{jx}	31.7^{hw}	23.1^{gw}	21.6^{gw}	21.1^{fw}	21.4^{fw}
	VAC	0.9^{ew}	0.8^{ew}	0.3^{ew}	1.6^{ew}	85.7^{hw}	90.9^{ix}	95.2^{hx}	94.0^{gx}
PM	HiOx	60.4^{fz}	52.9^{hy}	39.8^{hx}	19.2^{fw}	19.4^{fx}	27.2^{hy}	25.3^{gy}	8.5^{ew}
	HiOx-PVC	62.0^{fz}	51.2^{hy}	24.0^{gx}	17.8^{fw}	19.0^{fx}	21.2^{gx}	19.5^{fx}	7.4^{ew}
	PVC	61.6^{fy}	34.0^{fx}	18.6^{fw}	17.7^{fw}	18.8^{fx}	22.6^{gx}	18.1^{fx}	8.9^{ew}
	VAC	3.7^{ew}	0.7^{ew}	0.5^{ew}	1.7^{ew}	84.3^{hw}	96.2^{ix}	97.9^{hx}	96.7^{gx}
ST	HiOx	65.7^{gy}	60.3^{ix}	58.0^{kx}	29.0^{kw}	18.9^{fx}	24.5^{gy}	19.4^{fx}	8.4^{ew}
	HiOx-PVC	65.7^{gz}	59.9^{jy}	54.1^{jx}	23.7^{gw}	18.9^{fx}	17.8^{fx}	18.6^{fx}	11.7^{ew}
	PVC	61.0^{fy}	48.1^{gx}	46.9^{ix}	29.6^{hw}	14.8^{ex}	18.9^{fy}	18.8^{fy}	10.1^{ew}
	VAC	2.8^{ew}	0.8^{ew}	1.0^{ew}	2.2^{ew}	84.7^{hw}	98.2^{ix}	96.9^{hx}	95.6^{gx}

[a] efghijklm = Within a column across muscles, means with a different letter differ ($p < 0.05$). wxyz = Within a row and myoglobin redox form, means with a different letter differ ($p < 0.05$). Standard error for packaging display day or muscle = 3.06 - 3.74.

[b] LL = *Longissimus lumborum* = color stable, ST = *Semitendinosus* = intermediate color stability, PM = *Psoas major* = color labile.

[c] HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 h and was then converted to PVC; PVC = Polyvinyl chloride overwrap; VAC = Vacuum packaging.

[d] Myoglobin

[e] Oxymyoglobin

[f] Deoxymyoglobin

Table 6.4: *Estimated Percentage of Metmyoglobin[a] in Three Beef Muscles[b] in Four Packaging Formats[c] Evaluated by NIR Tissue Oximetry and Reflectance Spectrophotometry During 10 d of Display*

		Mb[d] redox form							
		NITO[e]				RS[f]			
		display day				display day			
muscle	packaging format	0	2	4	10	0	2	4	10
LL	HiOx	1.5	2.0	10.0	32.8	18.6	21.4	21.0	53.2
	HiOx-PVC	2.3	3.0	6.8	24.1	17.7	22.7	25.3	47.2
	PVC	1.8	6.9	11.2	23.0	15.3	21.9	23.2	46.9
	VAC	2.5	1.8	1.4	1.1	13.4	8.3	4.5	4.4
PM	HiOx	2.1	8.9	22.0	56.6	20.2	19.9	34.9	72.3
	HiOx-PVC	3.1	8.6	18.7	41.5	19.0	27.6	46.5	74.8
	PVC	3.8	12.1	16.4	37.0	19.6	43.4	53.3	73.4
	VAC	1.1	1.3	1.0	1.2	12.0	3.1	1.6	1.6
ST	HiOx	1.1	8.1	18.0	48.1	15.4	18.2	22.6	62.6
	HiOx-PVC	2.0	9.9	18.1	32.0	15.4	22.3	27.3	64.6
	PVC	1.2	13.1	17.1	26.0	24.2	33.0	34.3	57.3
	VAC	1.2	0.5	0.8	2.4	2.5	1.0	2.1	2.2

[a] % metmyoglobin = 100% - (% oxymyoglobin + % deoxymyoglobin). Standard error for packaging display day or muscle = 3.57 - 4.13.

[b] LL = Longissimus lumborum = color stable, ST = Semitendinosus = intermediate color stability, PM = Psoas major = color labile.

[c] HiOx = 80% oxygen and 20% carbon dioxide; HiOx-PVC = Sample remained in 80% oxygen and 20% carbon dioxide for 48 h and was then converted to PVC; PVC = Polyvinyl chloride overwrap; VAC = Vacuum packaging.

[d] Myoglobin

[e] NIR Tissue Oximetry

[f] reflectance spectrophotometry

Chapter 7

Conclusion

1. Reduction of MMb via malate-MDH activity is a new mechanism of nonenzymatic and enzymatic reduction of MMb via malate-MDH system.

2. Reduction of MMb via malate-MDH system will regenerate NADH at different rates in muscles of different physiological origin.

3. Cytochrome *c* oxidase activity of muscles of different physiological origin will relate to their reducing ability and the relative color stability differences.

4. Localized MDH and/or LDH in mitochondrial and cytoplasm will exhibit differences in their ability to reduce MMb: MDH > LDH.

5. Ability of compartmentalized enzymes to reduce MMb offers new insight into myoglobin redox chemistry.

6. Malate and/or lactate at 2% vs. 1% will provide better color stability and suggests that these metabolites can be used as enhancement ingredients.

7. Near-infrared tissue oximetry has potential as a non-invasive, continuous, and direct method to measure the Mb redox dynamics.

8. To obtain repeatable measurements fiber orientation, muscle, tissue oxygen exposure, and storage time will affect the data.

9. Future use of NIR measurements of Mb redox forms should expand our knowledge base of muscle color chemistry.

Appendix A

Postrigor Meat pH Measurement Procedure

Solutions:

Distilled water

Procedure:

1. Standardize pH meter with 4.0 and 7.0 buffer prior to use
2. Weigh 10 g of sample muscle tissue into a beaker
3. Add 100 ml of distilled water to the beaker
4. Blend sample until reasonably mixed in water, approximately 30 sec
5. Measure pH

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Appendix B

Determination of Myoglobin Concentration for Raw and Cooked Steaks

B.1 Solutions

40 mM potassium phosphate buffer, pH 6.8
KH_2PO_4 = 4.87 g
K_2HPO_4 = 2.48 g
1000 mL distilled/deionized water
Sodium hydrosulfite (dithionite)

B.2 Procedure

B.2.1 Sample pulverization

1. Cut sample into small cubes
2. Submerse cubes in liquid nitrogen until rapid boiling of liquid nitrogen is complete
3. Pour small amount of liquid nitrogen into Waring blender.
4. Turn blender on for 2 - 4 sec to chill the blender. Blender should be dry to avoid freezing rotor.
5. Pour pulverized sample onto a clean sheet of paper, and then use the paper to pour sample into a whirlpak bag removing as much air as possible
6. Store sample in ultra low freezer until ready for usage.

B.2.2 Myoglobin Determination

1. In duplicate, weigh 10 g of pulverized sample into a Waring blender bowl and record the exact weight.
2. Add 100 ml of cold potassium phosphate buffer
3. Blend the sample for 1 min
4. Pour blended sample into 50 ml centrifuge tube and store at 4 °C for 1 h for pigment extraction.
5. Centrifuge the samples at 15,000 x g for 30 min at 4 °C.
6. Pour off all the supernatant into a small beaker and syringe filter 3 ml into a cuvette.
7. Add 3 to 5 μg of sodium hydrosulfite (dithionite) to convert all myoglobin in the sample to deoxymyoglobin. The sample will have a greenish tint indicative of a dilute solution of deoxymyoglobin.
8. Scan the sample with a scanning spectrophotometer from 700 - 400 nm. The absorption peaks should be within 2 nm of 433 and 556 nm.

   *** All myoglobin in the sample must be in the deoxymyoglobin form. The soret peak at 433 nm is a good indicator of deoxymyoglobin. Analyze the sample only if the peak following scanning is within 2 nm of 433 nm.
9. Once the peak is found to be within 2 nm of 433 nm, read the absorbance of the peak at 433 nm. Use the following formulas to calculate mg of myoglobin / g of sample.

   (a) Abs_{433} / ϵ * b = molar concentration of myoglobin
   Where ϵ = 114000 for deoxymyoglobin and b = pathlength of cuvet (usually 1 cm)
   Abs_{433} / 114000 = molar concentration of myoglobin

   (b) Molar concentration of myoglobin * molecular weight of myoglobin = g/L myoglobin (16,800 g/mol of bovine myoglobin)

   (c) g/L of myoglobin * ml of buffer added to pulverized sample = mg of myoglobin in sample

   (d) mg myoglobin / g of sample blended = mg of myoglobin / g of sample

*** The above dilution scheme worked for cooked samples from steaks packaged in ultra-low oxygen modified atmosphere packaging. For raw samples, an additional dilution had to be performed following centrifugation. This dilution consisted of taking 1 ml of centrifuged sample and adding 2 ml of cold potassium phosphate buffer in a cuvette. Cooked samples from steaks packaged in high-oxygen modified atmosphere packaging required an initial 10

g of pulverized sample and 50 ml of cold phosphate buffer.

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2. Hunt MC, Sørheim O, Slinde E. 1999. Color and heat denaturation of myoglobin forms in ground beef. J Food Sci 64:847-851.

Appendix C

Myoglobin Concentration and Heme Iron

1. In duplicate, homogenize a 5 g muscle sample with 15 ml of deionized water (4 °C).
2. Centrifuge at 30,000 ×g for 30 min at 4 °C.
3. Decant the supernatant and filter 2-3 ml with a 0.45-micron syringe filter into a 1.5 ml polystyrene cuvette.
4. Measure the absorbance at 525 nm.
5. Myoglobin concentration is calculated using a molar extinction coefficient of 7.6 and a molecular weight of 16,110 for myoglobin.
6. Report myoglobin concentration as mg/g of muscle.
7. Heme iron is calculated by multiplying myoglobin concentration by 0.0035 (Myoglobin contains 0.35% heme iron).
8. Report heme iron as μg/g of muscle.

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Appendix D

Metmyoglobin Reducing Ability Determined by Reduction of Metmyoglobin

Reagents:

- 0.2 mM sodium phosphate buffer (pH 5.6)-24 ml of 0.2 mM HPO_4 + 976 ml of 0.2 mM H_2PO_4
- 5 mM disodium EDTA - 1.86 g/l
- 50 mM COOH-CH_2-COO
- 3.0 mM potassium ferrocyanide - 1.27 g/l
- 0.75 mM horse metmyoglobin (skeletal muscle, Sigma M-0630) - 12.75 g/l
- 1.0 mM NADH (Sigma N-8129) - 0.7 g/l

1. Remove a 5 g sample of muscle tissue that does not contain any visible fat or connective tissue.
2. Homogenize the sample in 20 ml of a 0.2 mM sodium phosphate buffer (pH 5.6) for 90 s or until the muscle tissue has been completely disrupted.
3. Centrifuge the homogenate at 25,000 × g for 30 min at 4 °C.
4. Decant the supernatant and filter 2-3 ml with a 0.45-micron syringe filter into a small test tube
5. Zero spectrophotometer with distilled water.
6. Add the following reagent amounts to a 1.5 ml polystyrene microcuvette:

- 100 μl 5 mM EDTA
- 100 μl 50 mM citrate buffer
- 100 μl 3.0 mM potassium ferrocyanide
- 200 μl 0.75 mM metmyoglobin and 200 μl deionized water
  or
- 400 μl 0.05 mM bovine metmyoglobin

7. Place the microcuvette in the spectrophotometer cell and simultaneously add:
   100 μl of 1 mM NADH
   200 μl of filtered muscle extract
   Quickly mix solution in the cuvette by pipetting twice

8. Begin measuring the absorbance increase at 580 nm immediately and continue for 120 s. As metmyoglobin is reduced by the extract the absorbance will increase.

9. The reducing activity is calculated using Beer's law: A=Ebc
   where:
   A=absorbance (or change in absorbance)
   b= Pathlength (1cm for the plastic microcuvettes)
   E= Extinction coefficient (12,000)
   C= Concentration in moles/Liter

10. The following calculations is used to report MRA as nanomoles reduced/min/g of muscle:
    $\frac{\text{Concentration in mol}/L * 0.001L(\text{volume in cuvette}) * 25(\text{dilution factor})}{\text{5 g of muscle}}$

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Appendix E

Metmyoglobin Reducing Ability Determined by Reduction of Nitric Oxide Metmyoglobin

1. Remove a 3×2×1.27 cm^3 sample of muscle tissue that does not contain any visible fat or connective tissue.
2. Submerge sample in 50 ml of 0.3% sodium nitrite in a 100 ml beaker at room temperature.
3. Oxidized for 20 min with occasional stirring.
4. Remove sample from beaker and blot to remove excess solution.
5. Vacuum package and immediately read reflectance from 400-700 nm using a Hunter LabScan 2000 (1.27 cm diameter aperture, Hunter Associates Laboratory, Inc., Reston, VA).
6. Place sample in an incubator set at 30 °C.
7. Take spectrophotometric readings every 30 min for 2 h.
8. The following K/S ratios and formula were used to determine MRA:

$$\%MMb = \frac{K/S572 \div K/S525(For100\%DMb) - K/S572 \div K/S525(ForSample)}{K/S572 \div K/S525(For100\%DMb) - K/S572 \div K/S525(For100\%MMb)} \quad \text{(E.1)}$$

MRA=(observed decrease in MMb concentration ÷ initial MMb concentration)×100

REFERENCES

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2. Adapted from:
   Watts BM, Kendrick J, Zipser MW, Hutchins B, Saleh B. 1966, Enzymatic reducing pathways in meat. J Food Sci 31: 855-861

Appendix F

Metmyoglobin Reducing Activity Determined by Reduction of Horse Metmyoglobin by NADH

Reagents:

- 5 mM disodium EDTA
- 50 mM citrate buffer pH 5.6
- 3.0 mM potassium ferrocyanide
- 0.5 mM equine MMb (Sigma M-0630) in 30 mM phosphate buffer pH 7.0
- 2.0 mM FMN
- 0.1 mM methylene blue
- distilled water
- 2.0 mM NADH (Sigma N-8129)

1. Assays of nonenzymatic MMb reduction were carried out at ambient temperature (22-23 °C) in 10 mm path length cuvettes with 1.0 mL final reaction volumes.
2. Zero spectrophotometer with distilled water.
3. Add the following reagent amounts to a 1.5 ml polystyrene microcuvette:
   100 μl disodium EDTA
   100 μl citrate buffer
   100 μl potassium ferrocyanide
   300 μl quine MMb
   100 μl FMN
   100 μl methylene blue
   100 μl distilled water

4. Add 100 μl NADH into cuvette and mix quickly.

5. Begin measuring the absorbance increases at 580 nm every 2 sec for 300 seconds. As MMb is reduced nonenzymatically, the absorbance will increase.

6. The reducing activity is calculated using Beer's law: A=Ebc
   where:
   A=absorbance (or change in absorbance)
   b= Pathlength (1cm for the plastic microcuvettes)
   E= Extinction coefficient (12,000 at 580 nm)
   C= Concentration in moles/Liter

7. The following calculation is used to report MRA as nanomoles reduced/min

$$\frac{\text{Concentration in mol}/L * 0.001L(\text{volume in cuvette})}{5min}$$

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1. Al-Shaibani KA, Price RJ, Brown WD.1977. Purification of metmyoglobin reductase from Tuna. J Food Sci 42:1013-1015.

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Appendix G

Metmyoglobin Reducing Activity Determined by Reduction of Horse Metmyoglobin by Lactate-LDH System

Reagents:

- 0.5 mM equine MMb (Sigma M-0630) in 30 mM phosphate buffer pH 7.0
- 2.0 mM FMN
- 0.1 mM methylene blue
- distilled water
- 2.0 mM NAD (Sigma N-1511)
- 200mM L-lactate (Sigma L-1750) with Tris 400 mM pH 8.0
- LDH (Sigma L-1378, from bovine muscle)

1. Assays of nonenzymatic MMb reduction were carried out at ambient temperature (22-23 °C) in 10 mm path length cuvettes with 1.0 mL final reaction volumes.
2. Zero spectrophotometer with distilled water.
3. Add the following reagent amounts to a 1.5 ml polystyrene microcuvette:
   100 μl NAD
   100 μl FMN
   100 μl methylene blue
   300 μl quine MMb
   200 μl Tris-lactate
   100 μl distilled water

4. Add 100 μl LDH into cuvette and mix quickly.

5. Begin measuring the absorbance increases at 580 nm every 2 sec for 300 seconds. As MMb is reduced nonenzymatically with the system, the absorbance will increase.

6. The reducing activity is calculated using Beer's law: A=Ebc
   where:
   A=absorbance (or change in absorbance)
   b= Pathlength (1cm for the plastic microcuvettes)
   E= Extinction coefficient (12,000 at 580 nm)
   C= Concentration in moles/Liter

7. The following calculation is used to report MRA as nanomoles reduced/min

$$\frac{\text{Concentration in mol}/L * 0.001L(\text{volume in cuvette})}{5min}$$

REFERENCES

1. Al-Shaibani KA, Price RJ, Brown WD.1977. Purification of metmyoglobin reductase from Tuna. J Food Sci 42:1013-1015.

2. Brown WD, Snyder HE. 1969. Nonenzymatic reduction and oxidation of myoglobin and hemoglobin by nicotinamide adenine dinucleotides and flavins. J Biol Chem 244:6702-6706.

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Appendix H

Protocol for the Reduction of Metmyoglobin by Skeletal Muscle Extract

Basic Premise of the Method:

This method directly measures the reduction of metmyoglobin to oxymyoglobin by reducing enzymes present and active in skeletal muscle.

1. Remove a five-gram (weigh to 0.1g) sample of muscle tissue that does not contain any visible fat or connective tissue and cut into small pieces.

2. Homogenize the sample in 20 mL of a 0.2 mM sodium phosphate buffer pH 5.6 (or the pH of the muscle) for ninety seconds or until the muscle tissue had been completely disrupted.

3. Centrifuge the homogenate at 35,000 x g in a Beckman ultracentrifuge for thirty minutes at 4 °C.

4. Decant the supernatant into a small beaker and filter 2-3 mL with a 0.4-micron syringe filter into a small test tube.

5. Prepare assay solutions

   a. 5mM Disodium EDTA

   b. 50 mM sodium citrate buffer pH 5.65 (adjust this pH to the desired pH of the mixture)

   c. 3.0 mM Potassium Ferrocyanide

   d. 0.75 mM Metmyoglobin (horse skeletal muscle, Sigma M-0630 or purified from pig or bovine, see purification protocol) in 30 mM sodium phosphate buffer

e. 1.0 mM NADH (Sigma N-8129)

6. Turn on the Hitachi UV-2010 spectrophotometer and warm-up for 10 minutes.
7. Load the MRA method file to measure the absorbance increase at 580nm for 180-240 seconds.
8. Place an empty cuvette in the spectrophotometer cell and zero the instrument.
9. Add the following reagent amounts to the plastic microcuvette

   a. 100 l 5mM EDTA

   b. 100 l 50mM Citrate Buffer

   c. 100 l 3.0mM Potassium Ferrocyanide

   d. 200 l of 0.75mM Metmyoglobin

   e. 200 l Deionized water

10. Place the microcuvette in the spectrophotometer cell and simultaneously add

    - 100 l of 1 mM $NADH$
    - 200 l of filtered muscle extract
    - Mix well by pipetting and releasing the solution at least two times

    ** Add the reagents and mix as quickly as possible because the reaction will begin immediately.**

11. Begin measuring the absorbance increase at 580 nm as soon as possible and continue for 180-240 seconds. As metmyoglobin is reduced by the muscle extract the absorbance at 580 nm will increase.
12. The reducing activity can then be calculated using Beer's law with the extinction coefficient of 12×10^3 for oxymyoglobin at 580nm.
13. Metmyoglobin reductase activity is expressed as nmoles of metmyoglobin reduced/minute/gram of muscle during the initial linear phase of the timecourse (usually the first minute or two).
    Example:

    If the absorbance at 580nm at 0 seconds is 0 and the absorbance at 60 seconds is 0.132.
    $\Delta Abs580nm = 0.132/minute$

    Use Beer's Law to calculate the change in the concentration of metmyoglobin to oxymyoglobin.
    $A = Ebc$
    where A=absorbance (or change in absorbance)

b= Pathlength (1cm for the plastic microcuvettes)
E= Extinction coefficient (12,000)
C= Concentration in moles/Liter

$0.132 = 12000 * 1 * c$
$c = 0.132/12000$
$c = 11.0 \times e^{-6}$ molar/minute/5grams of muscle or 11 micromolar/minute/5grams of muscle

Remember this is the change in concentration not the concentration

Multiply the change in concentration by the volume in the cuvette and that will change the concentration to moles.

11 e^{-6} moles/liter/minute/5grams * 0.0015 liters =
16.5 e^{-9} moles reduced/minute/5 grams of muscle=
16.5 nanomoles reduced/minute/5 grams of muscle=
3.3 nanomoles reduced minute/gram of muscle
3.3 nmoles of metmyoglobin reduced/minute/gram= the number you report

** If you take the change in absorbance over 120 seconds (2 minutes) then divide the final number by 2.

REFERENCES

1. Hagler, L.; Coppes, Jr., R.I.; and Herman, R.H. 1979. Metmyoglobin Reductase. J. Biol. Chem. 254:6505-6514.

2. Madhavi, D.L. and Carpenter, C.E. 1993. Aging and processing affect color, metmyoglobin reductase and oxygen consumption of beef muscles. J. Food Sci. 58:939-942,947.

Appendix I

Protocol for the Reduction of Metmyoglobin by Malate Dehydrogenase

Basic Premise of the Method: This method directly measures the reduction of metmyoglobin to oxymyoglobin by reducing NAD^+ to $NADH$ by Malate Dehydrogenase enzyme present and active in skeletal muscle cytoplasm and in mitochondria.

1. Remove a five-gram (weigh to 0.1g) sample of muscle tissue that does not contain any visible fat or connective tissue and cut into small pieces.

2. Homogenize the sample in 20 mL of a 0.2 mM sodium phosphate buffer pH 5.6 (or the pH of the muscle) for ninety seconds or until the muscle tissue had been completely disrupted.

3. Centrifuge the homogenate at 35,000 x g in a Beckman ultracentrifuge for thirty minutes at 4 °C.

4. Decant the supernatant into a small beaker and filter 2-3 mL with a 0.4-micron syringe filter into a small testtube.

5. Prepare assay solutions

   a. 5mM Disodium EDTA

   b. 40 mM sodium phosphate buffer pH 7. 5 (adjust this pH to the desired pH of the mixture) containing 100 mM of Glutamic Acid.

   c. 0.25 mM Metmyoglobin (horse skeletal muscle, Sigma M-0630 or purifed from pig or bovine, see purification protocol) in 40 mM sodium phosphate buffer

   d. 50 mM NAD^+ (Sigma N-8129)

   e. 25 mM Sodium Malate in distilled water

f. 1 mM Methylene Blue

g. 100 μL of Isolated Mitochondria

Note: Do not **ADD MDH at this time since you are using muscle extract as a source of MDH for MRA assay.

6. Turn on the Hitachi UV-2010 spectrophotometer and warm-up for 10 minutes.

7. Load the MRA method file to measure the absorbance increase at 580nm for 180-240 seconds.

8. Place an empty cuvette in the spectrophotometer cell and zero the instrument.

9. Add the following reagent amounts to the plastic microcuvette

   a. 1 mL of 40 mM Phosphate Buffer containing 100 mM Glutamic Acid

   b. 100 l 5mM EDTA

   c. 100 l Methylene Blue

   d. 200 l of 0.75mM Metmyoglobin

   e. 100 l of 25 mM Sodium Malate

10. Place the microcuvette in the spectrophotometer cell and simultaneously add

    - 200 l of 50 mM NAD^+
    - 200 l of Isolated Muscle Extract
    - Mix well by pipetting and releasing the solution at least two times

    ** Add the reagents and mix as quickly as possible because the reaction will begin immediately.**

11. Begin measuring the absorbance increase at 580 nm as soon as possible and continue for 180-600 seconds. As metmyoglobin is reduced by the muscle extract the absorbance at 580 nm will increase.

12. The reducing activity can then be calculated using Beer's law with the extinction coefficient of 12×10^3 for oxymyoglobin at 580nm.

13. Metmyoglobin reductase activity is expressed as nmoles of metmyoglobin reduced/minute/gram of muscle during the initial linear phase of the timecourse (usually the first minute or two).
    Example:

    If the absorbance at 580nm at 0 seconds is 0 and the absorbance at 60 seconds is 0.132.
    $\Delta Abs580nm = 0.132/minute$

Use Beer's Law to calculate the change in the concentration of metmyoglobin to oxymyoglobin.
$A = Ebc$
where A=absorbance (or change in absorbance)
b= Pathlength (1cm for the plastic microcuvettes)
E= Extinction coefficient (12,000)
C= Concentration in moles/Liter

$0.132 = 12000 * 1 * c$
$c = 0.132/12000$
$c = 11.0 \times e^{-6}$ molar/minute/5grams of muscle or 11 micromolar/minute/5grams of muscle

Remember this is the change in concentration not the concentration

Multiply the change in concentration by the volume in the cuvette and that will change the concentration to moles.

11 e^{-6} moles/liter/minute/5grams * 0.0015 liters =
16.5 e^{-9} moles reduced/minute/5 grams of muscle=
16.5 nanomoles reduced/minute/5 grams of muscle=
3.3 nanomoles reduced minute/gram of muscle
3.3 nmoles of metmyoglobin reduced/minute/gram= the number you report

** If you take the change in absorbance over 120 seconds (2 minutes) then divide the final number by 2.

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1. Hagler, L.; Coppes, Jr., R.I.; and Herman, R.H. 1979. Metmyoglobin Reductase. J. Biol. Chem. 254:6505-6514.

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Appendix J

Mitochondria Isolation from Skeletal Muscle - General Protocol

Product Description:

This kit enables the fast and easy isolation of an enriched mitochondria fraction from animal tissues. Most of the isolated mitochondria will contain intact inner and outer membranes. In addition, the kit enables, the assessment of the mitochondrial inner membrane integrity by testing of the elctrochemical proton gradient ($\Delta\psi$) of the inner mitochondrial membrane. This may be achieved by measuring the uptake of the flourescent carbocyanine dye JC-1 (supplied in the kit) into the mitochondria.

The outer membrane integrity may be measured by observing cytochrome c oxidase activity (using the Cytochrome c Oxidaes Assay kit, Catalog Number CYTOCOX1). This kit measures the activity in the presence and absence of the detergent n-dodecyl β-D-maltoside, and the ratio of the two activities provides a measure of the inegrity of the outer membrane.

The mitochondria isolation Kit is useful for mitochondria mediated apoptosis studies. Such studies are of central importance for the investigation of a number of major debilitating diseases including Parkinson's disease and cancer. In addition, the kit may be useful for isolating mitochondrial proteins for proteome studies.

Components:

The reagents are sufficient for extraction of up to 10-20 g of animal tissue and 50 JC-1 assays of 2 ml.

1.
   - Extraction Buffer A, 5x (50 ml)
   - Catalog Number E2778
   - 50mM HEPES, pH 7.5, containing 1 M mannitol, 350mM sucrose, and 5 mM EGTA
2.
   - Extraction Buffer B, 5x (50 ml)

- Catalog Number E6028
- 100mM MOPS, pH 7.5, containing 550 mM KCl and 5mM EGTA

3. • Storage Buffer, 5x (25 ml)
   - Catalog Number S9689
   - 50 mM HEPES, pH 7.5, containing 1.25 M sucrose, 5mM ATP, 0.4 mM ADP, 25 mM sodium succinate, 10mM K_2HPO_4, and 5 mM DTT

4. • Albumin Solution (10 ml)
   - Catalog Number A0474
   - 50 mg/ml delipidated bovine serum albumin (Catalog Number A7511) in water

5. • JC-1 serum (25 μg)
   - Catalog Number J4519

6. • JC-1 Assay Buffer, 5x (25 ml)
   - Catalog Number J4519
   - 100 mM MOPS, pH 7.5, containing 550 mM KCl, 50 mM ATP, 50mM $MgCl_2$, 50 mM sodium succinate, and 5 mM EGTA

7. • Trypsin (50 mg)
   - Catalog Number T9201

Reagents and Equipments Required but Not Provided:

- Cooled Eppendorf centrifuge (for small scale) or Sorvall RC-5C centrifuge with SS-34 head (for large scale)
- PTFE pestle and 3 ml glass tube (Catalog Number P7734) (for small scale) or PTFE pestle and 45 ml glass tube (Catalog Number P7984) (for large scale)
- Overhead electric motor
- Spectroflourometer with a suitable cuvette
- Scalpel and glass plate
- Ice bath
- 2 ml Eppendorf tubes
- Ultrapure water
- Dimethyl sulfoxide ($DMSO$, Catalog Number D8418)

Preparation Instructions:

Use ultrapure water for the preperation of reagents.

- 1x Extraction Buffer A (sotonic solution, 10 mM HEPES, pH 7.5, containing 200 mM mannitol, 70 mM sucrose, and 1mM EGTA)-Defrost Extraction Buffer A at 37 °C). A short heating time (15 seconds in a microwave oven) may be needed to achieve to a clear solution. Dilute an aliquot of the buffer 5-fold with water. Keep the diluted buffer at 4 °C) before use. The concentrated buffer may be refrozen.

- 1x Extraction Buffer B (ionic solution, 20 mM MOPS, pH 7.5, containing 110 mM KCl and 1 mM EGTA)- Dilute an aliquot of Extraction Buffer B 5-fold with water. Keep the diluted buffer at 4 °C) before use. The concentrated buffer may be refrozen.

- 1x Storage Buffer (10 mM HEPES, pH 7.4, containing 259 mM sucrose, 1mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4, and 1 mM DTT). Dilute an aliquot of the 5x Storage Buffer 5-fold with water. Keep the diluted buffer at 4 °C) before use. The concentrated buffer may be refrozen.

- Albumin Solution (50 mg/ml)- Dilute as needed in the appropriate buffer (see Procedure).

- JC-1 Stain - Dissolve the vial in 25 μl of dry $DMSO$ (D 8418). This will give a 1 mg/ml solution (1.53 mM; MW 652.2). The solution may be stored at - 20 °C at this concentration. For the assay, dilute an aliquot of the reconstituted solution 5-fold with $DMSO$

- 1x JC-1 Assay Buffer (20 mM MOPS, pH 7.5, containing 110 mM KCl, 10 mM ATP, 10mM $MgCl_2$, 10 mM sodium succinate, and 1 mM EGTA)- Dilute an aliquot of the buffer 5-fold with water. Keep the diluted buffer at 4 °C) before use. The concentrated buffer may be refrozen.

- Trpsin - Dissolve an aliquot of trypsin in the appropriate 1x Extraction Buffer at 0.25 mg/ml. Keep at 4 °C) until needed.

Storage/Stability
Store the kit at - 20 °C. When stored unopened, the components in the kit are stable for 24 months.
Procedure

- Mitochondria can be prepared easily from animal tissues by a simple method of homogenization followed by low ($600 \times g$) and high speed ($11,000 \times g$) centrifugation. The final pellet represents a crude mitocondrial fraction that may be used as the basis for further experiments. For a more purified "heavy" mitochondrial fraction that will be enriched in mitochondria as opposed to lysosomes and perisomes that normally contaminate this fraction, the low and high speed centrifugation steps can be changed to $1,000 \times g$ and $3,500 \times g$, respectively.

- The kit supplies the necessary solutions for the isolation of mitochondria from "soft" tissues such as liver or brain, or from "hard" tissues such as skeletal muscle or heart muscle.
- "Hard" tissues cannot be homogenized easily without pretreatment with a protease such as trypsin to promote breakdown of the cellular structure. The myofibrils in skeletal muscle tend to give a gelatinous consistency to the homogenate in non-ionic media (isotonic sucrose) and thus must be isolated in an ionic medium such as 1 × Extraction Buffer B.
- The mitochonrial pellet may be suspended in either 1 × Storage Buffer (for experiments that need respiring mitochondria) or in 1 × Extraction Buffer A.
- Perform all the isolation procedure at 4 °C with ice cold solutions

Note: The procedures described here are for small amounts of tissue samples (50-200 mg). The centrifugation is performed using a cooled Eppendorf centrifuge (Model 5417R). The homogenizer used is a PTFE pestle and 3 ml glass tube (Catalog Number P7734).

JC-1 uptake in mitochondria

The integrity of the inner mitochondrial membrane may be measured by observing the potential gradient ($\Delta\psi$) over this membrane. This can be achieved by measuring the uptake of the cationic carbocyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) into the matrix. The mitochondrial membrane potential, across the inner membrane, determines the redistribution of this dye. The distribution depends on the transmembrane electric fields (negative inside) and the concentration gradient of the dye.

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Appendix K

Protocol for the Isolation of Mitochondria and Cytoplasmic Protein for Malate Dehydrogenase assisted - MRA Assay

K.1 Isolation of Mitochondria and Cytoplasmic Protein

Reagent Setup

220 mM Mannitol: Dissolve 40.1 g of mannitol in 1 liter of distilled water; mix well and prepare 20 ml aliquots; store them at -20 °C.

80 mM sucrose: Dissolve 27.38 g of sucrose in 1 liter of distilled water; mix well and prepare 20 ml aliquots; store them at -20 °C.

100 mM Tris/MOPS: Dissolve 12.11 g of Tris in 500 ml of distilled water, adjust pH to 7.4 using MOPS powder bring the solution to 1 liter and store at 4 °C.

46 mM KCl: Dissolve 3.43 g of KCl in 1 liter of distilled water, adjust pH to 7.4 using MOPS powder bring the solution to 1 liter and store at 4 °C.

100 mM Tris/HCl: Dissolve 12.1 g of Tris in 500 ml of distilled water adjust pH to 7.4 using HCl; bring the solution to 1 liter and store at room temperature.

50 mM $MgCl_2$: Dissolve 4.8 g of MgCl2 in 1 liter of distilled water and store at room temperature.

10 mM EDTA: Dissolve 2.92g of EDTA in 1 liter of distilled water and store at 4 °C.

0.5% BSA: Dissolve 5 g of BSA in 1 liter of distilled water and store at -20 °C.

5 mM Pi: Dissolve 0.68 g KH2PO4 in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 °C.

Nagarse Protease: Prepare a 20 mg % Nagarse.

Isolation Buffer A for muscle mitochondria (IB_m1): Prepare 1 Liter of IB_m1 by mixing 80 mM sucrose, 100 mM Tris/MOPS, 46 mM KCl, 10 mM EDTA, 100 mM Tris/HCl and 0.5% BSA. Adjust the pH to 7.4. Bring the volume to 1 liter with distilled water.

Incubation Buffer B for muscle mitochondria (IB_m2): Prepare 1 Liter of IB_m2 by mixing 220 mM Mannitol, 80 mM sucrose, 0.02 mM EDTA, 20 mM Tris/HCl and 5 mM Pi. Adjust the pH to 7.4. Bring the volume to 1 Liter with distilled water.

Suspension/Experimental Buffer for skeletal muscle mitochondria (EB_m): Prepare 1 Liter of EB_m by mixing 220 mM Mannitol, 80 mM sucrose, 0.02 mM EDTA, 20 mM Tris/HCl and 5 mM Pi. Adjust the pH to 7.4. Bring the volume to 1 Liter with distilled water.

Procedure

1. Remove a five-gram (weigh to 0.1g) sample of muscle tissue of interest that does not contain any visible fat or connective tissue and cut into small pieces.
2. Immerse the tissue in a small beaker containing 20 mL of ice-cold Phosphate Buffer supplemented with 10 mM EDTA.
3. Mince the muscle into small pieces using scissors.
4. Wash the minced muscle twice or thrice using scissors with ice-cold Phosphate Buffer supplemented with 10 mM EDTA.
5. Re-suspend the minced muscles in 5 mL of ice-cold Phosphate Buffer supplemented with 10 mM EDTA.
6. Centrifuge at 200g for 5 min and discard the supernatant.
7. Resuspend the pellet in IB_m1.
8. Homogenise the muscles using a Teflon pestle operated at 1,600 r.p.m.; stroke the minced muscle ten times.

- **CRITICAL STE**P: The optimal ratio between tissue and isolation buffer ranges between 1:5 and 1:10 (w:v)
- **CRITICAL STEP**: Precool the glassware in an ice-bath 5 min before starting the procedure. Homogenization and the following steps, must be performed at 4 °C to minimize the activation of damaging phospholipases and proteases.

9. Transfer the homogenate to a 50 mL polypropylene Falcon tube and centrifuge at 700g for 10 min at 4 °C.

10. Transfer the supernatant to glass centrifuge tubes and centrifuge at 8,000g for 10 min at 4 °C.

11. Discard the supernatant and re-suspend the pellet containing mitochondria. You can use a glass rod to loosen the pellet paste. Avoid using ISOLATION BUFFER and try to re-suspend the mitochondria in the small amount of buffer that remains after discarding the supernatant. Use a 200 μL pippettor and avoid the formation of bubbles during the re-suspension process.

12. Transfer the mitochondrial suspension into a 14 mL Falcon tube and keep it on ice.

13. Measure the mitochondrial concentration using the Biuret methods.

K.2 Procedure for MRA Assay with Isolated Mitochondria and Cytoplasmic Protein

1. Prepare assay solutions

   a. 5mM Disodium EDTA

   b. 40 mM sodium phosphate buffer pH 7. 5 (adjust this pH to the desired pH of the mixture) containing 100 mM of Glutamic Acid.

   c. 0.25 mM Metmyoglobin (horse skeletal muscle, Sigma M-0630 or purifed from pig or bovine, see purification protocol) in 40 mM sodium phosphate buffer

   d. 50 mM NAD^+ (Sigma N-8129)

   e. 25 mM Sodium Malate in distilled water

   f. 1 mM Methylene Blue

   g. 100 μL of Isolated Mitochondria

   Note: Do not **ADD MDH at this time since you want to use mitochondrial MDH MRA assay.

2. Turn on the Hitachi UV-2010 spectrophotometer and warm-up for 10 minutes.

3. Load the MRA method file to measure the absorbance increase at 580nm for 180-240 seconds.

4. Place an empty cuvette in the spectrophotometer cell and zero the instrument.

5. Add the following reagent amounts to the plastic microcuvette

   a. 1 mL of 40 mM Phosphate Buffer containing 100 mM Glutamic Acid

   b. 100 l 5mM EDTA

   c. 100 l Methylene Blue

   d. 200 l of 0.75mM Metmyoglobin

   e. 100 l of 25 mM Sodium Malate

6. Place the microcuvette in the spectrophotometer cell and simultaneously add

   - 200 l of 50 mM NAD^+
   - 200 l of Isolated muscle Mitochondria/Muscle Extract
   - Mix well by pipetting and releasing the solution at least two times

   ** Add the reagents and mix as quickly as possible because the reaction will begin immediately.**

7. Begin measuring the absorbance increase at 580 nm as soon as possible and continue for 180-600 seconds. As metmyoglobin is reduced by the muscle extract the absorbance at 580 nm will increase.

8. The reducing activity can then be calculated using Beer's law with the extinction coefficient of 12×10^3 for oxymyoglobin at 580nm.

9. Metmyoglobin reductase activity is expressed as nmoles of metmyoglobin reduced/minute/gram of muscle during the initial linear phase of the timecourse (usually the first minute or two).
   Example:

   If the absorbance at 580nm at 0 seconds is 0 and the absorbance at 60 seconds is 0.132.
   $\Delta Abs580nm = 0.132/minute$

   Use Beer's Law to calculate the change in the concentration of metmyoglobin to oxymyoglobin.
   $A = Ebc$
   where A=absorbance (or change in absorbance)
   b= Pathlength (1cm for the plastic microcuvettes)
   E= Extinction coefficient (12,000)
   C= Concentration in moles/Liter

$0.132 = 12000 * 1 * c$
$c = 0.132/12000$
$c = 11.0 \times e^{-6}$ molar/minute/5grams of muscle or 11 micromolar/minute/5grams of muscle

Remember this is the change in concentration not the concentration

Multiply the change in concentration by the volume in the cuvette and that will change the concentration to moles.

11 e^{-6} moles/liter/minute/5grams * 0.0015 liters =
16.5 e^{-9} moles reduced/minute/5 grams of muscle=
16.5 nanomoles reduced/minute/5 grams of muscle=
3.3 nanomoles reduced minute/gram of muscle
3.3 nmoles of metmyoglobin reduced/minute/gram= the number you report

** If you take the change in absorbance over 120 seconds (2 minutes) then divide the final number by 2.

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Appendix L

Cytochrome *c* Oxidase Assay

Product Description

The Cytochrome *c* Oxidase Assay Kit is designed for the determination of cytochrome *c* oxidase activity in soluble and membrane bound mitochondrial samples. Cytochrome *c* oxidase [EC 1.9.3.1] is the principle terminal oxidase of high affinity oxygen in the aerobic metabolism of all animals, plants, yeasts, and some bacteria. The enzyme is present in mitochondria of the more highly developed cells and in the cytoplasmic membrane of bacteria. The enzyme is probably unique in providing the energy for the cell by coupling of the electron transport through the cytochrome chain with the process of oxidative phosphorylation. Cytochrome *c* oxidase is located on the inner mitochondrial membrane that divides the mitochondrial matrix from the intermembrane space and it has been used for many years as a marker for this mechanism.

The colorimetric assay in this kit is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochome *c* oxidase.

This kit is suitable for detection of mitochondria in subcellular fraction and for detection of mitochondria in subcellular fractions. For optimal preparation of mitochondria it is recommended to use the MITOISO1 kit., which enables the fast and easy isolation of an enriched mitochondria fraction from animal tissues. The MITOSO2 and MITOISO3 are available for preparation of mitochondria from cultured cells and yeasts, respectively.

Components
This kit is sufficient for 100 tests.

- Assay Buffer 5 × (Product Code) - 25 ml
  50 mM Tris-HCL, pH 7.0,
  containing 600 mM KCL
- Enzyme Dilution Buffer 2 × - 20 ml (Product Code E2903)
  20mM Tris-HCL, pH 7.0
  containing 500 mM sucrose

- Cytochrome c
- 1 M Dithiothreitol (DTT) Solution - 0.4 ml (Product Code D7059)
  1M DTT in deionized water
- Cytochrome *c* Oxidase - 1 vial (positive control, Product Code C8109)
- *n*-Dodecyl *β-D*-maltoside - 10 mg (Product Code D4641)

Reagents and Equipment Required but Not Provided

- Spectrophotometer
- 1 ml Cuvettes
- Analytical balance
- Ultrapure (minimum 17 MΩ.cm) water

Precautions and Disclaimer
The Cytochrome *c* Oxidase Assay Kit is for R & D use only, not for drug, household, or other uses. Consult the MSDS for information regarding hazards and safe handing practices.

Preparation of Reagents
Use ultrapure (minimum 17 MΩ.cm) water for the preparation of reagents.

1. 1 × Assay Buffer : 10 mM Tris-HCL, pH 7.0, containing 120 mM KCL - Dilute an aliquot of Assay Buffer 5 × (A0599) 5-fold with water. Keep at room temperature (25 °C)
2. 1 × Enzyme Dilution Buffer: 10 mM Tris-HCL, pH7.0, containing 250 mM sucrose - Dilute an aliquot of Enzyme Dilution Buffer 2 × (E2903) 2-fold with water. Keep at 2-8 °C.
3. Enzyme Dilution Buffer with 1 mM *n*- Dodecyle *β-D*- maltoside (for measurement of mitochondrial integrity): 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and 1 mM *n*-Dodecyl-*β-D*-maltoside (D4641; MW 510.6 Da) in 2 ml of 1 × Enzyme Dilution Buffer.
4. 0.1 M Dithiothreitol (DTT) Solution: Dilute an aliquot of the 1 M DTT Solution (D7059) 10-fold with ultrapure water to a concentration of 0.1 M.
5. Ferrocytochrome c Substrate Solution (0.22 mM):

   a. Dissolve 2.7 mg of cytochrome c (MW 12,384 Da) in 1 ml of water.

b. In order to reduce the protein, add 5 μl of the 0.1 M DTT Solution to a final concentration of 0.5 mM, mix gently, and wait for 15 minutes. The color of the solution should go from dark orange-red to pale purple-red.

c. Measure the A_{550}/A_{565} ratio of an aliquot diluted 20-fold with 1 × Assay Buffer to zero the spectrophotometer. The A_{550}/A_{565} should be between 10 and 20.
Note: If the A_{550}/A_{565} ratio remains less than 10, the substrate has not been sufficiently reduced and the enzyme activity will not be valid.

6. Cytochrome *c* Oxidase Positive Control:

   a. Dissolve the vial in th volume of water specified in the instructions on the label.

   b. For the enzyme assay, further dilute the sample 10-fold with 1 × Enzyme Dilution Buffer and use 20-40 μl for each control reaction mixture.

   c. The sample must be stored at 2-8 °C for at least 3 weeks or frozen in aliquots at -20 °C.

7. Enzyme sample: The best results are achieved when the enzyme activity is between 0.4 and 4.0 milliunits of cytochrome c oxidase per reaction. assay different amounts of enzyme to find a linear range for the sample.

Storage/Stability

The kit ships on wet ice and storage at -20 °C is recommended. When stored unopened, the components in this kit are stable for 24 months. After initial thawing of the 1 M Dithiothreitol Solution, divide the solution into undiluted working aliquots (still at 1 M concentration) and store at -20 °C.

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3. Rasmussen, U.F., and Rasmussen, H.N., Mol. Cell. Biochem., 208, 37-44 (2000).
4. Suarez, M.D., et al., J. Biol. Chem., 259, 13791-13799 (1984).

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Appendix M

Bicinchoninic Acid Protein Assay

Product Description

Protein determination is one of the most common operations performed in biochemical research. The principle of the bicinchoninic acid (BCA) assay is similar to the Lowry procedure,[1] in that both rely on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond[2] are able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.[3].

Reagents

Bicinchoninic Acid Solution (B9643 - Sigma)
Reagent A is a 1,000 ml solution containing bicinchoninic acid, sodium carbonate, sodium tartrate,and sodium bicarbonate in 0.1 N NaOH (final pH 11.25).
Copper(II) Sulfate Pentahydrate 4% Solution (C2284 - Sigma)
Reagent B is a 25 ml solution containing 4% (w/v) copper(II) sulfate pentahydrate.
Protein Standard (Bovine Serum Albumin - BSA)
Solution (P0914 - Sigma)

Preparation Instructions

The BCA Working Reagent is prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. Mix the BCA Working Reagent until it is light green in color.

Storage/Stability

Store Reagents A and B at room temperature. Reagent A, without Reagent B added, is stable for at least one year at room temperature in a closed container. The BCA Working

Reagent (Reagent A mixed with Reagent B) is stable for one day.

Store the Protein Standard at 2 - 8 °C.

Procedures

In the standard assay, 20 parts of the BCA Working Reagent are then mixed with 1 part of a protein sample. For the 96 well plate assay, 8 parts of the BCA Working Reagent are mixed with 1 part of a protein sample. The sample is either a blank, a BSA protein standard, or an unknown sample. The blank consists of buffer with no protein. The BSA protein standard consists of a known concentration of bovine serum albumin, and the unknown sample is the solution to be assayed.

BCA assays are routinely performed at 37 °C. Color development begins immediately and can be accelerated by incubation at higher temperatures. Higher temperatures and/or longer incubation times can be used for increased sensitivity. Incubation at lower temperatures can slow down color development (see Procedures A and B). The absorbance at 562 nm is recorded and the protein concentration is determined by comparison to a standard curve.

A. Standard 2.1 ml Assay Protocol

(Linear concentration range is 200-1,000 mg/ml or 20-100 mg of total protein) This is the standard assay that can be performed in a test tube. This procedure uses 0.1 ml of a protein sample and 2 ml of the prepared BCA Working Reagent. The instructions are a step-by-step procedure on how to perform the standard assay. If a nonstandard assay is used (96 well plate) adjust the volumes accordingly.

Note: It is necessary to create a standard curve during each assay, regardless of the format used.

1. Prepare the required amount of BCA Working Reagent needed for the assays (Table N.1). The final volume used in the assay depends upon the application and the equipment available. Table N.1 can be used to determine the volume of BCA Working Reagent to prepare, depending on how many blanks, BSA protein standards, and unknown samples are to be assayed. Combine the volumes of Reagents A and B specified in the table. Mix until the BCA Working Reagent is a uniform, light green color.

2. Prepare standards of different concentrations. These BSA protein standards can range from 200-1,000 μg/ml (20-100 μg of total protein). This is accomplished by making serial dilutions starting from the 1 mg/ml standard, and then using 0.1 ml of each diluted standard in the assay. It is best to make the dilutions in the same buffer as

the unknown sample . Deionized water may be used as a substitute for the buffer, but any interference due to the buffer will not be compensated for in the BSA protein standards.

3. Add 2 ml of the BCA Working Reagent to 0.1 ml of each BSA protein standard, blank, and unknown sample. Vortex gently for thorough mixing. The total liquid volume in the test tube is 2.1 ml.

4. The following incubation parameters may be used:
   60 °C for 15 minutes Or
   37 °C for 30 minutes Or
   25 °C (Room Temperature) from 2 hours to overnight

5. If required, allow the tubes to cool to room temperature.

6. Transfer the reaction solutions into a cuvet.

7. Measure the absorbance of the solution at 562 nm. Color development continues slowly after cooling to room temperature, but no significant error is seen if all the tubes are read within 10 minutes of each other. Create an assay table as needed and a standard curve based on either the BSA protein standard concentration or on the amount of protein present in the BSA protein standard.

8. Determine protein concentration by comparison of the absorbance of the unknown samples to the standard curve prepared using the BSA protein standards.

Results Based on the Standard Assay

Create a table with the absorbance results obtained during the assay. A separate standard curve should be generated for each assay performed. The amount of protein for tubes 1-6 was obtained from the known amount of BSA protein standard added.

Note: The data below should not be used as a replacement of a standard curve. The absorbance of the BSA protein standards (tubes 1-6) in each assay will differ from those presented here. The amount of protein recorded for tubes 7 and 8 was obtained from the standard curve.

After obtaining the results, create a standard curve to determine the protein concentration in the unknown sample. Plot the Net Absorbance at 562 nm versus the BSA protein standard concentrations (μg/ml, Tubes 1-6).

References

1. Lowry, O.H. et al, J. Biol. Chem., 193, 265-275 (1951).

2. Wiechelman, K. et al, Anal. Biochem., 175, 231-237 (1988).

3. Smith, P.K. et al, Anal. Biochem., 150, 76-85 (1985).

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Table M.1: *Volume of BCA Working Reagent to prepare. This is dependent on how many blanks, BSA protein standards, and unknown samples are to be assayed.*

Number of Assays		Amount of Each Reagent Used		
Number of 2.1 ml Standard Test Tube Assays	Number of wells in 96 well plate assay	Reagent A (ml)	Reagent B (ml)	Total volume of BCA Working Reagent (ml)
4	40	8	0.16	8.16
8	80	16	0.32	16.32
9	96	19	0.38	19.38
12	127	25	0.5	25.5

Appendix N

Tables Associated with Chapter 4

Table N.1: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Longissimus lumborum muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	21.6^{cm}	45.4^{gn}	58.3^{go}	79.4^{hp}	83.4^{kq}	85.4^{kp}
Malate	1M	15.2^{bm}	19.9^{bmn}	24.2^{bcn}	38.3^{eo}	52.3^{hip}	66.3^{hiq}
Lactate	1L	19.0^{bcm}	22.6^{bcdm}	26.5^{bcn}	44.8^{fgo}	61.8^{jp}	68.8^{iq}
Pyruvate	1P	19.6^{bcm}	23.4^{bcdm}	33.3^{efn}	48.4^{go}	63.4^{jp}	76.4^{jq}
Malate	2M	18.6^{bcm}	$27.4\mathrm{d}^{efn}$	34.7^{fo}	31.2^{bcno}	35.2^{cdo}	42.2^{dp}
Lactate	2L	17.7^{bcm}	22.7^{bcdn}	22.3^{bn}	29.7^{bco}	41.2^{efp}	47.8^{eq}
Pyruvate	2P	15.3^{bm}	21.9^{bcn}	28.2^{cdo}	39.1^{ep}	47.9^{ghq}	54.9^{fgr}
Malate + Lactate	1M+1L	15.2^{bm}	19.9^{bm}	26.9^{bcn}	38.3^{eo}	48.3^{ghp}	57.3^{gq}
Malate + Pyruvate	1M+1P	19.0^{bcm}	21.6^{bcm}	28.5^{cden}	45.6^{fgo}	55.7^{ip}	78.8^{jq}
Lactate+ Pyruvate	1L+1P	19.6^{bcm}	23.4^{bcdm}	33.3^{efn}	48.4^{go}	63.4^{jp}	76.4^{jq}
Malate + Lactate	2M+2L	8.6^{am}	11.4^{amn}	12.1^{amn}	13.2^{ano}	17.2^{ao}	24.2^{ap}
Malate + Pyruvate	2M+2P	17.7^{bcm}	22.7^{bcdn}	22.3^{bn}	27.2^{bo}	29.2^{bo}	33.2^{bp}
Lactate+ Pyruvate	2L+2P	15.3^{bm}	25.9^{cden}	28.2^{cdno}	32.4^{cdo}	40.2^{ep}	47.3^{eq}
Malate + Lactate	1M+2L	29.2^{dm}	29.9^{efm}	31.9^{defm}	37.3^{den}	45.3^{fgo}	51.3^{efp}
Malate + Pyruvate	1M+2P	17.5^{bcm}	24.2^{bcdn}	28.9^{cdeo}	41.8^{efp}	54.8^{iq}	63.8^{hr}
Lactate+ Pyruvate	1L+2P	29.6^{dm}	31.4^{fm}	33.3^{efm}	48.4^{gn}	51.4^{hio}	63.4^{hp}
Malate + Lactate	2M+1L	28.6^{dm}	26.4^{cdem}	28.5^{cdem}	33.2^{cdm}	39.4^{den}	42.2^{do}
Malate + Pyruvate	2M+1P	27.7^{dn}	26.2^{cdemn}	22.3^{bm}	31.8^{bco}	37.2^{cdep}	41.2^{cdq}
Lactate+ Pyruvate	2L+1P	15.3^{bm}	21.9^{bcn}	23.2^{bno}	26.9^{bo}	33.9^{bcp}	36.9^{bcp}

[a] a,b,c,d,e,f,g,h,i,j,k = means in a column within a trait with a different letter differ ($p < 0.05$). m,n,o,p,q,r = means in a row with a different letter differ ($p < 0.05$). Standard error for muscle type × incubation and display of muscle = 1.34 - 2.17.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.

Table N.2: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Psoas major muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	17.6^{bcdem}	39.4^{hin}	64.3^{io}	92.4^{lp}	94.4^{kp}	96.4^{jp}
Malate	1M	20.2^{dem}	19.9^{abcm}	44.9^{fgn}	72.3^{jo}	72.3^{hip}	72.3^{gp}
Lactate	1L	19.0^{cdem}	27.6^{den}	46.5^{fgo}	74.8^{jkp}	74.8^{ip}	74.8^{ghp}
Pyruvate	1P	19.6^{dem}	43.4^{in}	53.3^{ho}	78.4^{kp}	83.4^{jq}	86.4^{iq}
Malate	2M	18.6^{cdme}	29.4^{efgn}	34.9^{deo}	33.2^{co}	39.2^{bcp}	42.7^{bq}
Lactate	2L	17.7^{bcdem}	26.7^{den}	35.3^{doe}	47.2^{efp}	51.6^{deq}	53.2^{cq}
Pyruvate	2P	15.3^{abcdem}	26.3^{den}	38.2^{eo}	51.9^{fp}	58.9f^{gq}	63.9^{er}
Malate + Lactate	1M+1L	15.2^{abcdem}	33.9^{fgn}	43.2^{fo}	57.3^{gp}	63.3^{gq}	67.3^{efr}
Malate + Pyruvate	1M+1P	16.6^{bcdme}	31.6^{fgn}	48.5^{gho}	65.6^{ip}	75.7^{iq}	78.8^{hr}
Lactate + Pyruvate	1L+1P	19.6^{dem}	38.4^{hin}	47.3^{fgo}	77.4^{jkp}	83.4^{jq}	87.4^{iq}
Malate + Lactate	2M+2L	28.6^{fo}	21.4^{bnc}	16.1^{am}	13.2^{am}	15.2^{am}	22.2^{an}
Malate + Pyruvate	2M+2P	11.7^{abm}	22.7^{bcdn}	22.3^{bn}	26.2^{bn}	39.2^{bco}	47.2^{bp}
Lactate + Pyruvate	2L+2P	15.3^{abcdem}	35.9^{ghn}	38.2^{en}	47.4^{efo}	55.8^{efp}	58.3^{dp}
Malate + Lactate	1M+2L	18.2^{cdem}	39.9^{hin}	44.9^{fgo}	52.3^{fgp}	62.3^{gq}	72.3^{gr}
Malate + Pyruvate	1M+2P	15.0^{abcdem}	37.6^{ghin}	53.5^{ho}	64.8^{ip}	67.8^{hp}	71.8f^{gq}
Lactate + Pyruvate	1L+2P	14.3a^{bcdm}	33.4^{fgn}	49.7^{gho}	58.4^{ghp}	63.4^{gq}	76.4^{ghr}
Malate + Lactate	2M+1L	18.6^{cdem}	23.4^{bcdm}	31.9^{cdn}	33.2^{cn}	36.2^{bn}	42.7^{bo}
Malate + Pyruvate	2M+1P	12.7^{abcm}	16.7^{abm}	25.3^{bn}	37.2^{co}	41.6^{cp}	53.2^{cq}
Lactate + Pyruvate	2L+1P	10.3^{am}	16.3^{an}	28.2^{bco}	41.9^{dp}	48.9^{dq}	63.9^{er}

[a] a,b,c,d,e,f,g,h,i,j,k = means in a column within a trait with a different letter differ ($p < 0.05$). m,n,o,p,q,r = means in a row with a different letter differ ($p < 0.05$). Standard error for muscle type incubation and display of muscle = 1.54 - 2.79.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.

Table N.3: *Percentages*[a] *of metmyoglobin accumulation during 12 hr of incubation of tissue homogenates of the Semitendinosus muscle treated with different metabolites*

	Treatment[b] levels, %	Metmyoglobin, % (Incubation time, hr)					
Treatment		0	2	4	8	10	12
Control	0	14.6^{am}	32.8^{dn}	46.7^{fo}	63.4^{fp}	71.7^{iq}	82.2^{lr}
Malate	1M	25.2^{cm}	29.1^{dm}	34.7^{dn}	48.1^{deo}	56.4^{fp}	66.3^{iq}
Lactate	1L	14.0^{am}	25.8^{cn}	36.5^{do}	54.7^{ep}	65.2^{hq}	71.5^{jr}
Pyruvate	1P	12.8^{am}	30.4^{dn}	41.8^{eo}	49.8^{dep}	58.7^{fgq}	66.7^{ir}
Malate	2M	18.6^{bm}	21.1^{bm}	20.4^{bm}	25.2^{bn}	33.2^{bo}	41.8^{dpe}
Lactate	2L	10.4^{am}	12.7^{am}	14.7^{amn}	17.7^{an}	24.2^{ao}	28.1^{ao}
Pyruvate	2P	11.1^{am}	17.3^{bn}	28.2^{co}	38.3^{cp}	42.9^{dq}	47.9^{fgr}
Malate + Lactate	1M+1L	18.5^{bm}	19.9^{bcm}	22.4^{bcm}	35.7^{cn}	52.5^{efo}	64.4^{hip}
Malate + Pyruvate	1M+1P	12.8^{am}	21.6^{bn}	28.5^{co}	45.6^{dp}	55.7^{fq}	78.8^{klr}
Lactate + Pyruvate	1L+1P	19.6^{bm}	28.4^{dn}	38.3^{do}	55.4^{ep}	63.4^{ghq}	76.4^{kr}
Malate + Lactate	2M+2L	25.5^{cno}	19.3^{bcm}	20.4^{ab}	20.7^{am}	22.3^{amn}	29.8^{abo}
Malate + Pyruvate	2M+2P	17.7^{bm}	22.4^{bcmn}	22.3^{bcmn}	27.2^{bn}	39.2^{cdo}	48.2^{fgp}
Lactate + Pyruvate	2L+2P	16.7^{bm}	23.4^{cn}	36.9^{do}	46.6^{dp}	48.3^{eq}	52.7^{gr}
Malate + Lactate	1M+2L	14.4^{am}	15.5^{abm}	18.6^{abmn}	22.7^{abn}	36.4^{bco}	44.4^{efp}
Malate + Pyruvate	1M+2P	12.6^{am}	26.6^{cn}	35.5^{do}	48.6^{dep}	55.7^{fq}	61.4^{hr}
Lactate + Pyruvate	1L+2P	18.8^{am}	29.7^{cdn}	41.3^{eo}	52.8^{ep}	61.6^{ghq}	77.9^{kr}
Malate + Lactate	2M+1L	13.5^{am}	24.3^{cdn}	26.6cn	29.1^{bno}	31.3^{bo}	34.4^{bcp}
Malate + Pyruvate	2M+1P	27.7^{cm}	23.9^{cdm}	24.3^{bcm}	27.2^{bm}	36.1^{bcn}	38.2^{cdn}
Lactate + Pyruvate	2L+1P	16.7^{bm}	29.8^{dn}	39.9^{do}	52.6^{ep}	63.3^{ghq}	67.7^{ijr}

[a] aa,b,c,d,e,f,g,h,i,j,k = means in a column within a trait with a different letter differ ($p < 0.05$). m,n,o,p,q,r = means in a row with a different letter differ ($p < 0.05$). Standard error for muscle type incubation and display of muscle = 2.14 - 2.88.

[b] 1M = 1% malate; 2M = 2% malate; 1L = 1% lactate; 2M = 2% lactate; 1P = 1% pyruvate; 2P = 2% pyruvate; 1M+1L = 1% malate and 1% lactate; 1M+1P = 1% malate and 1% pyruvate; 1L+1P = 1% lactate and 1% pyruvate; 2M+2L = 2% malate and 2% lactate; 2M+2P = 2% malate and 2% pyruvate; 2L+2P = 2% lactate and 2% pyruvate; 1M+2L = 1% malate and 2% lactate; 1M+2P = 1% malate and 2% pyruvate; 1L+2P = 1% lactate and 2% pyruvate; 2M+1L = 2% malate and 1% lactate; 2M+1P = 2% malate and 1% pyruvate; 2L+1P = 2% lactate and 1% pyruvate.