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Metabolomics Approach to Investigate Oxidation Stabilities In Different Bovine Muscles With Postmortem Aging

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METABOLOMICS APPROACH TO INVESTIGATE OXIDATION STABILITIES IN DIFFERENT BOVINE MUSCLES WITH POSTMORTEM AGING

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of

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This thesis is dedicated to my families (include my little ones in the future) for them sharing all the love and beautifulness of life with me

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LIST OF ABBREVIATIONS

CD	Conjugated diene
DMb	Deoxymyoglobin
HPLC-MS	High performance liquid chromatography –mass spectrometry
LL	*Longissimus lumborum*
MMb	Metmyoglobin
MRA	Metmyoglobin reducing activity
OCR	Oxygen consumption rate
OMb	Oxymyoglobin
ORAC	Oxygen radical absorbance capacity
PM	*Psoas major*
SM	*Semimembranosus*
TBARS	2-Thiobarbituric acid reactive substances
WOF	Warmed-over flavor

ABSTRACT

Ma, Danyi. M.S., Purdue University, August 2016. Metabolomics Approach to Investigate Oxidation Stabilities In Different Bovine Muscles With Postmortem Aging. Major Professor: Y. H. B. Kim.

Postmortem aging has been extensively practiced in the meat industry for decades due to its beneficial impact on improving eating quality attributes. However, prolonged postmortem aging may adversely impact color and/or lipid oxidation stabilities of meat, leading to decreased display shelf-life and increased rancid off-flavor. As physical/biochemical changes occur during postmortem aging, metabolites that impart antioxidant or pro-oxidant activities are generated or consumed, which could be related to the oxidation stabilities of postmortem muscles with aging. However, the underlying mechanism of the aging-induced oxidation susceptibility has not been fully established. Therefore, the overall objective of this thesis was to understand the effect of postmortem aging on oxidation related metabolites in different bovine muscles.

The first chapter of this thesis is the literature review, and the second chapter focuses on determining the effect of postmortem aging on color and lipid oxidation stabilities in different bovine muscles. Three muscles, *longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM) were chosen (n=7) for the study due to

the distinct features in muscle fiber composition, oxidation stabilities, and metabolic properties of those muscles. At 1-day postmortem, the three muscles were separated from 7 beef carcasses, divided into 3 sections, vacuum-packaged, and assigned to 9, 16, and 23 days of aging. After each aging time, steaks made from each sections were overwrap PVC-packaged and displayed for 7 days. Instrumental and sensory color characteristics were measured. Lipid oxidation by measuring TBARS and conjugated dienes (CD), myoglobin content, and non-heme iron contents were determined. Intensified discoloration and decreased redness of meat were observed with prolonged aging, where LL was the most color-stable followed by SM and PM ($P<0.05$). Lipid oxidation increased with aging, and LL was more oxidative stable (lower TBARS and CD) compared to SM and PM. Lower myoglobin and non-heme iron contents were observed in LL compared to SM and PM ($P<0.05$). A significant increase in non-heme iron contents during aging was found. The results of the current study indicate that color and lipid oxidation stability could be negatively associated with aging, and different muscles have different susceptibilities to oxidation. This observation suggests that developing a post-harvest aging strategy to track optimal aging times for different muscles would be beneficial for the meat industry to minimize the oxidation-related quality defects while maintaining consistent eating attributes.

The third chapter of this thesis was a study to identify key metabolites that could be associated with oxidation stabilities of aged bovine muscles using metabolomics approach. Selected raw samples (n=4; at day 0 of display of each aging) from the previous study (Chapter 2) were analyzed using the HPLC-ESI-MS metabolomics. The metabolomics data were analyzed using PCA and split-plot ANOVA. Spearman

correlations between metabolites and oxidation related quality attributes were conducted using R software. The metabolomics platform detected 1012 compounds, among which 243 were significantly responsive to either aging or muscle treatments. Most distinct metabolites being identified were carnitines, free amino acids, nucleotides, vitamins /coenzymes, and glucuronides. NAD showed a positive correlation to the redness of meat color ($r = 0.672$) and negative correlations to discoloration ($r = -0.535$), TBARS ($r = -0.554$), and non-heme iron ($r = -0.667$), indicating its relevance to myoglobin redox form stability and/or lipid oxidation stability. A group of carnitines that decreased with aging was associated with decreased redness ($r = -0.67$) and intensified discoloration ($r = 0.70$), which may be explained by their roles in the mitochondria matrix and/or their potential antioxidant properties. Glucuronides increased with extended aging and associated with discoloration ($r = 0.56$) and non-heme iron accumulation ($r = 0.65$). Some nucleotides, nucleosides and free amino acids were more liberated with aging and positively correlated to chemical/phenotypic oxidation indicators.

The results from the current study suggest that some metabolites could be associated with oxidation stabilities of beef muscles. In particular, our study confirmed the relevance of the NAD/NADH system in myoglobin redox stability. Further, we identified potential compounds, such as carnitines and glucuronides, which could be related to color/lipid oxidation stabilities of aged beef muscles. The identified key metabolites should be further investigated to reveal the chemical basis of oxidation stabilities of beef muscle and could be validated into biomarkers for industry to track the quality development of beef during aging.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to meat color

The surface color of fresh meat is one of the most important factors that affect consumers' purchasing decision. A bright cherry-red color is perceived to be associated with desirable quality and freshness of meat (Jeremiah, Carpenter, & Smith, 1972). A study found that when 30%-40% of the meat surface is discolored with brown pigments, purchasing decisions would be reconsidered (Hood & Riordan, 1973). Annual economic loss due to discolored beef could be as high as $1 billion for the US beef industry (Smith, Belk, Sofos, Tatum, & Williams, 2000).

1.1.1 Myoglobin structure and chemistry

In meat, myoglobin comprises the major portion of the pigment compounds (about 90%) (Warriss & Rhodes, 1977). The remaining portion is composed by cytochromes, hemoglobin, and flavins, which could marginally affect meat color (Mancini & Hunt, 2005). The structure and chemical properties of myoglobin are closely related to meat color change and have been well described by several reviews (Mancini & Hunt, 2005; Suman, Hunt, Nair, & Rentfrow, 2014; Suman & Joseph, 2013). Myoglobin, whose molecular weight is approximately 17 kDa, is a monomeric, globular protein formed by

140 to 160 amino acid residues with a heme group in a crevice of the molecule (Renerre, 2000). The structure of the myoglobin (Figure 1.1) reveals a hydrophobic pocket that holds a prosthetic group, which contains a heme-ring structure in the center. The heme ring has six binding sites, four of which bind to an iron atom in the center, while the fifth binds to the proximal histidine 93 on the peptide chain. The sixth site is the free binding site for ligands including but not limited to O_2, CO_2, NO, CO, or H_2O (Suman & Joseph, 2013).

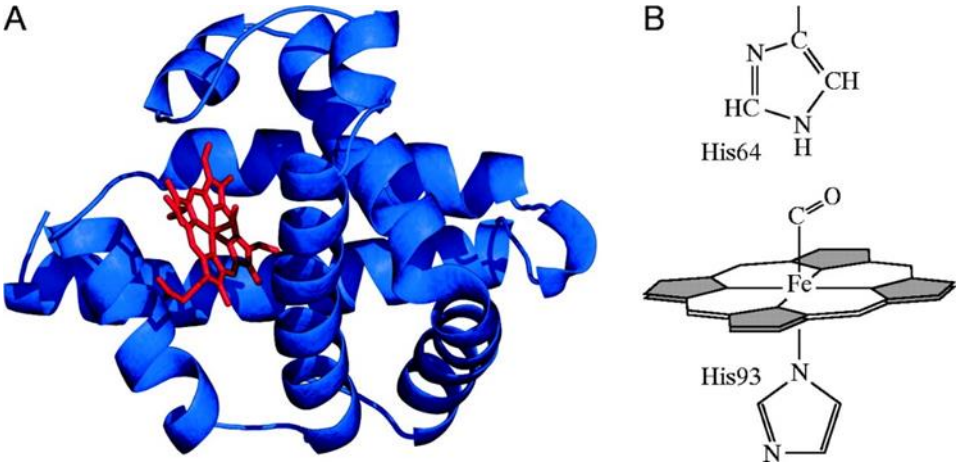

Figure 1.1 Myoglobin structure.
Reproduced from Ordway & Garry, 2004

Specifically, the oxidation status of the iron atom and the binding ligands would determine the chemical status of myoglobin and furthermore its coloration (Suman & Joseph, 2013). The concentration of myoglobin is affected by difference in species, sex, breed, age (Kim & Hunt, 2011), muscle types, and anatomic location within the muscle (Seideman, Cross, Smith, & Durland, 1984). In fresh meat, the three most common

myoglobin derivatives are deoxymyoglobin (DMb), oxymyoglobin (OMb), and metmyoglobin (MMb) (Figure 1.2).

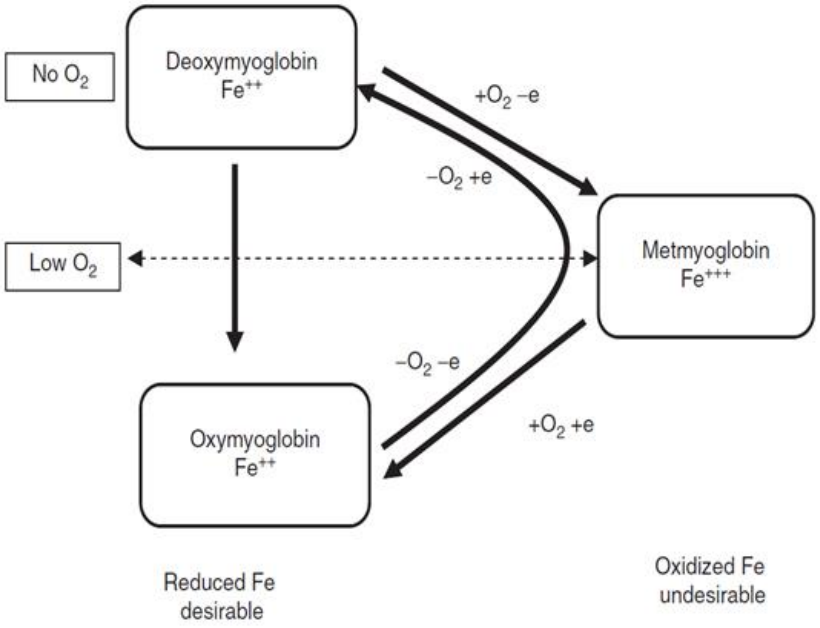

Figure 1.2 Myoglobin redox form triangle.
Adapted from Encyclopedia of Meat Science Vol.3 (Keeton, Eddy, & Werner Klinth, 2004).

DMb, which presents as purplish-red color, is free from ligand binding with ferrous (Fe^{2+}) iron status, which can be seen in freshly cut or vacuum packaged meat (Cornforth, 1989). Conversion between DMb and OMb is reversible through the process of oxygenation and deoxygenation, and this transition is the natural base for the oxygen transporting activity of myoglobin. Oxygenation implies the ligand binding of an O_2 molecule to the myoglobin with the ferrous status of iron, and deoxygenation is the reverse. OMb has a bright cherry-red color, which is most attractive to red meat consumers (Livingston & Brown, 1981). MMb refers to a form of myoglobin in which

the iron is oxidized to the ferric status (Fe^{3+}). MMb has a brownish-red color, and the presence of MMb on the meat surface commonly indicates the discoloration of meat (Mancini & Hunt, 2005).

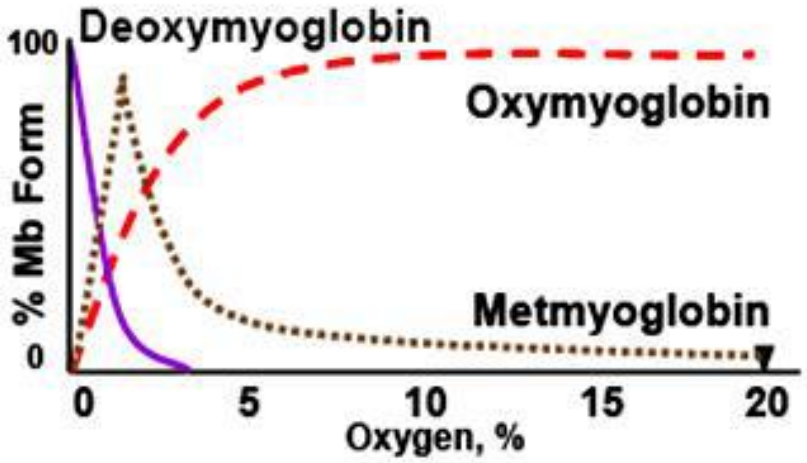

Figure 1.3 Dominant Myoglobin redox forms under different partial oxygen pressure. (Adapted from Kropf, 1980).

MMb formation is favored under relatively low oxygen partial pressure (2% to 3%). As oxygen content increases, OMb would gradually become the stable, dominant form of myoglobin (Figure1.3, Kropf, 1980). MMb is an innate form of myoglobin in live muscles, which is steadily maintained at 2-3% level (Halliwell & Gutteridge, 1999). The presence of MMb is induced by the low oxygen partial pressure in working muscles, but it can be quickly reduced by the innate metmyoglobin reducing activity (MRA) to maintain the function of oxygen transportation. During postmortem storage, MMb gradually increases due to the loss of MRA, changes in oxygen partial pressure, pH, and other factors, leading to meat discoloration (Bekhit & Faustman, 2005).

1.1.2 Factors affecting meat color

1.1.2.1 Metmyoglobin reducing activity (MRA)

Fresh muscle tissues have the limited ability to reduce MMb to DMb, which is called metmyoglobin reducing activity (MRA) (Stewart, Bobbie K Hutchins, Zipser, & Watts, 1965). MRA was originally observed in pork muscle mince by Walters and Taylor (1963), who found a slow enzymatic reduction of pure MMb in anaerobic conditions. Substantial MRA was observed after ferricyamide treatment of ground beef, inspiring the technique for MRA measurement that is widely used nowadays (Stewart et al., 1965).

Further studies in this field revealed that different muscle tissues have different levels of MRA. Seyfert and others (2006) investigated the color stability, MRA, total reducing activity (TRA) and, cytochrome c oxidase activity from five different types of beef muscles: *psoas major* (PM), *longissimus lumborum* (LL), superficial *semimembranosus* (SSM), deep *semimembranosus* (DSM), and *semitendinosus* (ST). The results showed that LL and ST had a higher color stability than that of PM and DSM, while the color stability of SSM was intermediate. The MRA was also higher in LL and ST and lower in DSM and PM ($P<0.05$). They observed a strong correlation between color stability and MRA among muscle types. On the contrary, differences in TRA and cytochrome c oxidase activity were not significant (Seyfert et al., 2006).

MRA requires reducing substrates and/or co-factors to initiate the conversion process. NADH is one of the well-known reducing equivalents that are involved in the reducing system (Bekhit & Faustman, 2005). During the early postmortem stage when the muscle to meat conversion process is initiated, muscle cells still retain enzyme activity and glycogen to undergo anaerobic respiration and produce lactic acid to lower

the pH of postmortem muscles. It was proposed by Watts in 1966 that lactate dehydrogenase (LDH) can replenish NADH from NAD+ by consuming lactic acid (Watts, Kendrick, Zipser, Hutchins, & Saleh, 1966). Then NADH can reduce MMb either enzymatically or non-enzymatically. Kim and others (2009) investigated three different bovine muscles: LL, SM, and PM, and found that LL was the most color stable muscle with highest LDH-B activity, LDH1 expression, NADH concentration, and MAR activity (Kim, Keeton, Smith, Berghman, & Savell, 2009).

The enzymatic reduction of MMb depends on metmyoglobin reductase, which can be attributed to several enzymes including NADH-cytochrome b5 reductase, erythrocyte NADH cytochrome b5 reductase, diaphorase, and cytochrome c reductase (Bekhit & Faustman, 2005). Extensive studies have been conducted to investigate the underlying mechanisms of metmyoglobin reductase. These enzyme activities were found in both mitochondria fraction and microsomal fraction of the cells (Keizo Arihara, Itoh, & Kondo, 1989). The study showed that MRA may use cytochrome b5 both on the outer membrane of the mitochondria and in the sarcoplasmic reticulum, which explained the observation that both the mitochondria fraction and microsomal fraction of the muscle extracts exhibited enzymatic reduction activity (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995).

Non-enzymatic reduction of MMb has also been suggested as an additional mechanism of the reducing system of muscle (Brown & Snyder, 1969; Koizumi & Brown, 1972). However, most experimental settings of non-enzymatic reduction were EDTA dependent. EDTA was proposed to inhibit transient-metal-induced myoglobin autoxidation, which was necessary for observing MRA in non-enzymatic system (Brown

& Snyder, 1969; Koizumi & Brown, 1972). Earlier studies observed non-enzymatic reduction activity in porcine (Mikkelsen, Juncher, & Skibsted, 1999), but not in bovine or ovine muscles (Faustman, Cassens, Schaefer, Buege, & Scheller, 1989; Hagler, Coppes, & Herman, 1979; Reddy & Carpenter, 1991). However, most recently, Elroy and others did a detailed study on non-enzymatic MRA in vitro (Elroy, Rogers, MafiVanOverbeke, Hartson, & Ramanathan, 2015). They observed a strong species-specific effect on the rate of non-enzymatic MRA, where myoglobin from bovine samples was significantly higher in MRA than that from equine and porcine samples. Additionally, pH significantly affected the rate of non-enzymatic MRA, where MRA at pH 5.6 was stronger than that at pH 7.4 (Elroy et al., 2015). Furthermore, lipid oxidation products that are commonly found in meat, such as 4-hydroxy-2-nonenal (HNE), could inhibit enzymatic MRA by reacting with myoglobin. The authors also investigated the role of HNE in non-enzymatic MRA and reported a decreased non-enzymatic MRA due to mono-adducts of HNE with myoglobin revealed in MS spectrum (Elroy et al., 2015).

A third form of MMb reduction was proposed to be the direct reduction by the electron transportation chain located in mitochondrion (Watts et al., 1966). Reduction by electron transport chain requires an anaerobic environment and supplementation of succinate as the reducing agent. An *in vitro* study substantiated the involvement of electron transportation chain in MMb reduction. The inhibitor analysis revealed that the working electrons donors for MRA were between complex III and IV of the electron transport chain (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005).

1.1.2.2 Myoglobin content

As myoglobin contributes to the major pigment compounds in the meat, different levels myoglobin concentration would affect meat color. It is generally established that muscles with higher myoglobin content appear redder/darker in color, whereas a recent study has confirmed a moderate correlation ($r = 0.43$) between myoglobin content and lightness value (McKenna et al., 2005). Myoglobin contents varies among muscles with different anatomic locations, physiological functions and different species. Other intrinsic/extrinsic factors affecting myoglobin concentration include animal species and genetics (King et al., 2010; Meng, Bentley, & Pittman, 1993), sex states (Seideman et al., 1984), age (Kim, Stuart, Black, & Rosenvold, 2012), muscle fiber composition (Hunt & Hedrick, 1977), living environments such as altitude of the habitat (Anthony, Ackerman, & Strother, 1959), and diet/nutritional conditions (Kim & Hunt, 2011).

1.1.2.3 Non-heme iron

In meat, iron is the most abundant trace mineral, which primarily presents as the form of heme iron. The minor portion of iron fragment in meat, the non-heme iron, is critically involved in postmortem muscle biochemistry due to its significant pro-oxidative activity (Allen, 2009). The non-heme iron is present as iron sulfur clusters in metalloproteins or forms organic salt with amino acids (Decker & Hultin, 1992). Given the existence of reducing compounds in meat, such as ascorbic acid and NAD (P)H, the non-heme iron is present in redox form (coexist of Fe^{2+} and Fe^{3+}), which makes it highly reactive as a pro-oxidant (Decker & Hultin, 1992).

Several studies attempted to assess the involvement of non-heme iron in myoglobin oxidation in postmortem muscle. Rhee and Ziprin (1987) proposed that higher heme pigment content may catalyze OMb auto-oxidation. Moderate to high correlation between non-heme iron content and color traits have been found in various bovine muscles and other meat products as well (Estevez & Cava, 2004; Purohit, Singh, Kerr, & Mohan, 2015). It was also reported that the decrease of OMb was partially attributed to two simultaneous reactions, the formation of MMb and the removal of the heme group (Prasad, Engelman, Jones, & Das, 1989). The later reaction was facilitated by the hydrogen peroxide generated by the former reaction and was then followed by the release of free iron, causing non-heme iron accumulation.

In previous studies, the redox activity of non-heme iron catalyzing lipid oxidation has been well described (Gorelik & Kanner, 2001; Kanner, Shegalovich, Harel, & Hazan, 1988). As various lipid oxidation products can be facilitators of myoglobin oxidation (Faustman, Sun, Mancini, & Suman, 2010), Gorelik and Kanner (2001) proved that accelerated lipid oxidation due to non-heme iron catalysis in turn can promote OMb oxidation.

1.1.2.4 Muscle type

Effects of muscle type on meat color can be mainly attributed to the effects of muscle fiber compositions. Muscle fibers can be mainly categorized into 4 types: type I, type IIA, type IIX, and type IIB. Type I fibers are characterized as “slow twitch, oxidative” fiber. They are higher in myoglobin level and mitochondrial activity, which make them more efficient in oxygen transportation and consumption (Close, 1972). As a

result of oxidative metabolism preference, the type I fibers can "burn" glucose completely into CO_2 and H_2O, thus less lactic acid will be accumulated during muscle exercise, resulting in high resistance to fatigue in live animals (Close, 1972). Type IIB fibers, on the other hand, are fast twitch, glycolytic type with lower myoglobin content and mitochondria intensity. The main metabolism activity in these fibers is glycolysis, namely anaerobic respiration, which is more efficient in providing immediate energy to support explosive intense activity; however, the following lactic acid accumulation renders the fibers limited in fatigue resistance. Meanwhile, the remaining two types are the intermediate fiber types (Gerrard & Grant, 2003).

Muscles higher in type I fiber composition in general appear darker/redder due to more myoglobin content (Hunt, 1977), which are categorized as red muscles. In bovine, *psoas major* (PM) as a representative red muscle, is a high value meat cut that has premium tenderness but low color stability (Seyfert et al., 2006; Decker & Hultin, 1992; Lanari & Cassens, 1991; McKenna et al., 2005). Although MRA has been proposed to be a good indicator of color stability (Seyfert et al., 2006), higher MRA (either enzymetical or non-enzymatical) was repeatedly observed in red muscles like PM (Echevarne, Renerre, & Labas, 1990; Renerre, & Labas, 1987). It is also reported that PM muscles have accelerated myoglobin auto-oxidation rate in comparison with other white muscle representatives such as *longissimus* or *tensor fasciae latae* (Renerre, Anton, & Gatellier, 1992; Renerre, & Labas, 1987). The inconsistency between the observed color stability and MRA in red muscles could be partially explained by the metabolic properties of red muscles. As a result of elevated type I fiber composition, red muscles have more mitochondria that may consume oxygen, which will compete with myoglobin for oxygen

binding. Such competition may eliminate the oxygen content that is available to the myoglobin and reduce the level of myoglobin oxygenation (Seideman et al., 1984). However, the different color stabilities and/or oxidation stabilities due to muscle type in postmortem muscles have not been fully understood.

1.1.2.5 Postmortem aging

Postmortem aging is the indispensable management practice referring to a period of storage time under the chilling environment after animal slaughter. During aging, meat palatability can be improved due to the degradation of meat structure and accumulation of flavor related compounds. Wet aging under vacuum packaging conditions is the most commonly practiced aging regime, during which vacuum packaging minimizes the contact of meat to oxygen to preserve myoglobin oxidation. In retail practice, the aged meat cuts are commonly displayed in foam trays wrapped with polyvinyl chloride (PVC) film, which forms an oxygen permeable environment. Such a condition allows the formation of OMb layer on the meat surface, giving the product a cherry-red appearance. It has been reported that short-term aging (around one week) improved meat color (increased redness and color intensity) during display compared with non-aged meat (Abdullah & Qudsieh, 2009; Teixeira, Pereira, & Rodrigues, 2011). Such improvement was suggested to be associated with decreased oxygen consumption rate (OCR) (Seideman et al., 1984). During postmortem aging, loss of mitochondria enzyme activity results in decreased OCR, therefore, more oxygen is available to penetrate into a deeper layer of the meat and forms a thicker layer of OMb. Such a situation rendered the meat with increased redness and/or color intensity compared to early postmortem muscles with

high OCR (Seideman et al., 1984). On the contrary, some studies found that long-term aging (more than 2 weeks) impairs initial color development and results in decreased redness and color intensity (D. King, Shackelford, Kalchayanand, & Wheeler, 2012; Suman et al., 2014). Some studies found that prolonged aging time (i.e. up to 3 weeks) did not significantly change the initial color, but surely impaired color stability during the following display section (King et al., 2012; Lindahl, Lagerstedt, Ertbjerg, Sampels, & Lundström, 2010). According to Descalzo and colleagues (2008), significant decrease of innate reducing compounds such as α-tocopherol and β-carotene in Argentina buffalo meat was observed from 15 d to 25 d postmortem (Descalzo, Rossetti, Sancho, Garcia, Biolatto, Carduza, & Grigioni, 2008). These current findings are in consistent with an early study in which the drastic decrease of NAD concentration, OCR, and MMb reductase activity were observed in beef LL and PM muscles throughout a 21-day aging process (Madhavi & Carpenter, 1993).

1.1.2.6 Packaging system

Diversified packaging techniques have been developed to minimize the post processing spoilage to extend shelf-life. Overwrap packaging is the cheapest and most commonly adopted method during commercial display where meat is packed in disposable plastic foam trays wrapped with oxygen-permeable polyvinylchloride (PVC) films. This packaging method allows for OMb formation on the meat surface, and it is good for the initial red color development. However, it results in less display and microbiological shelf-life, since normal atmosphere (21% oxygen partial pressure) will provide a favorable aerobic condition for bacterial growth (Kim & Hunt, 2011), but

neither minimizes the formation of MMb nor maintains a thick, stable OMb layer to prolong the color-life (Kim & Hunt, 2011).

Modified atmosphere packaging (MAP) became popular because it favors a better color stability and a longer shelf-life with more efficient bacterial control (McMillin, 2008). High oxygen atmosphere (80% O_2 plus 20% CO_2, Hi-Oxi MAP) promotes color stability, because it produces a thicker and more stable bright-red OMb layer under high oxygen partial pressure (refer to Figure 1.3), with an up to 10 days' longer shelf-life was observed (Phillips, 1996). Bacterial control is also provided by this method, because high oxygen partial pressure prevents growth of anaerobes, and CO_2 inhibits Gram-negative bacteria by penetrating into the bacteria cells to change the pH and enzyme activity (Phillips, 1996). However, this method has several drawbacks including accelerated bone discoloration (Mancini et al., 2005), lipid oxidation and/or lean discoloration at the end of display, and reduction in tenderness and juiciness due to protein oxidation and polymerization (Aaslyng, Torngren, & Madsen, 2010; Grobbel, Dikeman, Hunt, & Milliken, 2008; Hague et al., 1994). Steaks stored under Hi-Oxi MAP are more likely to exhibit "premature browning" during cooking, which refers to the accelerated browning of meat surface color. Such a condition may be more likely to result in under-cooked meat and brings food safety concern (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007).

To address the shortcomings of the Hi-Oxi MAP method, an ultra-low oxygen MAP (CO-MAP) was developed. The CO-MAP used 0.4% CO to provide a cherry-red color to the meat, because CO binds with myoglobin with high affinity to form carboxymyoglobin (CO-Mb), which is a bright-red pigment (Wilkinson, Janz, Morel,

Purchas, & Hendriks, 2006). The remaining portion of the gas comprises of CO_2 (30-60%) and N_2 (40-70%) without O_2. This method enhances MRA (Seyfert, Mancini, Hunt, Tang, & Faustman, 2007; Mark Seyfert et al., 2006) and is very useful for long-term transportation before shelf display (Hunt et al., 2004; Jayasingh, Cornforth, Brennand, Carpenter, & Whittier, 2002). Moreover, CO-MAP could overcome the irradiation-induced color deterioration, thus the combined usage of irradiation and CO-MAP is recommended (Kusmider, Sebranek, Lonergan, & Honeyman, 2002). However, CO-Mb may lead to the "persistent pinking", which refers to the situation that cooked meat color may still remain pink even under over-cooked temperature (King & Whyte, 2006). The concentration of CO was also strictly regulated, for the fact that high concentration of CO may form a thicker, more stable CO-Mb layer that can mask spoilage (Hunt et al., 2004).

Vacuum packaging is the most effective form of meat packaging for storage/aging purposes. It provides both anti-bacteria effect and high efficiency in storage space. However, it leads to a dark purplish-red color of the meat due to the removal of oxygen from myoglobin. To off-set this downside, a new method called Fresh Case was invented. Fresh Case uses sodium nitrite on the inner side of meat package, which can react with myoglobin to form bright red nitrosylmyoglobin. Several conditions are needed for the optimized results with Fresh Case, but it is generally considered an attractive choice of meat package to consumers (Siegel, 2010).

1.2 Introduction to lipid oxidation in meat

The lipid portion of muscle food affects meat quality to a large extent because its composition and involved chemical reactions are closely related to meat color, flavor, and

tenderness (Kanner, 1994; Wood et al., 2004), which are the three most important quality traits that determine consumers' satisfaction (Gray, Gomaa, & Buckley, 1996).

Meat lipids can be categorized into two types, the structural lipids, which refer to the phospholipids and cholesterols that compose the cell membrane, and the storage fat, meaning adipose tissues that deposit closely to the muscles. The phospholipids in cell membrane (sarcolemma), although they represent a minor portion of the total lipids, contribute to a large percentage of the total polyunsaturated fatty acids (PUFA); while the storage fat mainly comprises with saturated fatty acids and monounsaturated fatty acids (MUFA). Both PUFA and MUFA are main substrates of lipid oxidation reactions. The lipid oxidation reactions are the main deleterious reactions in muscle food that negatively affect meat surface color, flavor, and texture (Gray et al., 1996).

1.2.1 Basic mechanism of lipid oxidation

Lipid oxidation was defined as "a multi-step, multifactorial process", in which the common effective factors that can be found in food systems include molecular structure and physical status of the lipids, presence of catalysts (metals or oxidative free radicals), and application of antioxidants (Jackson, Knize, & Morgan, 2012). Lipid oxidation primarily comprises of 3 steps: initiation, propagation, and termination (Min & Ahn, 2005). In the initiation stage, a methylene group of a lipid molecule (LH) is attacked by an activated oxygen species, loses a labile hydrogen and forms a lipid radical (L•, reaction1). The lipid radical tends to go through intramolecular rearrangements and stabilizes into a conjugated diene, which can be an important marker for primary oxidation assessment (Buege & Aust, 1978).

$$LH+O_2 \rightarrow L\bullet+\bullet OOH \quad (1)$$

In the propagation stage, the conjugated diene reacts with oxygen to form a more reactive species called lipid peroxyl radical (LOO•, reaction2). The labile hydrogens on the intact lipid molecules are more susceptible to LOO• attack, which consequentially leads to the formation of lipid hydroperoxides (LOOH) and lipid radical (L•, reaction 3). In this regard, lipid radicals will be generated easier and faster with existence of LOO•, such that the propagation of the chain reaction will be maintained (Min & Ahn, 2005).

$$L\bullet+O_2 \rightarrow LOO\bullet \quad (2)$$

$$LOO\bullet+LH \rightarrow LOOH+L\bullet \quad \Delta H=-9 \text{ kcal/mol} \quad (3)$$

LOOH can induce further oxidation reactions and forms various secondary derivatives, such as alcohols, aldehydes, and ketones. The chain reactions will be terminated when the radicals react to each other to form stabilized compounds (Min & Ahn, 2005), which can be summarized as follows (reactions 4-6):

$$L\bullet+L\bullet \rightarrow L\text{-}L \quad (4)$$

$$L\bullet+ LOO\bullet \rightarrow LOOL \quad (5)$$

$$LOO\bullet+ LOO\bullet \rightarrow LOOL + O_2 \quad (6)$$

1.2.2 Adverse impacts of lipid oxidation on meat quality

It has been proposed that except microbial spoilage, lipid oxidation is the primary process that leads to quality deterioration in muscle foods (Buckley et al., 1989). Lipid oxidation products decrease nutrition value of meat product and bring food safety/ human health concerns. Lipid oxidation negatively affects meat quality attributes such as color, tenderness, and flavor. Reactive lipid oxidation products, especially aldehydes, are

considered to induce or promote common diseases such as cancer and cardiovascular diseases (Graham et al., 2012; Ritota, Casciani, Failla, & Valentini, 2012).

Rancid oxidation generates volatile compounds with unpleasant odors that are responsible for flavor deterioration, among which some pro-oxidative compounds may also be generated and cause toughness and discoloration issues by inducing protein polymerization/denaturation (Lynch, Faustman, Silbart, Rood, & Furr, 2001) and myoglobin oxidation (Lynch & Faustman, 2000).

The two terms, warmed-over flavor (WOF) or rancid taste, are often used to describe the flavor deterioration in cooked, refrigerated, and precooked meat products. Aldehydes are most common volatiles that are perceived as WOF even at low concentration (ppb level) (Ladikos & Lougovois, 1990), including pentanal, pentenal, hexnal, and 2, 4-decadienal hexenal (HNE). Given such low concentrate, instrumental analysis such as gas chromatography can be used to detect the existence of volatiles and assess the extent of WOF development (St Angelo et al., 1987).

Reactive oxygen species (ROS) produced in lipid oxidation are suggested to be major factors that promote discoloration by reacting either directly to facilitate pigment auto-oxidation or indirectly to damage the MRA systems (Gray et al., 1996). According to Hutchins et al. (1967), lipid oxidation and MMb formation were moderately correlated ($r = 0.73$). In addition, lipid oxidation end-products could evolve in promoting myoglobin oxidation. In a series of *in situ* model system studies, the researchers found that HNE, a representative aldehyde compound generates from food lipid oxidation, can react with OMb to form adducted complexes and accelerate OMb auto-oxidation (Faustman, Liebler, McClure, & Sun, 1999). Also, pre-incubation of MMb with HNE “rendered the heme

protein a poorer substrate for enzymatic MMb reduction" (Lynch & Faustman, 2000). Some aldehyde compounds may react with structural proteins to form adducts and induce protein polymerization and denaturation (Lynch et al., 2001), which may further link to the increased toughness and decreased water holding capacity of meat.

1.2.3 Measurement of lipid oxidation

Lipid oxidation can be assessed according to sensory, physical, and chemical dimensions (Lai, Gray, Booren, Crackel, & Gill, 1995). As lipid oxidation is a complex process with multiple inner mechanisms and various intermediates and end products, a single method cannot present all the major oxidative reactions at one time to evaluate the quality deterioration of food.

1.2.3.1 2- Thiobarbituric acid reactive substance (TBARS)

TBARS refers to the 2- thiobarbituric acid reactive substance, which is the most commonly adopted method to assess the extent of lipid oxidation, particularly in food systems (Ahn, Grün, & Fernando, 2002; Carpenter, O'Grady, O'Callaghan, O'Brien, & Kerry, 2007; Kim, Nam, & Ahn, 2002)**.** The reactive substance malondialdehyde (MDA), a common lipid oxidation end product, can react with 2-thiobarbituric acid (TBA) to form red-colored complexes. Spectrophotometric absorbance of such colored complex will reveal the abundance of MDA in the system and indicate the extent of lipid oxidation.

1.2.3.2 Conjugated diene (CD)

At the initiation stage of lipid oxidation, one labile hydrogen that adjacent to the carbon-carbon double bond is abstracted by ROS, resulting in a lipid radical. The lipid radical converts into conjugated diene (CD) structure through intramolecular rearrangements, which can be measured at 233 nm absorption. Determination of conjugated diene formation is considered to represent primary lipid oxidation (Srinivasan, Xiong, & Decker, 1996), while TBARS values indicate secondary lipid oxidation or end product accumulation. As CD is one of the intermediates of lipid oxidation, the retained CD content in muscle could be affected by both formation and decomposition processes. As a result, changes of CD values in muscles are not always consistently correlated to TBARS in a similar manner. Therefore, combination of both methods is usually adopted to describe a full picture of the lipid oxidation process (Chaijan, Benjakul, Visessanguan, & Faustman, 2006; Kanner, Harel, & Jaffe, 1991; Roldan, Antequera, Armenteros, & Ruiz, 2014).

1.2.3.3 Oxygen radical absorbance capacity (ORAC)

ORAC (Oxygen radical absorbance capacity) is a standard method to measure antioxidant activity of a biological system in many disciplines, particularly in food science (Cao, Alessio, & Cutler, 1993). The ORAC method makes use of a chemical called AAPH (2, 2’ –Azobis (2–Amidinopropane) hydrochloride), which produce peroxyl radicals that can be scavenged by the intrinsic antioxidant activity of the experimental subject. The extent of radical scavenging activity can be indicated by the fluorescence generation. By measuring and quantifying the fluorescence generation of the

experimental samples, and by comparing the quantified result to the working curve that obtained from the standard solutions of positive control (an antioxidant called Trolox, 6-hydroxy-2 5 7 8-tetramethychroman-2-carboxylic acid), the total antioxidant activity of the samples can be calculated accordingly (Scott 2012). The ORAC assay measures both the degrees and effective time windows of free radical scavenging activities (Prior & Cao, 1999); therefore, it could be appropriate for total antioxidant activity measurement in food and meat.

1.2.4 Relationship between myoglobin and lipid oxidation

As a transient metal catalyst, the effects of different forms of iron on lipid oxidation have been studied (Baron & Andersen, 2002; Carlsen, Møller, & Skibsted, 2005). One of the most frequently described mechanisms for heme iron to catalyze lipid oxidation is the Fenton-like mechanism (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013; Carlsen et al., 2005). The Fenton reaction is a common way for simple transient metal to generate free oxygen radicals (reaction 7 and 8).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (7)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^{+} \quad (8)$$

In the Fenton-like process (Figure 1.4), ferrous iron (heme-Fe^{2+}) will be oxidized into ferric iron (heme-Fe^{3+}) through heamoprotein (or myoglobin) auto-oxidation, during which the ROS hydroperoxides (HOO•) are formed to initiate and propagate lipid oxidation (Carlsen et al., 2005).

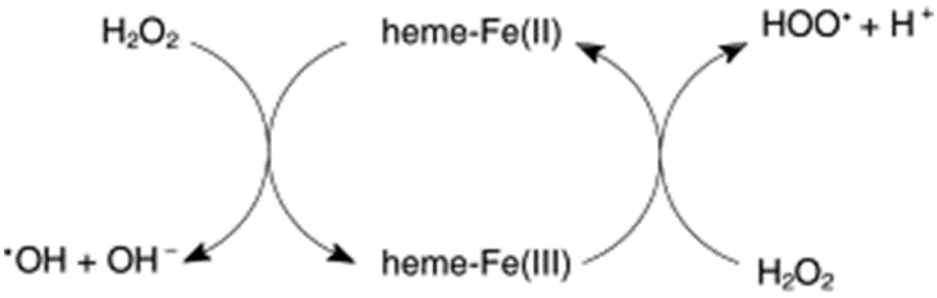

Figure 1.4 Fenton-like process oxidizes heme iron and generates reactive oxygen spicies

In general, because of the protein-binding/heme-chelating system, the catalytic activity of iron in *vivo* is suggested to be insignificant due to limited availability (Baron & Andersen, 2002). In fact, under the biophysical pH conditions in muscle, many experiments were tried but failed to observe a catalytic role of MMb in lipid oxidation (Kanner & Harel, 1985; Mikkelsen, Sosniecki, & Skibsted, 1992; Stewart, 1990). However, after slaughter and during postmortem storage, these metal ions may become more available due to biochemical changes such as pH decline, proteolysis and/or heme release, therefore, can be more involved in oxidation related quality deterioration (Baron & Andersen, 2002). In the *in vivo* studies, the pro-oxidative activity of hemoglobin has been found in low pH environments such as inflammation sites and ischemic sides (Fantone, Jester, & Loomis, 1989). In general, the existed evidence supports a high possibility of ferric-iron (Fe^{3+}) dependent, low-pH (5.3-6.2) promoted, pro-oxidative activity toward lipid oxidation (Baron, Skibsted, & Andersen, 1997; Hogg et al., 1994).

In addition, ferric status of myoglobin plus peroxides were proven to have pro-oxidative activity for lipid oxidation (Hogg et al., 1994). Many scientists thus proposed a

lipid-hydroperoxides dependent heme protein-catalyzed lipid oxidation (Baron et al., 1997; Hogg et al., 1994; Yoshida, Kashiba, & Niki, 1994). Degradation of myoglobin and release of the heme group is reported to promote lipid oxidation. Baron and colleagues (2002) reported that the denatured MMb with exposed heme group would instantly initiate lipid peroxidation in an environment that was rich in lipid double bond. They therefore concluded that myoglobin disintegration/denaturation may result in the release or exposure of the heme group, which would induce hematin-dependent lipid peroxidation, particularly in a lipid rich environment (Baron, Skibsted, & Anderson, 2002).

1.3 Potential of metabolomics to determine meat quality attributes

Metabolites are defined as all the products generated by the cellular activity of a biological organism (Johanningsmeier, Harris, & Klevorn, 2016). Ranging from biochemical intermediates to end products, the level of metabolites in a biological organism fluctuates, and the part of the shown dynamic is the biological response to genetic and/or environmental stimuli (Fiehn, 2002). The study of all the metabolites as a whole, which is normally referred to as metabolomics, is one of the popular ‘omics’ technologies that aims at providing detailed and complete information regarding to the live activities of cells, tissues, organs or bio-fluids (Nicholson & Lindon, 2008). Data in large scales are produced in a high throughput manner for the ‘omics’ study, such as genomics and proteomics (Rochfort, 2005). Compared to the other ‘omics’ technologies, metabolomics is relatively new, with a grand goal to provide comprehensive information of endogenous metabolites at the molecular level and demonstrate global and dynamic

changes of metabolites in a complex multi-cellular system in respond to the challenge of biological and environmental stimuli (Whitfield, German, & Noble, 2004). The actual application of metabolites has flourished in many research areas, such as disease diagnosis (Zhang, Sun, & Wang, 2012), environmental science (Lankadurai, Nagato, & Simpson, 2013), nutrition (Jones, Park, & Ziegler, 2012), and food sciences (García-Cañas, Simó, Herrero, Ibáñez, & Cifuentes, 2012).

1.3.1 Classification of metabolomics studies

1.3.1.1 Classification of metabolomics by the research objective

Depending on the method and purpose of the experiment, metabolomics typically can be categorized as targeted and untargeted studies (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). Untargeted analysis refers to a procedure in which the maximum coverage of metabolites can be simultaneously detected from the complex biological matrix without bias to get a complete picture of metabolic distribution with the knowledge of as many chemical compounds as possible. Targeted metabolomics, on the other hand, focuses on a group of metabolites from particular classes or metabolic pathways through selective purification and extraction methods (Patti, Yanes, & Siuzdak, 2012).

Metabolomics can also be categorized as discriminative, informative, and predictive (Cevallos-Cevallos et al., 2009). Discriminative studies aim at discriminating groups of raw materials, such as diseased population vs healthy population, or spoiled food vs. safe food. In the discriminative studies, no model or pathways are involved, and quantification of the metabolites can be raw and relative. Informative study tries to

provide as much intrinsic information of metabolites as possible, therefore the compound identification needs to be most accurate, and quantification is recommended to be absolute. Informative studies can be used for database establishment, identification of possible pathways, discovery of novel bioactive compounds, and validation of biomarkers. Predictive studies utilize multivariate statistic models generated from the metabolite profiles, such that a variable that is hard to measure can be predicted (Osorio et al., 2013).

1.3.1.2 Classification of metabolomics by the technology platform

The usage of metabolomics is dominated by two methods, nuclear magnetic resonance (NMR) and mass spectrometry (MS). NMR is capable of detecting metabolites from a large category and has the reputation of being robust and reproducible (Mayr, 2008). But the NMR platform has low sensitivity over metabolites at low concentration (lower than the level of 10 μmol/L), with relatively high initial investment of purchasing the instrument (Wishart, 2008). In comparison, the MS based platform is very sensitive, capable of detecting picogram level for some metabolites (Wishart, 2008). As the prevailing technology, the current applications of metabolomics flourish on two platforms: GC-MS (gas chromatography MS) and LC-MS (liquid chromatography MS) (Wishart, 2008). Features of each major platform were well summarized by Wishart et al. (2008).

1.3.2 Basic procedure of metabolomics

A typical metabolomics study includes the following steps: sample preparation/extraction, instrumental separation/detection, data treatment, and potential compound identification (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; D'Alessandro & Zolla, 2013).

1.3.2.1 Sample preparation and extraction

The main goal of sample preparation is to homogenize samples, break down sample structures for release of contents, and concentrate metabolites for further analysis. Common methods involved in sample preparation are grinding in liquid nitrogen, lyophilization, and solid phase micro-extraction. It has to be noted that the method of sample preparation could be varied significantly, based on the samples to be analyzed and the method of detection. Liquid samples generally require less preparation, while some detection techniques do not require sample preparation because they are non-destructive (near inferred spectrometry, NIR).

Metabolic extraction is one of the key steps for metabolomics, since subtle changes in the choice of extraction buffers and methods can dramatically affect the extracted metabolites, hence the result. Targeted metabolomics may use a specific solution and method to extract metabolites, due to the fact that the research interest will be targeted to a specific group of chemicals with given biochemical features (Cevallos-Cevallos, et al., 2009). For untargeted metabolomics, a rather complete extraction is generally carried out by sequential and selective extractions (MeOH-H_2O-$CHCl_3$), to group the extracted metabolites into hydrophilic vs. hydrophobic categories and to obtain

the information about as many metabolites as possible. Especially for meat science, a novel method for metabolic extraction from frozen meat using desorption gas has been reported (Cevallos-Cevallos et al., 2009).

1.3.2.2 Instrumental separation

Separation with detection is the core step of metabolomics workflow. MS – based separation/detection platform is more sensitive than the NMR based platform (Wishart, 2008). In the MS system, the term mass to charge ratio (m/z) is referred to a value obtained by dividing the molecular mass of an ion by its net charge, which can be measured during separation step (Wishart, 2008). The m/z values are consequentially converted into high accuracy molecular weights, which are accurate up to 4 decimals and can be used in compound identification (Rochfort, 2005). Many techniques are available for MS-based separation, including LC (liquid chromatography), HPLC (high performance liquid chromatography), UPLC (ultra-performance liquid chromatography), GC (gas chromatography), CE (capillary electrophoresis) among others (Wishart, 2008).

1.3.2.3 Data analysis

Data analysis step includes two main parts, data pre-processing and variable analysis. Initial data obtained from the mass spectrum is the raw data that is mixed with unwanted variations and machine errors, so the raw data needs to be pretreated before the actual multivariate analysis (Brown, Dunn et al., 2005). There is no best way to do data preprocessing, but the procedure could be generally divide into two steps: scaling and normalization (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006).

Scaling is essential due to the large variation of the content of metabolites in a biological system. The general rule of scaling is to treat the data in such a way that all the metabolites are scaled to the equal importance. Scaling by the standard deviation (divide the number by standard deviation) is called auto-scaling (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006), and scaling by the square root of the standard deviation is called Pareto-scaling (van den Berg et al., 2006). Auto-scaling provides all features with the same standard deviation, thus significance, while the Pareto-scaling will still keep the original dimension of all values. Normalization is to erase the unwanted variations caused by environmental and instrumental factors. The actual normalization processing highly depends on the individual contexts and situations (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006).

The pre-processed metabolomics data could be analyzed by both univariate analysis and multivariate analysis. Techniques of univariate analysis are common statistical methods including ANOVA and student t test, which are used to test whether concentration of a compound is significantly different in control and tested groups or not (Muroya, Oe, Nakajima, Ojima, & Chikuni, 2014; Warner et al., 2015). Univariate data analysis also includes paired correlation, which provides insights about whether two compounds or a compound and a measurable biological feature are closely associated (D'Alessandro, Rinalducci, et al., 2012; Straadt, Aaslyng, & Bertram, 2014). Multivariate data analysis allows analyzing all the collected data simultaneously by generating a model from all the included variables to make discriminations among groups and draw scientific conclusions. Multivariate data analysis can be unsupervised (to explore the innate difference among groups without providing the group identity) or supervised (the

group identity are pre-considered in the model). Generally speaking, supervised data analysis is more powerful in discriminating groups, but it may be biased over the data collected from samples and limited in generalization ability.

Different multivariate models are employed in metabolomics data analysis, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA), and other models (Ben-Hur & Guyon, 2003; Barker & Rayens, 2003; Bylesjö, Rantalainen, Cloarec, et al., 2006). PCA is an unsupervised (meaning without presumptions of grouping categories) model for compact visualization of multivariate dataset. It is very useful in finding clusters or groups of data entries that share similar features and/or outliers of the dataset (Ben-Hur & Guyon, 2003). PCA works by orthogonally transforming the dataset to a new set of uncorrelated variables called principal components, with the top components (PC1, PC2, and so on) keeping the most of the variance within the dataset. The correlation coefficients between the PC scores and the original variables are calculated as PC loadings, which represents the relative importance of each variable in a given PC. PLS is a supervised modeling method, which is commonly used to find the quantitative relationship between the two matrixes that share same sample list (Barker & Rayens, 2003). OPLS extends the supervised PLS model by adding an orthogonal signal correction (OSC) filter (Bylesjö et al., 2006).

1.3.2.4 Metabolites identification

Compound identification generally requires three steps: peak alignment, database matching and comparison with standards (Goodacre & Broadhurst, et al., 2007).

Database matching is the key consideration for this step, because different separation techniques use different databases, and the availability and richness of the database for a given separation technique determines the amount of data output from the method of choice. Compound identification should give the approximate identity of compounds in samples, and the relative or absolute concentration or content of the identified compounds (Goodacre et al., 2007).

1.3.3 Current limitation

Although metabolomics is intended to study the "metabolome", which is the collection of all the metabolites in a biological system, it should be noted that the current techniques are not good enough to provide the information of the full coverage of metabolome (Yu, 2013). One reason for such limitation of metabolomics platforms roots in the chemical complexity and wide concentration range of the metabolites (from pM to mM) (Brown et al., 2005). In comparison to other omics technologies, the chemical basis of genomics and transcriptomics are limited to a number of nucleobases (A, T, G, C, and U), whereas the basic analytical units of proteomics are about 20 amino acids with several methylation and/or phosphorylation derivatives (Yu, 2013). However, the chemical basis of metabolomics analysis is much more complicated. Even in human being, which is happened to be the most well-studied species by metabolomics, a simple question as how many metabolites do humans have is still a matter of debate, and the proposed answers vary from a few thousands to tens of thousands (Kaddurah-Daouk, Kristal, & Weinshilboum, 2008).

Moreover, it is very difficult to preserve all the metabolites in a single sample preparation procedure, since most extraction procedures are biased on either the polar or non-polar compounds, which is mostly due to the polarity of the extraction solvent (Yu, 2013). It is also very difficult to extract and preserve the instable intermediates. Under such circumstance, targeted techniques, such as lipidomics, have been emerged study the subgroups of the metabolome (Shevchenko & Simons, 2010). Therefore, the appropriate extraction and separation techniques needs to be wisely chosen to meet the objective(s) of research and metabolites of interest.

1.3.4 Metabolomics application in food science

Metabolomics has been extensively applied in food science, which forms a sub-area of the discipline named foodomics. Foodomics uses various omics technologies including genomics, transcriptomics, proteomics, and especially metabolomics, to investigate food composition, safety, and nutritional value (Hu & Xu, 2013). For example, metabolomics have been applied to beer production (Vautz et al., 2006) and other fermented food (Choi, Yoon, Kim, & Kwon, 2007) to monitor the process of fermentation. Food safety is another important research topic that can be analyzed by metabolomics. Microbial infection and spoilage of food change the chemical composition of food, which produce volatiles that can be detected by both MS and NMR metabolomics platforms (Vikram, Hamzehzarghani, & Kushalappa, 2005; Vikram, Lui, Hossain, & Kushalappa, 2006; Chen, Wortmann, & Zenobi, 2007). Since metabolomics reveals the very nature of food product at the chemical composition level, food authentication of geographical of origin (Donarski, Jones, & Charlton, 2008), organic/ non-organic production system (Levandi, Leon, Kaljurand, Garcia-Canas, & Cifuentes,

2008), and ingredients and additives (Tikunov et al., 2005; Son et al., 2008) are emerging research topics that can be effectively analyzed by metabolomics.

1.3.5 Metabolomics application in meat science

The earliest attempt to apply the idea of metabolomics in the field of meat science can be traced back to 1995, when King and his colleagues tried to assess the full coverage of volatile compounds of beef generated during cooking using GC-MS and flame ionization to gain detailed understanding of compounds that are related to flavor and taste (King, Matthews, Rule, & Field, 1995). 107 compounds were identified and the differences in relative abundance of the compounds were reported. This study initiated the idea that in such a complicated food system as meat, constituents should be assessed as a whole to elucidate their interactions and relationships to the quality attributes. With the development of both instrumental analysis technologies and chemometric theories, application of metabolomics has profound the critical insights for both meat industry and academia on the postmortem muscle metabolome and its potential implications to improve meat qualities. As an emerging new technology, application of metabolomics in meat science needs to be encouraged and is reviewed in the following section. Summaries of the studies are presented in Table 1.1.

1.3.5.1 Pre-slaughter processing

In 2010, Bertram and colleagues introduced metabolomics study into the meat science discipline (Bertram, Oksbjerg, & Young, 2010). The study assessed the effects of pre-slaughter stress on the antemortem blood serum metabolome and its relationship to

the meat qualities. Before slaughter, the researchers randomly separated 40 pigs into the following 4 groups: no pre-slaughter stress, pre-slaughter exercises on a treadmill without rest, pre-slaughter exercises with 1 hour of rest, and pre-slaughter exercises with 3 hours of rest. Blood samples were subjected to 1H NMR analysis and up to 9 peaks were used for statistical analysis. Multivariate analysis including PCA and PLS-DA showed that the control samples (no pre-slaughter stress) clustered to each other while separated from those stressed samples. The exercise-rest groups were intermediate and showed a trend of recovery as the resting time increased. The single metabolite that was most relevant to this change was found to be lactate, which was associated to pH decline, muscle temperature, and water holding capacity (WHC).

Country of origin labeling is a pre-slaughter factor that may not necessarily impact meat quality, however, it needs to be well regulated for the avoidance of the BSE ("Mad Cow" disease) concern and the implementation of Free Trade Agreement (Umberger, 2003). A good example is to use metabolomics to distinguish the country of origin of fresh beef (Jung, Lee, Kwon, Lee, Ryu, & Hwang, 2010). To address this issue, Jung and others explored the NMR based metabolomics on raw beef samples from four countries (Australia, Korea, New Zealand, and the United States). Multivariate analysis model (OPLS-DA) showed separation among different groups of samples; and separation and predictability were high enough for application. From the loading analysis of OPLS-DA, succinate and various amino acids (isoleucine, leucine, methionine, tyrosine, and valine) were found to be most responsible for discriminating the geological origin of beef.

Similarly, feed authentication is another issue that is closely related to labeling administration and inspection, which is crucial to maintain a well-regulated market. As

intra-species feeding (i.e. supply boilers with chicken bone meat) is strictly forbidden to minimize the risk of prion infection, feed fraudulence needs to be inspected in every step of animal production. However, when animals entered slaughter/packing process, it is very difficult to monitor such feed fraudulence. In order to address this issue, Cajka and others (2013) employed HPLC (High Performance Liquid Chromatography) and DART-MS to assess the metabolome of chicken muscle samples from normal diet and diet with 5% chicken bone meal (Cajka, Danhelova, Zachariasova, Riddellova, & Hajslova, 2013). The identified metabolites were pre-selected for OPLS-DA analysis. The established OPLS-DA model provided feasible differentiation between normal chicken muscles and feed-fraudulent groups. Potential biomarkers were proposed including anserine, glutamine, and TAGs.

Another similar example is to authenticate different diet based beef cattle production system. Osorio and others (2012) tried an NMR-based metabolomics approach on the bovine urine and muscle samples (*M. Longissimus*) collected from beef cattle fed either purely on pastures or on different combination of silage, grain and pasture diet (Osorio, Moloney, Brennan, & Monahan, 2012). Urine samples and muscle samples were subjected to 1H NMR analysis, in which the identified metabolites were used to conduct multivariate data analysis including PCA and PLS-DA. The PLS model was effective in distinguishing each production system (Osorio et al., 2012). As the muscle-sample-based models did not show much power, further research used different tissues (both muscle and adipose tissue) that was analyzed by multiple omics platform (include GC based lipidomics, HPLC based non-targeted metabolomics, and EA-IRMS based isotopic ratio analysis) (Osorio et al., 2013). PCA, PLS-DA and SIMCA (soft

independent modelling of class analogy, similar to PCA) modeling were performed, and the discrimination of the experimental groups was much more efficient than the earlier study, which provides a new and reliable way to identify beef from different diet based production system.

1.3.5.2 Post-slaughter processing

Biochemical changes in muscles during postmortem aging directly affect meat palatability. As numerous metabolites were identified as possible flavor precursors, there has been growing interest in using metabolomics to address the effects of post slaughter processing on metabolomics profiles and meat quality, especially eating quality attributes.

Alessandro and colleagues conducted multiple metabolomics studies coupled with proteomics, which used *M. Longissimus* muscles (24 hours postmortem) of different species and breeds of livestock. Key regulators at the early postmortem stage that affect meat tenderness have been studied (D'Alessandro, Marrocco, et al., 2012; D'Alessandro, Rinalducci, et al., 2012; Marrocco, Zolla, & Zolla, 2011). The omics data from porcine (Casertana and Large White breed) and bovine (Maremmana and Chianina breed) both samples elucidated that the faster glycolytic rate could be related to improved tenderness, because higher levels of glycolytic intermediates (such as PEP, lactate, NAD+ or NADH) were associated with higher myofibril fragmentation index and/or lower Warner Bratzler shear force value. Interestingly they also reported that the more tender meat showed higher oxidative stress at early postmortem stage, which was indicated by glutathione activity, glucose phosphorylation enzyme activity, and glycolytic enzyme activity (D'Alessandro, Marrocco, et al., 2012; D'Alessandro, Rinalducci, et al., 2012).

Correspondingly, metabolomics research conducted on postmortem muscles that came from different aging time point confirmed that aging improves tenderness through structural protein degradation and improves palatability by generating glutamate and/or nucleotide related flavor precursors (Graham et al., 2010; Graham et al., 2012). NMR based metabolomics profiling from Beef *longissimus* muscle aged for 3, 7, 14 and 21 days showed decrease/depletion of ATP and ADP that coupled with an increase of free amino acids, antioxidant dipeptide, lactate, pyruvate, ribose and xanthine during aging (Graham et al., 2010). GC-MS based metabolomics of porcine muscles not only provided a consistent observation, but also proposed possible nucleotide degradation pathway in postmortem muscle (Muroya et al., 2014).

Effects of various postmortem processing techniques on muscle metabolome and meat quality attributes were studied, including different aging regimes, chilling regimes, irradiation treatments, and mechanical recovery (Surowiec, Fraser, Patel, Halket, & Bramley, 2011; Kim, Kemp, & Samuelsson, 2016; Zanardi, Emanuela et al., 2015; Xu, Cheung, Winder, Dunn, & Goodacre, 2011; Bertram, Dønstrup, Karlsson, Andersen, & Stødkilde-Jørgensen, 2001). For example, mechanical recovery is a frequently used technology to collect muscle protein residuals from bones. However, in European Union, using mechanically recovered meat (MRM) as a food ingredient must be regulated/monitored in terms of labeling and maximum level allowed (Surowiec, Fraser et al., 2011). To develop a reliable method to distinguish MRM from hand boned muscle, GC-MS based metabolomics profiling was conducted on extractions from MRM, hand boned meat (HB) and desinewed meat (DN) in both pork and chicken (Surowiec, Fraser et al., 2011). The identified compounds were selected to build up multivariate models

PCA and OPLS-DA. The both models exhibit separation of 3 products (MRM, HB and DN) in a specific manner, thus made accurate classification possible. As for different aging regimes, recently Kim et al. (2016) reported that dry-aged beef (air velocity 0.2 m/s under 3 °C for 3 weeks) had higher amounts of flavor compounds and flavor precursors such as glutamate, leucine, isoleucine, tryptophan and leucine as assessed by 1H NMR based metabolomics (Kim, Kemp, & Samuelsson, 2016). As irradiation is a common way to minimize microbial contamination in ground meat products, the metabolome of the highly irradiated ground beef (4.5 or 8 kGy) was investigated to differentiate from the slightly or non-irradiated samples (0 or 2.5 kGy) (Zanardi et al., 2015). Proposed biomarkers responsible for the differentiation between irradiated and non-irradiated ground beef include glycerol, lactic acid esters, and polyphenolic compounds (Zanardi et al., 2015).

Another typical example of post-slaughter processing that affects the muscle metabolome and meat quality is the chilling process. Bertram and others (2001) reported that different chilling regimes would affect postmortem muscle energy metabolism significantly. Porcine *M. longissimus* was assessed by 31P-NMR spectrum from 20 min until 12 hours postmortem, during which the changes of phosphocreatine indicated that postmortem muscle to meat conversion was significantly delayed by fast cooling environments (below 5 °C within 3 hours compared with below 5 °C within 8 hours). Interestingly, a recent metabolomics study on ovine muscles found that a very fast chilling (VFC) regime (down to -1.6 °C within 1.5 hours) would accelerate the postmortem metabolism process (Warner et al., 2015), as the PCA analysis showed that the VFC samples at early postmortem were overlapped with normal chilling samples at

later aging time. Metabolites identification also found that VFC samples had higher levels of intermediates from glycolytic pathway, which further confirmed corresponding metabolism acceleration (Warner et al., 2015). As the VFC regime was proposed to improve meat tenderness, those results were able to offer supportive an elucidation that it is probably due to the earlier onset of rigor mortis that prevents of shortening.

Application of metabolomics also has profound advantages in food safety assessment. As in muscle foods, it is necessary to distinguish between naturally spoiled meats vs. pathogen contaminated meats. In a project done by Xu and others (2011), naturally spoiled meat and *Salmonella typhimurium* contaminated meat (exogenously inoculated) were sampled at different time point of storage and analyzed in GC-TOF-MS. Multivariate data analysis, the PARAllel FACtor analysis 2 (PARAFAC2) method showed that the contaminated samples significantly differentiated after 32 to 36 hours of pathogen inoculation. Univariate analysis found 17 metabolites including valine, creatinine, tetradecanoic acid, hexadecanoic acid, and octadecenoic acid to be significantly different between two groups (Xu et al., 2011). As for naturally spoiled meat, lactic acid bacteria growth was reported to be associated with butanoic acid production, which is a volatile compound that determined by GC-MS (Xu et al., 2011). Another study used 1H NMR analysis to demonstrate a decrease in lactate, glycogen, IMP, and ADP levels and increase in 3-methylindole, betaine, creatine, and other amino acids during natural meat spoilage caused by general microbial growth (Ercolini et al., 2011).

In summary, recent studies indicate that metabolomics could be a promising technique to provide in depth information on the physiological/biochemical changes of postmortem muscle. Therefore, the objective of this thesis was to investigate the effects

of postmortem aging on oxidation stabilities in different bovine muscles and to profile oxidation related key metabolites by means of HPLC-MS based global metabolomics. Consequentially a deeper understanding of the underlying molecular and chemical mechanism of oxidation related quality defects will be elucidated. The results from this study will provide critical insights for the meat industry to develop a strategic quality monitoring system for beef muscles during aging practice.

Table 1.1 Summary of literatures of metabolomics application in meat science

Research topic and metabolomics platform	**Statistical analysis**	**Other measurements**	**Main findings**	**Study**
Effects of aging, packaging, display and color stability on ovine muscles metabolome using HILIC-MS/MS	PCA ANOVA	color stability	**NADH, glutathione and taurine** (antioxidant) were significantly higher in color stable groups and **sugar phosphate** (may replenish NADH) were lower in color stable groups. 12 **free AA** were more abundant after 8 weeks aging.	Arvind et al，2016
Effect of aging period and muscle type on pork metabolome using CE-TOF MS/MS	PCA ANOVA	pH Glycogen Glucose Myosin heavy chain isoform	Muscle effect*:* **carnosine, anserine, Ala, Gly, hydroxyproline (hPro), Gln, His.** Aging, decreased with**: ATP, glutathione divalent (GSSG), citric acid, GTP, O-acetylcarnitine, glycerol 3-phosphate, isovaleric acid.** Aging, increased with**: ribose 5-phosphate, lactic acid, 6-phosphogluconic acid, gluconic acid, GMP, and UMP.**	(Muroya et al., 2014)
Effects of pre-slaughter exercise (stress) vs rest after exercise on porcine blood plasma metabolome using 1HNMR	PCA PLS regression	pH decline and Water holding capacity	Control samples (no pre-slaughter stress) separated from stressed samples in The stressed-rest samples were intermediate and showed a trend of recovery. **Lactate** closely associated with pH decline and WHC.	(H. Bertram et al., 2010)

Table 1.1 continued

Research topic and metabolomics platform	**Statistical analysis**	**Other measurements**	**Main findings**	**Study**
Effect of aging time on beef metabolome Using 1H NMR and 1H13C NMR	PCA and loading analysis	no	PCA showed separation between each aging treatment. **ATP/ADP** depleted with increased aging. **Free amino acids, acetate, adenosine, carnosine, creatine, glucose, inosine, lactate, pyruvate, ribose and xanthine** increased with aging time..	(S. Graham et al., 2010)
Metabolome of ground beef Irritated at different doses (0, 2.5, 4.5 and 8 kGy), analyzed by 1H NMR	PCA and CT(classification tree)	no	PCA showed 0/2.5kGy irritated sample separated with 4.5/8kGy irritated sample. **Glycerol, lactic acid esters, and tyramine** were important biomarkers for detecting irritated beef.	(Zanardi et al., 2015)
Effect of aging time and suspension technique on beef metabolome, analyzed by different metabolomics platform, NMR , GC-MS and HPLC-MS	PCA and loading analysis	no	PCA plot showed separation based on aging difference, but not suspension techniques. Aging, affect **Free AA** and **nucleotides.** Suspension: no metabolites were found to be affected. Combination of different metabolomics platform provide a more complete description of metabolome.	(S. F. Graham et al., 2012)
Effect of Chilling regime on lamb muscle metabolome with postmortem aging, analyzed by both NMR and HPLC-MS platform	PCA ANOVA	no	PCA clustering showed that VFC samples at early postmortem stage overlapped with normal chilling sample at later postmortem stage. Adenosine nucleotides and sugar phosphate are affected by chilling regime.	(Warner et al., 2015)

Table 1.1 continued

Research topic and metabolomics platform	**Statistical analysis**	**Other measurements**	**Main findings**	**Study**
Effect of different pig breed on porcine muscle metabolome using frozen/thaw drip samples, analyzed by 1H NMR and 1H13C NMR	PCA, PLS Pearson correlation ANOVA	Sensory odor and juiciness perceptions	Multivariate analysis did not show much insights. Crossbreed affect short **peptide** and **free AA** as well **as IMP, inosine, glycero**l and **choline-containing** compounds. High content of **carnosine** associated with low sensory attributes.	(Straadt et al., 2014)
Detect feed fraudulent (feed with 5% chicken bone meal vs normal feed) in chicken using HPLC-MS	PCA OPLS-DA	no	**Anserine, glutamine** and **TAGs** could be potential biomarker for feed fraud in chicken.	(Cajka et al., 2013)
Biomarker for geographical origin (country) of the beef using 1H NMR and 1H13C NMR	PCA and OPLS-DA		PCA showed nice cluster, OPLS-DA successfully maximize the separation. Succinate and free AA including isoleucine, leucine, methionine, tyrosine, and valine are most responsible for discriminating the geographical origin of beef.	(Jung et al., 2010)
Biomarker for natural spoiled vs *Salmonella.* contaminated pork using GC-TOF- MS	PARAllel FACtor analysis Two-way ANOVA		Staring from 32 hrs post pathogen inoculation, samples begins to significantly separate from control groups. **Valine, creatinine, tetradecanoic acid, hexadecanoic acid,** and **octadecenoic acid** significantly different between naturally spoiled or pathogen contaminated samples.	(Xu et al., 2011)

Table 1.1 continued

Research topic and metabolomics platform	**Statistical analysis**	**Other measurements**	**Main findings**	**Study**
Biomarker indicating mechanically recovered meat (MRM), desinewed meat and hand-deboned meat using GC-MS	PCA OPLS-DA		OPLS revealed nice separation. No single metabolites stood out as biomarker to identify. MRM, due to low confidence in t-test or unable to identify a good peak.	(Surowiec et al., 2011)
Effects of wet vs. dry aging regime on Beef metabolome, affected by 1H NMR	t test Fold - change	Sensory pH Color WHC	Flavor precursors such as **glutamate, leucine, isoleucine, tryptophan and leucine are more abundant in dry aged beef.**	(Kim et al., 2016)
Effects of pig breed on intra-muscular fat deposition and muscle metabolome, analyzed by HPLC-MS	Fold change		CA breed has significantly more lactate and ATP content in muscle compared with LA breed. CA muscles also have significantly higher glycerol-3-phosphate and glycerol content, agreeing with higher G3P activity in CA breed from proteomic study.	(Marrocco et al., 2011)
Effects of postmortem aging on beef	ANOVA Pearson Correlation	Tenderness Proteomics Phosphor-proteomics	Metabolomics analysis confirmed higher glycolysis rate in tender meat due to higher phosphoenolpyruvate (PEP), lactate, NADH, NAD+ content. Metabolomics study also revealed higher oxidative stress in tender meat due to higher GSSG/GSH ratio.	(Angelo D'Alessandro, Rinalducci, et al., 2012)
Effects of postmortem aging on beef	Pearson Correlation	Tenderness Proteomics and Phosphor-proteomics	Higher level of glycolytic enzymes was correlated with tender meat. Higher glycolytic intermediates were also observed, along with higher oxidative stress from GSSG/GSH ratio.	(Angelo D'Alessandro, Marrocco, et al., 2012)

Table 1.1 continued

Research topic and metabolomics platform	**Statistical analysis**	**Other measurements**	**Main findings**	**Study**
Effects of commercial vs. tunnel chilling on porcine muscle metabolome. analyzed by 31P NMR	t-test		Time-dependent changes were observed in PME, Pi, phosphocreatine (PCr), and ATP. The Degradation of PCr was slowed down in tunnel chilled samples, indicating that antemortem energy metabolome processes was significantly reduced by fast cooling environments.	(Bertram et al., 2001)
Beef diet authentication and biomarker validation using muscle and urine samples, analyzed by 1H NMR	PCA PLS-DA		PLS-DA in urine samples provide strong signal to discriminate samples from diet system; PLS-DA in muscle is not that strong; single metabolites as biomarker was not found.	(Osorio et al., 2012)
Beef diet authentication and biomarker validation using muscle and adipose tissue, analyzed by NMR HPLC and GC and EA-IRMS platform	PCA PLS-DA SIMCA		Signals from different samples measured by different technologies were preselected and 83 signals were included on PLS-DA model. The model has 91.8% overall classification rates to authenticate different diet system.	(Osorio et al., 2013)
Effect of different packaging system on microbial growth in beef, analyzed by NMR and GC-MS		PCR pyrosequencing	Lactic acid bacteria growth was associated with butanoic acid production, which was a volatile compound that can be discovered in GC-MS. 1H NMR analysis also demonstrated a decrease in lactate, glycogen, IMP, and ADP levels and increase in 3-methylindole, betaine, creatine, and other amino acids during general microbial growth.	(Ercolini et al., 2011)

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CHAPTER 2. EFFECTS OF POSTMORTEM AGING ON COLOR AND LIPID OXIDATION STABILITIES OF THREE BOVINE MUSCLES

2.1 Abstract

The objective of this study was to determine the effect of postmortem aging on color and lipid oxidation stabilities of bovine muscles. Three muscles (*longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM)) from 7 beef carcasses were obtained at 1-day postmortem. Each muscle was divided into 3 sections and assigned for 3 aging periods (9-, 16- and 23-day postmortem). After aging, cuts were made, overwrap-repackaged, and displayed at 3°C for 7 days. Instrumental and sensory color and shear force values were measured. Myoglobin and non-heme iron contents, and lipid oxidation by measuring TBARS and conjugated dienes (CD) were evaluated. Discoloration was intensified with aging for all muscles, and LL was the most color-stable, followed by SM and PM ($P<0.05$). Lower myoglobin and non-heme iron contents were observed in LL compared to SM and PM ($P<0.05$). A significant increase in non-heme iron contents during aging was found. Lipid oxidation increased with aging, and LL was more oxidative stable (lower TBARS and CD) compared to SM and PM ($P<0.05$). The results indicate that color and lipid oxidation stability is negatively associated with aging, and different muscles have different susceptibilities to oxidation.

Key words: Beef, aging, meat color, lipid oxidation, myoglobin

2.2 Introduction

During postmortem aging, substantial chemical/biophysical changes occur in all muscle tissues, which results in beneficial impacts on eating quality attributes of meat. In particular, significant improvements in meat tenderness, flavor, or juiciness occur through cytoskeletal myofibrillar protein degradation by endogenous proteases during extended aging (Huff-Lonergan & Lonergan, 2005; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Kim, Warner, & Rosenvold, 2014; Kristensen & Purslow, 2001). In this regard, postmortem aging is a very common practice in the meat industry, where various aging periods are used for a range of 3 to 83 days from packing plants/local meat processors to the retail levels (Voges et al., 2007).

However, a prolonged postmortem aging process may have an adverse impact on color and lipid oxidation stability. A few studies reported that long-term chilled meat products (under vacuum packs) had inferior oxidation stability, resulting in a decrease in display shelf-life and an increase in rancid off-flavor development when repackaged into a retail display format (Kim, Frandsen, & Rosenvold, 2011; Kim, Stuart, Black, & Rosenvold, 2012; King, Shackelford, Kalchayanand, & Wheeler, 2012; Pouzo, Descalzo, Zaritzky, Rossetti, & Pavan, 2016). This can be a significant economic problem, since consumers heavily rely on surface meat color to determine the degree of freshness for their meat purchasing decision (Kim & Hunt, 2011). Consumers would likely discriminate fresh meat products appearing brown-red pigments (due to the formation of metmyoglobin; the oxidized form of myoglobin), even if meat products have been chilled for the long-term to enhance eating quality characteristics (Kim, Stuart, Rosenvold, & Maclennan, 2013).

Numerous studies were conducted to determine the effects of aging on meat quality attributes in general. However, it has not been fully understood how postmortem aging impacts oxidation stability of beef muscles and the underlying mechanistic factors associated with the postmortem aging-induced oxidation, such as release of non-heme iron and/or reducing capacity. In particular, while it has been well-known that different muscles have different aging responses in terms of protein degradation and subsequent eating quality attributes (Anderson, Lonergan, & Huff-Lonergan, 2012; Bratcher, Johnson, Littell, & Gwartney, 2005; Gruber et al., 2006), there is little information about how different muscles observe oxidation stability in respond to extended postmortem aging. Furthermore, considering physiologically different muscles have different color stability (e.g. *longissimus* muscle is more color stable compared to *psoas major*) (Canto et al., 2016; Kim, Keeton, Smith, Berghman, & Savell, 2009; King, Shackelford, & Wheeler, 2011; McKenna et al., 2005), it would be reasonable to hypothesize that different muscles will have different extents of oxidation susceptibility in relation to postmortem aging.

This postulation warrants further examination, since understanding the underlying mechanisms of oxidation stability of beef muscles is a crucial step to develop a practical aging strategy to prevent oxidation-related quality defects, while maximizing beneficial aging impacts on eating quality attributes. Therefore, the objective of this study was to determine the effects of postmortem aging on color and lipid oxidation stability and oxidation-related chemical attributes in three different muscles. We chose *longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM), since those muscles

were known to have distinct differences in fiber composition, color stability and metabolic properties (Kim et al., 2009).

2.3 Materials and methods

2.3.1 Raw materials and processing

A total of seven beef carcasses (USDA Low Select to Low Choice; A maturity) were slaughtered at Purdue University Meat Laboratory. At 1-day postmortem, three muscles (*longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM)) were separated from one side of each carcass. Each muscle was divided into three sections with equal length, vacuum packaged, and randomly assigned to three aging periods (9, 16, and 23 days postmortem). After completion of each assigned aging, two steak cuts (2.54 cm thickness) were made from each section, placed on a plastic tray, packaged with oxygen permeable polyvinylchloride film (23,000 $cm^3/O_2/m^2$/24h at 23 °C) and displayed under fluorescent white light (approximately 1,450 lx, Color temperature 3,500 K) for 7 days at 2.5 °C. After display, the steak samples were vacuum packaged and stored at -80 °C until being used for further chemical analyses.

2.3.2 pH

The pH of each muscle before and after aging was measured in triplicate using an insertion type pH meter (HI 99163, Hanna Instruments Inc., Woonsocket, RI, USA) by inserting a pH probe (calibrated to pH range 4 to 7) directly into the meat.

2.3.3 Instrumental color measurement

During the simulated retail display, instrumental color characteristics of the steak surface were measured using a Minolta CR-400 colorimeter (D65, 1 cm diameter aperture 10° standard observer; Konica Minolta Photo Imaging Inc., Tokyo, Japan) at display days 1, 4, and 7. Calibration was performed by using a standard white tile (CIE L^*=97.06, CIE a^*=0.41, CIE b^*=1.72) prior to the color measurement. CIE L*, a* and b* values were obtained by the average of 3 spot measurements per steak. Chroma [$(a^{*2}+b^{*2})^{1/2}$] and hue angle [$(b^*/a^*)\ \tan^{-1}$] values were estimated from CIE L^*, a^* and b^* values (AMSA, 2012).

2.3.4 Visual color evaluation

Visual color evaluation of steak samples was conducted according to the AMSA Color Measurement Guideline (AMSA, 2012). At display days 1, 4 and 7, lean color and discoloration scores were evaluated by a trained color panel (n=10). The sensory color panelists were selected based on the previous Farnsworth—Munsell 100 Hue screening test and were trained multiple-times to consistently determine the extent of lean color and discoloration of steak samples. The extent of lean color change was evaluated using eight scale points (1=extremely dark red, 2=dark red, 3=moderately dark red, 4=slightly dark red, 5=slightly bright red, 6=moderately bright red, 7=bright red, and 8=extremely bright red). The extent of discoloration was assessed based on seven scale points (1=no discoloration (0%), 2=slight discoloration (1-19%), 3=small discoloration (20-39%), 4=modest discoloration (40-59%), 5=moderate discoloration (60-79%), 6=extensive discoloration (80-99%), 7=total discoloration (100%)).

2.3.5 Conjugated diene (CD)

The extent of diene conjugation (primary lipid oxidation product) was determined after 7 days of retail display of beef samples by following the procedure described by (Srinivasan, Xiong, & Decker, 1996) with minor modifications. In duplicate, 0.5 g of meat sample was homogenized with 5 ml of distilled water. 0.5 ml of homogenate was mixed with 5 ml of extraction solution (isopropanol and hexane, 3:1 ratio) and centrifuged at 2,000 × g for 5 min. The absorbance of the supernatant was measured against a blank using the extraction solutions at 233 nm using a microplate spectrophotometer (Epoch, BioTek Instrument Inc.). The concentration of CD was calculated using a molar extinction coefficient of 25,200 $M^{-1}cm^{-1}$ and the results were expressed as μM/mg meat lipid sample.

2.3.6 2-Thiobarbituric acid reactive substances (TBARS)

The extent of secondary lipid oxidation products was measured in duplicate by following 2-thiobarbituric reactive substances acid (TBA) assay described by (Buege & Aust, 1978). After 7 days of retail display of beef samples, powdered meat samples were homogenized (Ultra-Turrax T25, Janke & Kunkel IKA-Labortechnik, Staufen, Germany) in distilled water (1:3 ratio) with butylated hydroxyl anisole (BHA) solution (10% v/v with 90% ethanol solution) for 30 s using an Ultra Turrax at 6,000 rpm and centrifuged at 2,000 × *g* for 10 min at 4 °C. The supernatant (2ml) was mixed with 4 ml of TBA/TCA reagent (20 mM TBA with 15% trichloroacetic acid solution) and heated for 15 min in a water bath (80°C) to develop the chromogen and cooled for 10 min in ice water. The absorbance of each sample at 538 nm was measured using a microplate

spectrophotometer (Epoch, BioTek Instrumrtnts Inc.) against a blank (2ml dH_2O + 4ml TCA/TBA solution). TBARS values were expressed as mg MDA/kg muscle.

2.3.7 Myoglobin content

Myoglobin content of the beef samples was measured in duplicate by following the procedure of Warris (1979) and Trout (1989) with a few modifications. In brief, samples (2.5 g) were homogenized in 40 mM potassium phosphate buffer (pH 6.8) and held on ice for 1 h. After centrifuging the homogenates at 35,000 × g for 30 min at 4 °C, supernatant was collected and filtered. The filtered supernatant was scanned in a cuvette for the absorbance spectra at 400 to 700 nm measured by VWR UV-1600 PC spectrophotometer (VWR International, San Francisco, CA). The myoglobin concentration (mg/g) was calculated using the absorbance difference between 525 nm and 700 nm multiply by 2.303 and dilution factor.

2.3.8 Non-heme iron

Non-heme iron content was determined by following the procedure described by Ahn, Wolfe and Sim (1993) with minor modifications. After 7 days of retail display of beef samples, powdered meat samples were homogenized in 0.1M citrate-phosphate buffer (pH 5.5) and mixed with ascorbic acid (1 % in 0.2 N HCl, w/v) and 11.3% TCA solution (w/v, 1 ml) and placed for 5 min at room temperature. The mixture was centrifuged at 3000 × g for 15min at 20°C, and supernatant was mixed with 10% ammonium acetate (w/v) and ferrozine at 22°C for 10 min for color development. The

absorbance was determined at 562 nm against blank. The non-heme iron content was expressed as mg non-heme iron per 100 gram of meat.

2.3.9 Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) was determined after D7 of the display. For sample extraction (Prior et al., 2003), muscle samples (1g) were homogenized with 10 volumes of dH_2O and centrifuged. The supernatant (400 µl) was mixed with an equal volume of ethanol and washed twice with 800 µl of hexane. The hexane layer was separated by centrifugation and discarded. The remaining extraction was mixed with 800 µl 0.5 M perchloric to precipitate and remove protein, stored at – 20 ◦C until analysis.

ORAC determination was used as previously described (Min, McClung, & Chen, 2011). Samples (25 µl) and Trolox standards (25 µl, concentration range from 6.25 to 50 nM) were each mixed with 150 µl fluorescein (67.95 mM) and 25 µl APPH (37.71 mM) and added on to a light impermeable, black polyester 96 well plate (Corning, NY). The fluorescence was determined every 2 minute up to 60min using a fluorescence microplate reader (Fluostar Omega, BMG Labtech, Cary, NC). The ORAC value of each sample was calculated by the regression equations obtained from Trolox standards, which was expressed as nM Trolox equivalent (TE) / g meat with dilution factor adjustment.

2.3.10 Data analysis

The experimental design of this study was split plot design, with muscle type effect (LL, SM and PM) as the whole plot and aging time effect (9, 16 and 23 days of

aging) as subplot. For color indexes, the display treatment (D1, D4, and D7) was set as sub-sub plot. Animal was considered as a random effect. All the data were analyzed by the PROC MIXED procedure of SAS 9.4 software (SAS Institute Inc.). Least significant difference was adopted to separate least square means for all traits in a pair-wise manner. The correlation coefficients between each trait were obtained by using PROC CORR procedure of SAS 9.4.

2.4 Results and discussion

2.4.1 Color and color stability

Instrumental color (CIE L*, a*, b*, chroma and hue angle) and visual color (lean color and discoloration) attributes were assessed to determine the effects of postmortem aging on color and color stability of different beef muscles. No significant interactions between muscle, aging and display day were found in L* values (indication of lightness). The lightness values were only affected by muscle ($P<0.05$), where SM had the highest L* values (lightest) followed by LL and PM (darkest). In contrast, significant main effects (muscle, aging, and display) and interactions of muscle × display and aging × display on CIE a* (redness), CIE b* (yellowness), hue angle (discoloration) and chroma (color intensity) were found (Table 2.1; Figure 2.1).

In general, LL and SM maintained higher redness and color intensity and lower hue angle values compared to PM throughout display ($P<0.05$), irrespective of aging periods (Table 2.1; Figure 2.1). A distinct muscle difference in color stability was also observed in the visual color evaluation. The trained color panel found that LL had the highest lean color scores followed by SM, and PM had the lowest scores, indicating LL

maintained more bright red color compared to other muscles during retail display ($P<0.05$; Table 2.1).

Furthermore, a significant interaction between muscle and display day was found in the extent of discoloration. The panel found no discoloration on the surface of steaks from three beef muscles at the initial display time ($P > 0.05$; Figure2.1). However, with an increase in display time, distinct differences in discoloration were observed in the steaks from different beef muscles, where PM showed the most discoloration score (above 5; moderate to extensive discoloration; 60-79%) followed by SM, and LL were the least discolored (score of 2; slight discoloration; 1 -19%) at day 7 of display ($P<0.05$; Figure2.1).

The observed muscle differences in color stability are in agreement with several other studies. In particular, LL is well-known as a color stable muscle, whereas PM is color labile indicating meat color stability is a muscle-specific characteristic (Kim et al., 2009; McKenna et al., 2005; O'Keefe & Hood, 1982). Various biochemical/biophysical differences in muscles, such as myoglobin and lipid contents, heme and non-heme iron contents, or mitochondrial metabolism and subsequent differences in redox systems are considered to be related to the muscle-specific color attribute. Some of the chemical traits of beef muscle in accordance with postmortem aging will be discussed in more detail in later sections of the current study.

Postmortem aging significantly affected color and color stability of beef muscles. As aging time increased, a decrease in redness of surface beef color was found as indicated by a*, chroma, and visual lean color ($P<0.05$; Table 2.1 and Figure 2.1). Moreover, a significant increase in surface discoloration with increasing aging time was

observed (Figure 2.1). In particular, there was a significant aging × display interaction in a*, chroma, hue angle, lean color and discoloration (Table 2.1 and Figure 2.1). The steaks from the muscles showed similar redness and/or color intensity at the initial display time, regardless of aging period. However, at the end of display (day 7), the steak samples from muscles that were aged for 23 days showed a greater extent of decline in redness or intensity compared to the steaks from the muscle aged at 9-day or 16-day aging samples. Decline of color stability with increasing postmortem aging period was reported by several studies. King et al. (2012) evaluated color change on beef loin steaks aged for 14 and 35 days. They found that rapid decreases in redness and chroma but increases in hue angle and overall color change of beef loin aged for 35 days was observed after 7 days of display. Lindahl et al (2011) also reported that CIE a* values in beef LL and SM muscles aged for 25 days was lower than that aged for 5 or 15 days, and 25-day aging samples exhibited extensive discoloration.

However, in the current study, a significant muscle by aging interaction was found in hue angle and discoloration, where PM showed significantly higher discoloration and hue angle values after 16 days of aging, while LL and SM maintained the low values over the postmortem aging ($P > 0.05$; Figure 2.1). This observation indicates that the adverse aging impacts on beef color stability is a muscle-specific trait, where color labile muscles (such as PM) would be more susceptible to aging-induced discoloration compared to other color stable muscles.

2.4.2 Lipid oxidation

Changes in primary oxidation by measuring conjugated diene (CD) formation and secondary lipid oxidation by measuring 2-thriobarbituric acid reactive substances (TBARS) were assessed after D7 of the simulated retail display. TBARS was significantly affected by aging time ($P<0.05$) and muscle type ($P<0.01$), as an incline of TBARS was observed in 23-day aging group compared with 9-day and 16-day samples, and highest TBARS was found in SM muscle, followed by PM and LL (Figure 2. 2). A trend of aging × muscle interaction ($P=0.06$) showed that LL was lower in MDA value than the other two muscles in 9-day and 16-day aging groups. This is partially in agreement with McKenna et al (2005) that TBARS measurement after simulated retail display was followed LL<SM<PM. A recent study reported that extensive aging (8 weeks) significantly accelerated lipid oxidation of beef during 5 days of retail display (Pouzo, Descalzo, Zaritzky, Rossetti, & Pavan, 2016). Previous studies also noted that prolonged aging period could promote lipid oxidation in fresh meat (Ismail, Lee, Ko, & Ahn, 2008; Vitale, Pérez-Juan, Lloret, Arnau, & Realini, 2014). According to Descalzo, Rossetti et al. (2008), an increase of TBARS in buffalo meat with increased aging time could be related to the depletion of antioxidant vitamins such as α-tocopherol and β-carotene.

CD concentration was significantly affected by muscle type ($P<0.001$) but not aging ($P=0.1$, Figure 2. 2). Consistent with the result of TBARS, LL maintained lower CD value than SM and PM after display regardless of aging treatment, indicating improved oxidation stability. A significant muscle × aging interaction was observed, where CD formation in SM and PM was not affected by aging, but LL muscle aged for 16 or 23 days showed significantly higher CD compared to 9- or 16-day groups ($P<0.05$,

Figure 2. 2). Such a phenomenon could be attributed to that CD is one of the intermediates. CD accumulation is affected by both the formation and the decomposition processes and is less robust in responding to aging treatment (Andreo, Doval, Romero, & Judis, 2003).

2.4.3 Myoglobin and non-heme iron contents

Myoglobin content was affected by muscle type ($P<0.05$), but not postmortem aging (Table 2.2). Myoglobin content was significantly higher in PM muscle compared with LL and SM ($P<0.001$), which was likely due to high content of type I red muscle fiber in PM muscle (Hunt & Hedrick, 1977).

Non-heme iron was significantly affected by muscle type ($P<0.001$) and postmortem aging ($P<0.001$). Non-heme iron was lowest at 9-day of aging and accumulated as aging time prolonged. During aging, exposure of heme group caused by myoglobin deformation would further lead to non-heme iron accumulation (Prasad, Engelman, Jones, & Das, 1989). LL maintained the lowest non-heme iron level followed by SM and PM regardless of the aging treatment (Table 2.2). Considering that the total myoglobin content of PM was about 1.2 times of that of LL or SM, while the non-heme iron content of PM was about 2 times of that of LL, myoglobin in postmortem PM muscles may be more prone to degradation due to higher oxidative distress and could in turn further impact color and lipid oxidative stabilities in PM muscles.

2.4.4 ORAC

Antioxidant activities measured by ORAC (oxygen radical absorbance capacity) in all samples were determined after 7-day simulated retail display. ORAC values significantly varied among all three muscle types, as PM was lowest followed by LL and SM ($P<0.001$; Table 2.2), indicating that PM might have a lower level of antioxidant components that could scavenge free radicals. ORAC values were increased with aging period ($P<0.001$), which is contradictory to the current results of lipid oxidation and discoloration assessment. However, it should be noted that ORAC value might not be the best indicator of the metmyoglobin reducing activity nor the antioxidant activity that protects lipid molecule from being oxidized. In the previous reports about buffalo meat, antioxidant activities measured by FRAP (ferric reducing/antioxidant power) assay did not change during aging (Descalzo et al., 2008), which probably suggests that such assays measured reducing fractions that were not responsive to postmortem aging or not closely related to metmyoglobin reducing system (Descalzo et al., 2008). In the current study, ORAC was assessed only in hydrophilic phase but not non-polar phase, which may potentially remove a considerable amount of cofactors/substrates that were reactive to pro-oxidative iron species. Moreover, free amino acids and antioxidant bi-peptides such as carnosine or anserine can be substantially increased during postmortem aging, which are known to have free radical scavenging abilities (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013; Faustman, Sun, Mancini, & Suman, 2010). This may partially explain the observed increase in ORAC with aging.

2.4.5 Correlation

For oxidation related quality defects, CD was moderately correlated to all the color indexes except CIE L* value (Table 2.3), suggesting a potential involvement of primary oxidation in meat color deterioration. Previous studies showed that lipid-oxidation-induced myoglobin oxidation was more likely due to primary rather than secondary products (O'Grady, Monahan, & Brunton, 2001). Moreover, lipid hydroperoxide was reported to trigger myoglobin into a transient ferryl status, then lead to a covalent bond formed between heme group and protein moiety (Catalano, Choe, & de Montellano, 1989). Such oxidative modification could enhance catalytic activity of the myoglobin to oxidize liposomes (Reeder, Svistunenko, Cooper, & Wilson, 2004). Also, myoglobin the pre-incubated with lipid oxidation products was reported as a "poorer" substrate to the metmyoglobin reducing system (Lynch & Faustman, 2000), thus rendering compromised color stability to the meat.

Myoglobin content was moderately correlated with CIE a* ($r = -0.49$, $P<0.001$), hue angle ($r = 0.59$, $P<0.01$), lean color score ($r = 0.57$, $P<0.001$) and discoloration score ($r = 0.57$, $P<0.001$) and strongly correlated to CD ($r = 0.78$, $P<0.001$) (Table 2.3). It is well known that myoglobin is the main pigment compound that is responsible for meat color, where higher myoglobin content in general results in darker meat (Kim & Hunt, 2011). Since myoglobin contains heme-iron which is highly reactive to oxidation process, it may further facilitate meat darkening/discoloration. In addition, non-heme iron was moderately correlated to myoglobin content ($r = 0.51$, $P<0.01$), CD ($r = 0.66$, $P<0.001$), and color indexes including CIE a* ($r = -0.76$, $P<0.001$), chroma ($r = -0.76$, $P<0.001$), hue angle ($r = 0.63$, $P<0.001$), lean color ($r = -0.7$, $P<0.001$), and discoloration ($r = 0.73$,

P<0.001) (Table 2.3). A recent study reported that the heme and non-heme iron in aged beef muscle were not closely associated with color or lipid oxidation, which was contradictory to the current observation (Purohit, Singh, Kerr, & Mohan, 2015). However, iron species was well known to promote lipid oxidation in muscle food system through various mechanisms (Carlsen, Møller, & Skibsted, 2005). It is also evident that various lipid oxidation products in turn may facilitate myoglobin oxidation (Baron & Andersen, 2002). The current results provide strong supportive evidence to elucidate the interactions between lipid oxidation and myoglobin oxidation.

2.5 Conclusion

The results from the present study found that oxidation stability could be differently influenced by muscle type and aging time. In particular, steak samples from PM showed a further extent of lipid oxidation coupled with more rapid discoloration, lower free radical scavenging activity, and higher non-heme iron content when compared with LL and SM muscles. These results suggested that developing muscle-specific aging strategies that track optimal aging times for different muscles could be beneficial to minimize oxidation-related quality defects, while maximizing aging impact on improving eating quality attributes. Interactions between myoglobin oxidation, non-heme iron accumulation and lipid oxidation processes would need to be further investigated to profound the understanding of muscle food quality preservation.

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Table 2.1 Effects of aging time and muscle type on CIE L*, CIE a*, CIE b*, hue angle and sensory lean color values.

Trait	Display (D)	Muscle (M) †††			Aging (A)			P-values			
		LL	SM	PM	9d	16d	23d				
CIE L*	1	44.58	46.71	42.47	44.72	43.94	45.10	M	<.001	M × A	0.217
	4	44.10	45.60	43.52	44.01	44.85	44.37	A	0.580	M × D	0.307
	7	44.19	45.71	43.54	43.93	44.91	44.60	D	0.930	A × D	0.455
	SEM	0.64	0.64	0.64	0.64	0.64	0.64	M × A × D	0.163		
CIE a*	1	19.27	21.30	19.98	20.41	20.28	19.86	M	<.001	M × A	0.217
	4	17.28	16.87	13.65	16.40	15.53	15.87	A	<.001	M × D	<.001
	7	14.88	12.83	9.98	13.68	12.57	11.44	D	<.001	A × D	0.003
	SEM	0.51	0.51	0.51	0.51	0.51	0.51	M × A × D	0.479		
CIE b*	1	8.86	10.74	9.73	9.81	9.95	9.57	M	<.001	M × A	0.178
	4	8.42	9.63	8.52	9.08	8.63	8.86	A	0.002	M × D	<.001
	7	7.30	8.85	8.82	8.79	8.18	8.01	D	<.001	A × D	0.027
	SEM	0.15	0.15	0.15	0.15	0.15	0.15	M × A × D	0.017		
Hue Angle †	1	24.71	26.77	25.92	25.59	26.10	25.71	M	<.001	M × A	0.052
	4	26.01	29.82	32.19	29.26	29.28	29.48	A	0.268	M × D	<.001
	7	26.29	35.22	41.99	33.64	34.08	35.78	D	<.001	A × D	0.372
	SEM	0.93	0.93	0.93	0.93	0.93	0.93	M × A × D	0.439		
Lean Color ††	1	6.73	6.20	5.23	6.09	6.04	6.04	M	<.001	M × A	0.001
	4	6.07	5.06	3.83	5.02	5.01	4.93	A	0.004	M × D	<.001
	7	5.33	3.69	2.40	4.12	3.97	3.33	D	<.001	A × D	0.007
	SEM	0.24	0.24	0.24	0.24	0.24	0.24	M × A × D	0.983		

† Hue angle= $[(b^*/a^*) \tan^{-1}]$

†† Lean color: 1=extremely dark red, 2=dark red, 3=moderately dark red, 4=slightly dark red, 5=slightly bright red, 6=moderately bright red, 7=bright red, and 8=extremely bright red

†††LL: *longissimus lumborum*, SM: *semimembranosus*, PM: *psoas major*

Table 2.2 Effects of aging time and muscle type on pH, myoglobin, non-heme iron and ORAC.

	Muscle*					Aging				
	LL	SM	PM	SE	P-value	9d	16d	23d	SE	P-value
pH	5.52 [a]	5.54 [ab]	5.64 [b]	0.01	<.001	5.57 [a]	5.58 [a]	5.55 [b]	0.01	0.009
Myoglobin (mg/g meat)	7.63 [a]	9.02 [b]	10.87 [c]	0.29	<.001	9.03 [a]	9.25 [a]	9.25 [a]	0.29	0.642
Non-heme iron (μg/g meat)	2.00 [a]	2.98 [b]	3.86 [c]	0.22	<.001	2.04 [a]	3.29 [b]	3.51 [b]	0.22	<.001
ORAC ** (μM TE/g meat)	3.69 [a]	4.20 [b]	3.12 [c]	0.11	<.001	3.09 [a]	3.70 [b]	4.23 [c]	0.11	<.001

a-c Indicating that numbers in the same row with different letters are significantly different.

* LL: *longissimus lumborum*, SM: *semimembranosus*, PM: *psoas major*

** ORAC: Oxygen Radical Absorbance Capacity

Table 2.3 Pearson correlation coefficients (R-values) between color characteristics and chemical oxidation attributs.

	ORAC	CD	TBARS	Non heme Iron	Mb	CIE L*	CIE a*	CIE b*	Chroma	Hue Angle	Lean Color	Discoloration
ORAC	1											
CD	0.15	1										
TBARS	0.04	0.49*	1									
Non-heme Iron	0.16	0.66**	0.36*	1								
Mb	-0.30*	0.78**	-0.07	0.51**	1							
CIE L*	0.34*	0.09	0.28*	0.05	-0.26	1						
CIE a*	0.00	-0.66**	-0.45**	-0.76**	-0.49**	-0.15	1					
CIE b*	-0.23	0.43*	0.03	0.16	0.46*	0.36*	-0.37	1				
Chroma	-0.05	-0.55*	-0.46**	-0.76**	-0.43*	-0.03	0.95**	-0.11	1			
Hue Angle	-0.11	0.64**	0.36*	0.63**	0.59**	0.25	-0.90**	0.71**	-0.75**	1		
Lean Color	0.05	-0.69**	-0.39*	-0.70**	-0.57**	-0.08	0.92**	-0.51**	0.84**	-0.92**	1	
Discolo-ration	-0.03	0.64**	0.35*	0.73**	0.57**	0.10	-0.93**	0.51**	-0.84**	0.92**	-0.95**	1

* P<0.05

** P<0.001

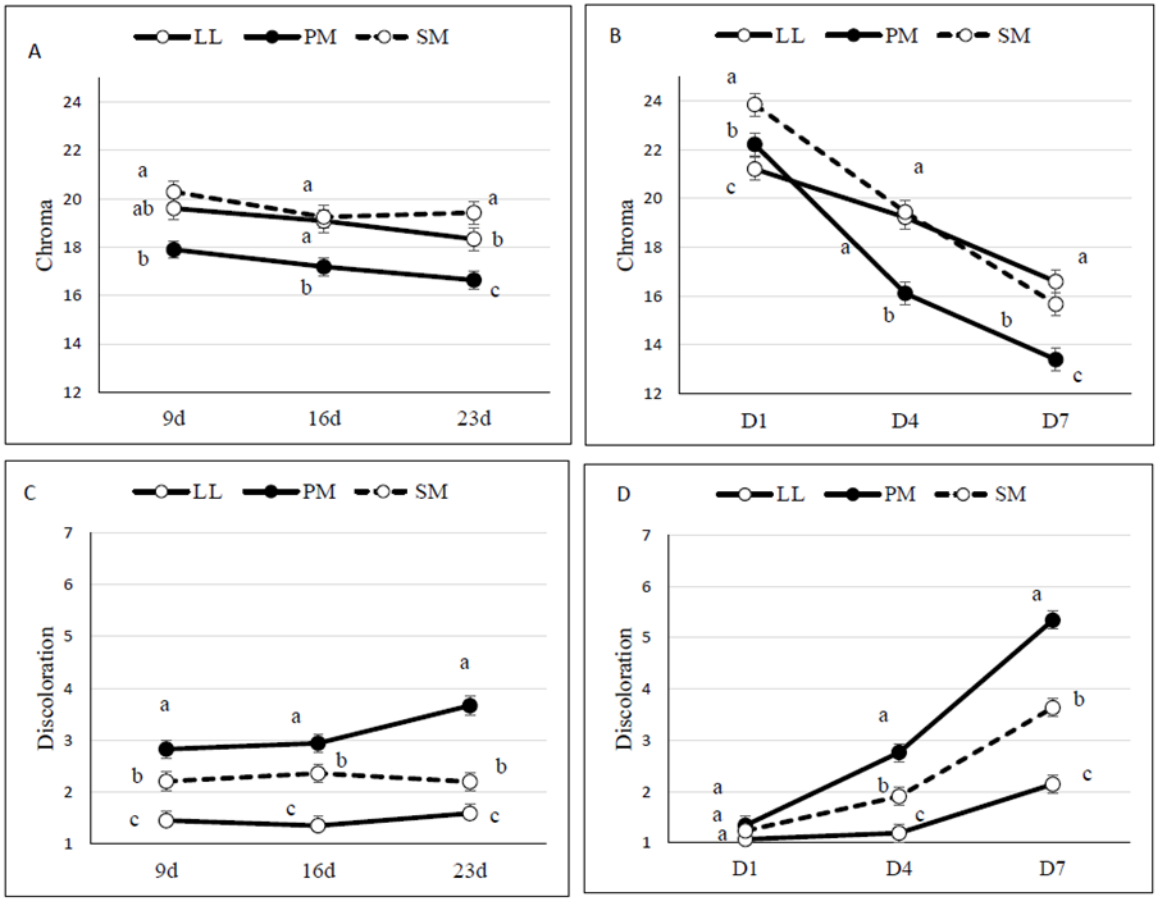

Figure 2.1 Chroma or discoloration affected by aging time or display time in the three bovine muscles.

LL: *longissimus lumborum*, SM: *semimembranosus*, PM: *psoas major*

Bars indicate standard errors of differences of the means. (A) Aging time × muscle type on Chroma; (B) Display time × muscle type on Chroma; (C) Aging time × muscle type on visual discoloration; (D) Display time × muscle type on discoloration.

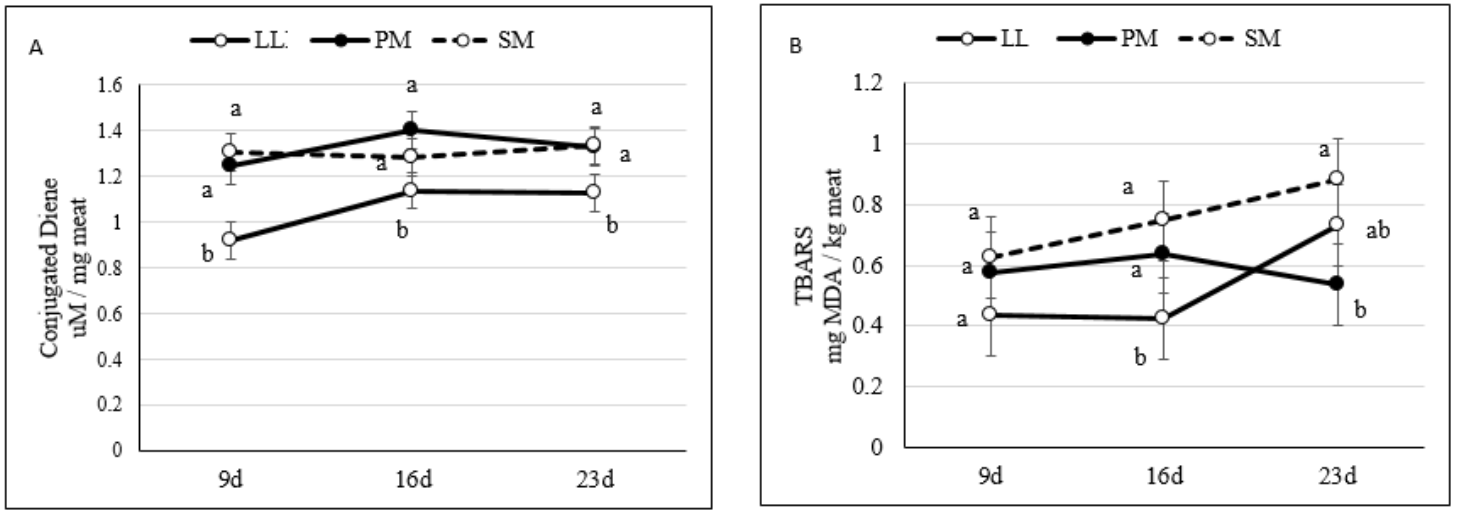

Figure 2.2 Lipid oxidation affected by aging time and muscle types.

LL: *longissimus lumborum*, SM: *semimembranosus*, PM: *psoas major*
Bars indicate standard errors of differences of the means. (A) Aging time × muscle type on diene conjugation as indicator of primary oxidation process; (B) Aging time × muscle type on TBARS as the indicator of secondary oxidation process

CHAPTER 3. METABOLOMICS PROFILING TO DETERMINE THE EFFECT OF POSTMORTEM AGING ON COLOR AND LIPID OXIDATION STABILITIES OF THREE BOVINE MUSCLES

3.1 Abstract

The objective of this study was to identify the key metabolites that could be associated with oxidation stabilities of aged bovine muscles by using a novel HPLC-MS based global metabolomics approach. Three muscles (*longissimus lumbrum* (LL), *semimembranosus* (SM), and *psoas major* (PM)) from 7 beef carcasses were divided into 3 sections and vacuum-packaged at 1-day postmortem and were assigned to 9, 16 and 23 days of aging. After each aging time, selected samples (n = 4) were analyzed by HPLC-ESI-MS metabolomics platform. Unsupervised PCA plots showed separation among 3 aging treatments or muscle types, which revealed effects of aging and muscle on metabolite profiles of samples. Most distinct metabolites that were significantly responsive to aging time and/or muscle type and were closely associated with oxidation stabilities were acyl carnitines, free amino acids, nucleotides, nucleosides, and glucuronides. Particularly, NAD was highly related to antioxidant properties and had high relevance to myoglobin redox stability. Carnitines, free amino acids, purine metabolites and glucuronides were related to the pro-oxidant chemical attributes of the muscles. The results from the current study suggested that some metabolites (either well-

acknowledged or newly found) could be related to oxidation and color stability of beef muscles with aging. Further research into the identified key metabolites and their underlying relevance to the chemical basis of oxidation stability of beef muscle is warranted.

Key words: Beef, aging, metabolomics, meat color, lipid oxidation

3.2 Introduction

Postmortem aging has been extensively practiced in the meat industry mainly due to its beneficial impacts on improving eating quality attributes. However, during prolonged aging, postmortem muscles become more oxidation susceptible, resulting in several oxidation related quality defects, such as reduced blooming potential, decreased color stability, accumulated lipid oxidation products or rapid off-flavor development (Adeyemi, Shittu, Sabow, Ebrahimi, & Sazili, 2016; King, Shackelford, Kalchayanand, & Wheeler, 2012; Ma, Choe, Min, Trinderup, & Kim, 2015).

While the underlying mechanism by which aging affects oxidation stability of postmortem muscles has not been fully understood, a few studies suggested that the accumulation of pro-oxidants, such as heme and non-heme iron (Ma et al., 2015; Purohit, Singh, Kerr, & Mohan, 2015), and/or the depletion of endogenous antioxidants or reducing enzyme activity, such as NADH, α-tocopherol and glutathione peroxidase (Insani et al., 2008; Madhavi & Carpenter, 1993), could be attributed to the aging-induced oxidation. Further, our recent study found that the aging-induced oxidation processes were muscle-specific, where *M. psoas major* had more rapid discoloration, lipid oxidation and non-heme iron accumulation compared to *M. longissimus lumborum*

during extended aging periods (Ma et al., 2015). This observation suggests that the extent of compounds or metabolites that impart antioxidant or pro-oxidation properties may be varied among specific muscle types (Muroya, Oe, Nakajima, Ojima, & Chikuni, 2014) and thus might result in different levels of oxidation stability of muscles during postmortem aging. This postulation would require further confirmation, as there is little or no information surrounding the effects of postmortem aging on oxidation related compounds in muscles was known.

Metabolomics is an emerging technique to analyze small molecule compounds (M.W. less than 2 kDa) in complex biological systems such as cell, tissue or bio-fluids (Johanningsmeier, Harris, & Klevorn, 2016). Mass spectrometry (MS) based metabolomics, in particular, is gaining wide acceptance in the food science discipline due to its potential in analyzing molecular composition, safety and quality properties, and health and nutritional properties of food matrix (Hu & Xu, 2013). In muscle foods, several studies have been recently published in obtaining metabolome profiles of meat samples including beef, pork, chicken and lamb. Those studies determined effects of various pre- and post-harvest factors on meat metabolites, such as packaging (Subbaraj, Kim, Fraser, & Farouk, 2016), chilling (Warner et al., 2015), suspension technique (Graham et al., 2012), feed authentication (Cajka, Danhelova, Zachariasova, Riddellova, & Hajslova, 2013; Osorio et al., 2013), genetics (Marrocco, Zolla, & Zolla, 2011), muscle type (Muroya et al., 2014) and postmortem processing conditions (Graham et al., 2012; Kim, Kemp, & Samuelsson, 2016; Subbaraj et al., 2016). However, no studies have been conducted to identify muscle specific metabolites associated with oxidation stability of different muscles during postmortem aging.

Therefore, the objective of the current study was to identify the metabolites that are related to oxidation stability of different beef muscles during postmortem aging. The present study was a further investigation of our previous study determining effects of postmortem aging on oxidation stability of beef muscles and focused on the metabolomics profiling approach (Ma et al., 2015). In the current study, key metabolites that were significantly responsive to the main treatments were correlated to the results from the previous study, such as color stability, lipid oxidation and oxidation related chemical attributes. Identifying key metabolites that are related to oxidation stability will provide critical insights for the meat industry to develop practical postmortem aging strategies to prevent oxidation-related quality defects.

3.3 Materials and methods

3.3.1 Raw materials and processing

As described above, the results of meat quality and chemical attributes from our previous study (Ma et al., 2015) were used as the foundation of the current study for further metabolomics analyses and statistical analyses. Detailed information regarding raw material sample processing and measured assays was reported in the study (Ma et al., 2015). In brief, seven beef carcasses (USDA Select; A maturity) were harvested at Purdue University Meat Laboratory. Three bovine muscles (*longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM)) were separated from each carcass at 1-day postmortem, divided into three sub-cuts, vacuum packaged and randomly assigned to 9d, 16d, and 23d aging times. After the assigned aging period, 1g of muscle samples from

each cut were snap frozen, powdered, vacuum packaged, and stored at -80 °C until sample extraction for metabolomics profiling.

3.3.2 Color, lipid oxidation and reducing capacity assessment

After each assigned aging time, steaks from each muscle were overwrap packaged by polyvinyl chloride film (23,000 $cm^3/O^2/m^2$/24 hours at 25°C) in Styrofoam trays and displayed under fluorescent light (color temperature 3,500 K) at 3 °C for seven days. Color traits measurements by a Minolta CR-400 colorimeter and a trained visual color panel were conducted on D1, D4, and D7 of the display. Lipid oxidation (TBARS), myoglobin content, and non-heme iron concentrate were determined on the displayed meat samples as previously reported (Ma et al., 2015).

3.3.3 Metabolomics sample preparation and extraction

Protein removal and sample extraction were performed using a Bligh-Dyer extraction protocol (Bligh & Dyer, 1959). Chloroform (200 uL) mixed with an equal volume of methanol was added to 100 mg of meat powder. Samples were extracted in a Precellys 24 tissue homogenizer. 200 uL of water was mixed with the extraction and was centrifuged at 16,000 × g for 8 minutes. The upper methanol and water phase contained the polar metabolites, which was transferred to separate vials and were evaporated to dryness in a SpeedVac Concentrator. The dried polar fraction was reconstituted in 50 uL of a diluent composed of 95% water and 5% acetonitrile containing 0.1% formic acid.

3.3.4 HPLC-MS analysis and data pre-process

Separations were performed on an Agilent 1100 system (Palo Alto, CA), with a mobile phase flow rate of 0.3 mL/min. The metabolites were assayed using a Waters Atlantis T3 column (3 µm, 2.1 × 50 mm), where the mobile phase A and B were 0.1% formic acid in ddH_2O and acetonitrile, respectively. Initial conditions were 100:0 A:B and followed by a linear gradient to 5:95 at 21 min and was held until 26 min

Peak deconvolution was performed using Agilent MassHunter ver. B.06. Chromatographic peaks were aligned across all samples. Peak areas were normalized by sample weights, converting to log2 and applying a 75% percentile shift. Peak annotations were performed based on mass assignment and retention behavior using the METLIN (www.metlin.scripps.edu) and HMDB (www.hmdb.ca) metabolite databases with a mass error of less than 10 ppm.

3.3.5 Data analysis

Univariate statistics were performed by split-plot ANOVA or Kruskal-Wallis test (KWT) using R software (www.r-project.org) with Benjamini–Hochberg FDR correction. Metabolites with $P<0.05$ were considered significantly responsive to aging or muscle treatment and were introduced into multivariate modeling. The corresponding FDR values were reported as further reference. Associations of metabolites and color characteristics, lipid oxidation, non-heme iron and myoglobin content and were analyzed by using paired Spearman correlation. Correlation with statistical significance ($P<0.05$) were considered for further biological interpretation. Unsupervised principal component

analysis (PCA) was performed using R software, relevance of each metabolite to each PC was assessed by the corresponding loadings.

3.4 Results

3.4.1 Metabolome profiles

The untargeted metabolome profiling initially detected 1695 different compounds in meat samples. Metabolites were checked with the assumption of equal variance and normality of error term to determine the applicability of conducting ANOVA, where 702 metabolites that satisfied the two assumptions were tested for ANOVA, and the remaining metabolites were tested using the Kruskal – Wallis method. For the effect of aging, 222 out of 702 metabolites were significantly responsive to the aging treatment ($P<0.05$); 150 of which showed strong signals ($FDR<0.05$, $P<0.05$) after Benjamin-Hochberg multiple testing correction, which is an adjustment of P-values to control the level of type I error.

From the 222 compounds, the major groups included free amino acids, fatty acetyl carnitines and nucleotide related metabolites (Table 3.1). For the effect of muscle type, 35 of the metabolites exhibited signals under the $P<0.05$ criterion, but none of them satisfied the $FDR<0.05$ criterion after P-value adjustment. Primary metabolites being identified from this group were presented in Table 3.2. Overall, the LL and SM muscles were characterized with more abundant in β-alanine-histidyl dipeptides, fatty acetyl carnitines, phenylalanine and niacinamide. The results of KWT was summarized in Table 3.3, where L-carnitine, xanthine and hypoxanthine were affected by aging or muscle type as indicated by a fold change larger than 2.

3.4.2 Principal component analysis (PCA)

The principal component analysis (PCA) was used to visualize the distinctiveness of the metabolome profile of the aged bovine muscles. Two PCA models have been built based on the different subsets of the original dataset, namely the 243 metabolites that showed statistical significance indicated by ANOVA or KWT under $P<0.05$ criterion (Figure 3.1 A and 3.1B), and the 150 metabolites that showed significances in responding to aging effect indicated by ANOVA under both $P<0.05$ and $FDR<0.05$ criteria (Figure 3.1C and 3.1D). Based on the further selected data, the PCA plots (Figure 3.1C and 3.1D) clearly showed improved efficacy compared to the initially selected data (Figure 3.1A and 3.1B), as the total variance explained by PC1 increased from 33% in the first model to 41% in the second model. As showed in the both figures, the first two PCs have already specified more than 50% of the observed variance. In particular, PC1 is mostly accounted for the difference of aging treatment and clearly separated muscle samples that aged for 9 days, 16 days, and 23 days respectively. By definition, PC1 is the axis where the data set has the largest variation (Ben-Hur & Guyon, 2003). Therefore, in the current study, the major differences of the metabolomes among muscle samples were caused by postmortem aging. In addition, PC2 could explain 21% of the total variance of the data set, which separated the PM samples from the cluster formed by overlapped LL and SM samples.

3.4.3 Correlation

The heatmaps demonstrated the correlations of all the metabolites that were significantly affected by either muscle type or aging time. Specifically, Figure 3.2A showed the correlations to the color characteristics obtained on D7 of the display, and

Figure 3.2B showed the correlations to pro-oxidation related indexes, include lipid oxidation (TBARS), and non-heme iron content. Metabolites that significantly correlated to color characteristics (Table 3.4) or to at least one of the pro-oxidation related indexes (Table 3.5) mainly included NAD, acyl-carnitines, free amino acids, assorted nucleotides, and glucuronides. In particular, NAD showed positive correlation to redness of meat color ($r = 0.672$) and negative correlation to discoloration ($r = -0.535$), TBARS ($r = -0.554$), and non-heme iron ($r = -0.667$), reflecting its relevance to myoglobin redox stability and/or antioxidant activity in general. A number of fatty acetyl carnitines were associated with decreased lean color ($r = -0.74$ to -0.37) and intensified discoloration ($r = 0.35$ to 0.70). Nucleotides xanthine and hypoxanthine, free amino acids phenylalanine and tryptophan, and glucuronides were in general positively associated with discoloration characteristics and non-heme iron accumulation, which implied potential pro-oxidative activities of those compounds in aged beef muscles.

3.5 Discussion

3.5.1 The effect of aging on muscle metabolome and oxidation stabilities

3.5.1.1 Free amino acids

Free amino acids phenylalanine, tyrosine, and tryptophan were identified based on the m/z values 165.0782, 181.0736, and 204.0902 respectively. Phenylalanine was more abundant in the 16d aging group, while both tyrosine and tryptophan were higher in the 23d group. Free amino acids and oligo peptides were most likely generated through postmortem proteolytic process, while the dynamics of the phenylalanine might suggest a

further degradation mechanism when extended aging was applied. Moreover, there were moderate negative correlations between phenylalanine or tryptophan and color characteristics include CIE a*, chroma, and visual lean color ($P<0.05$, Table 3.4). Consistently, the correlations between the two amino acids and discoloration indexes (Table 3.4), non-heme iron (Table 3.5) and TBARS (Table 3.5) were positive. Amino acids, especially phenylalanine, are common substrates of reactive oxygen species (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). The reactive activity of amino acids to ROS could be exhibited as either radical scavenging capacity or pro-oxidative activities due to the generation of oxidative susceptible products (Bekhit et al., 2013), which may in turn affect the color characteristics of meat. Moreover, the side chains of phenylalanine and tryptophan are both reactive to the heme groups (Sirangelo, Tavassi, Martelli, Casadio, & Irace, 2000). The potential interactions between AAs and heme groups could form heme complexes that may be detrimental to the meat color stability (Bekhit, Geesink, Ilian, Morton, & Bickerstaffe, 2003). The observed correlations between free amino acids and color stability would require further investigation.

3.5.1.2 NAD/NADH

Nicotinamide adenine dinucleotide (NADH) and its oxidized form NAD+ was identified by the m/z values 665.1249 and 663.1092 respectively. The ANOVA results indicated that both NADH and NAD+ were significantly decreased with prolonged aging period ($P<0.05$, $FDR<0.05$, Table 3.1). Moreover, NAD+ showed moderate correlation to both color and chemical oxidation indexes include CIE a*, chroma, hue, TBARS and

non-heme iron among other weak correlations (Table 3.4 and Table 3.5). Similarly, Subbaraj et al. (2016) found higher NADH content in color stable ovine steaks compared to color labile samples by using the HILIC –MS metabolomics. The significance of NAD/NADH system on meat color stability was well established (Mancini & Hunt, 2005), where it acts as electron donor to various metmyoglobin reducing systems, either enzymatically or non-enzymatically (Bekhit & Faustman, 2005). Bekhit et al. (2003) indicated that the amount of NADH retained in the postmortem muscles was more influential to meat color maintenance than metmyoglobin reducing activity (MRA). In thoroughly washed porcine muscles, MRA could not be observed without additional exogenous NADH (Mikkelsen, Juncher, & Skibsted, 1999). Regeneration of NADH in postmortem muscle could be achieved by adding exogenous metabolism intermediates, such as pyruvate, lactate and malate, which could improve color stability or myoglobin redox stability (Kim et al., 2006; Mohan, Hunt, Barstow, Houser, & Muthukrishnan, 2010; Ramanathan, Mancini, & Dady, 2011). Depletion of NADH during postmortem aging was consistent with other studies (Sammel et al., 2002) and might be a partial explanation for the aging related color stability deterioration.

3.5.1.3 Nucleotide degradation

Nucleotides and related catabolism metabolites were identified according to the m/z values. Adenosine 5’-diphosphate (ADP, m/z=427.0285) was not significantly responsive to the muscle type but was marginally affected by aging treatment ($P=0.07$, 9d<16d=23d, data not shown). ADP can be degraded into adenosine monophosphate

followed by inosine monophosphate (IMP), inosine, and hypoxanthine (Terasaki, Kajikawa, Fujita, & Ishii, 1965). IMP and inosine, which were indicated by m/z values 348.0471 and 268.0808 respectively, were both significantly responsive to the aging treatment, whereas the both compounds showed higher abundance in 16d aging group ($P<0.05$, FDR 0.006 to 0.112, Table 3.1). Hypoxanthine (m/z=136.0389), on the other hand, has been tested by KWT and affected marginally by aging treatment ($P<0.05$, Table 3.3). According to Muroya et al. (2014), in porcine *longissimus lumborum* (LL) and *vastus intermedius* (VI) muscles, IMP and inosine started to generate 24-hour postmortem and kept accumulating up to 7 day postmortem, while ATP exhaustion was observed within 24 hours. The current result did not detect the existence of ATP but observed its degradation products, including ADP, IMP, inosine, and hypoxanthine, were more liberated in the muscle as aging time extended. Particularly, hypoxanthine was moderately correlated to discoloration indexes and non-heme iron with positive coefficient, indicating its potential relationship with oxidation processes. It is known that in live animals, hypoxanthine was one of the pro-oxidative cofactors that reacted with oxygen (O_2) to generate superoxide ($O_2 \cdot^-$), a well-known reactive oxygen species that cause oxidative distress in live cells or tissues (Mesquita Casagrande et al., 2013; Rodrigues et al., 2014).

Overall, the results of the present study suggested that even after the ATP depletion (onset of the rigor mortis), liberation of ATP catabolism products that retain pro-oxidative activities may affect a decrease in oxidation stabilities of aged beef muscles.

3.5.2 The effect of muscle type on muscle metabolome and oxidation stabilities

3.5.2.1 Acyl-carnitines

Most identified fatty acetyl carnitines were significantly responsive to the aging effect, where muscle samples at 9 days of aging showed more fatty acetyl carnitines compared to those at longer aging times (Table 3.1). Some fatty-acetyl carnitines were higher in LL muscles, indicating difference in metabolome caused by muscle types (Table 3.2). L-carnitine was identified by m/z value161.1052 (Table 3.3). L-carnitine acts as transportation vessel to facilitate long-chain fatty acids to across the inner mitochondrial membranes. The carnitine-fatty-acid-chain complex is the substance of β-oxidation to produce biological energy (Rebouche, 2012). The catabolism of carnitines generates malate (Rebouche, 2012), which is an intermediate of the TCA cycle that were previously reported to improve color stability of bovine muscles (Mohan et al., 2010).

Previous study using extraneous L-carnitines treatment to enhance the color of fresh beef cuts found no effect on the meat color stability (Djenane, Martínez, Sánchez-Escalante, Beltrán, & Roncalés, 2004). The current study mainly assessed relative abundance of endogenous L- or fatty-acetyl carnitines. No obvious correlation was observed between L-carnitine and any of the meat quality characteristics, but some fatty-acetyl carnitines were moderately correlated to color traits during display (Table 3.4). The negative correlation between fatty–acetyl carnitines and meat color might be partially attributed to the effect of mitochondria activity. Higher carnitines could indicate a higher level of energy metabolism and mitochondria enzyme activity of antemortem muscles. Such a condition might cause an increase of oxygen consumption rate of the

aged muscle and eliminate the free oxygen penetration during muscle to meat conversion, under which circumstance thin layer of oxymyoglobin was not able to fully mask the underlying deoxymyoglobin layer, resulting in dark purple appearance of the meat (Tang et al., 2005).

3.5.2.2 β-Alanyl dipeptides

Carnosine (m/z=226.1062) and anserine (or homocarnosine, m/z 240.1214) were increased with postmortem aging ($P<0.05$; Table 3.1) and were at a higher level in LL compared to SM and PM muscles (Table 3.2). Both carnosine and anserine exhibit their antioxidant activity by chelating transition metals (Brown, 1981). These peptides have been reported to play critical roles in meat quality preservation. Djenane et. al (2004) reported that surface application of carnosine provided antioxidant protection against meat oxidation related deterioration during postmortem aging. Consistent with previous studies, carnosine content was different according to the type of muscle metabolism. Muroya et al. (2014) reported higher carnosine content in porcine LL muscles compared to VI muscles. In broilers, breast muscles retained seven times higher carnosine compared to thigh muscles (Intarapichet & Maikhunthod, 2005). In general, carnosine tended to be more abundant in glycolytic muscles, i.e. LL, than the muscle of oxidative metabolism such as PM (Mora, Sentandreu, & Toldrá, 2008).

3.6 Conclusion

The results from the current study suggest that metabolomics can be effectively used for profiling compounds associated with oxidation stabilities of beef muscles with aging. In particular, the current study confirmed the relevance of NAD and NADH in myoglobin redox stability. Other metabolites showed high association with oxidation characteristics including carnitines, (hypo) xanthine and glucuronides, which could be related to color and/or lipid oxidation stabilities of aged beef muscles. Those metabolites have the potential to be further validated as biomarkers, which could provide critical insights for the meat industry to predict oxidation related quality defects during aging practice.

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Figure 3.1 PCA plots separated samples with different aging time or muscle type.

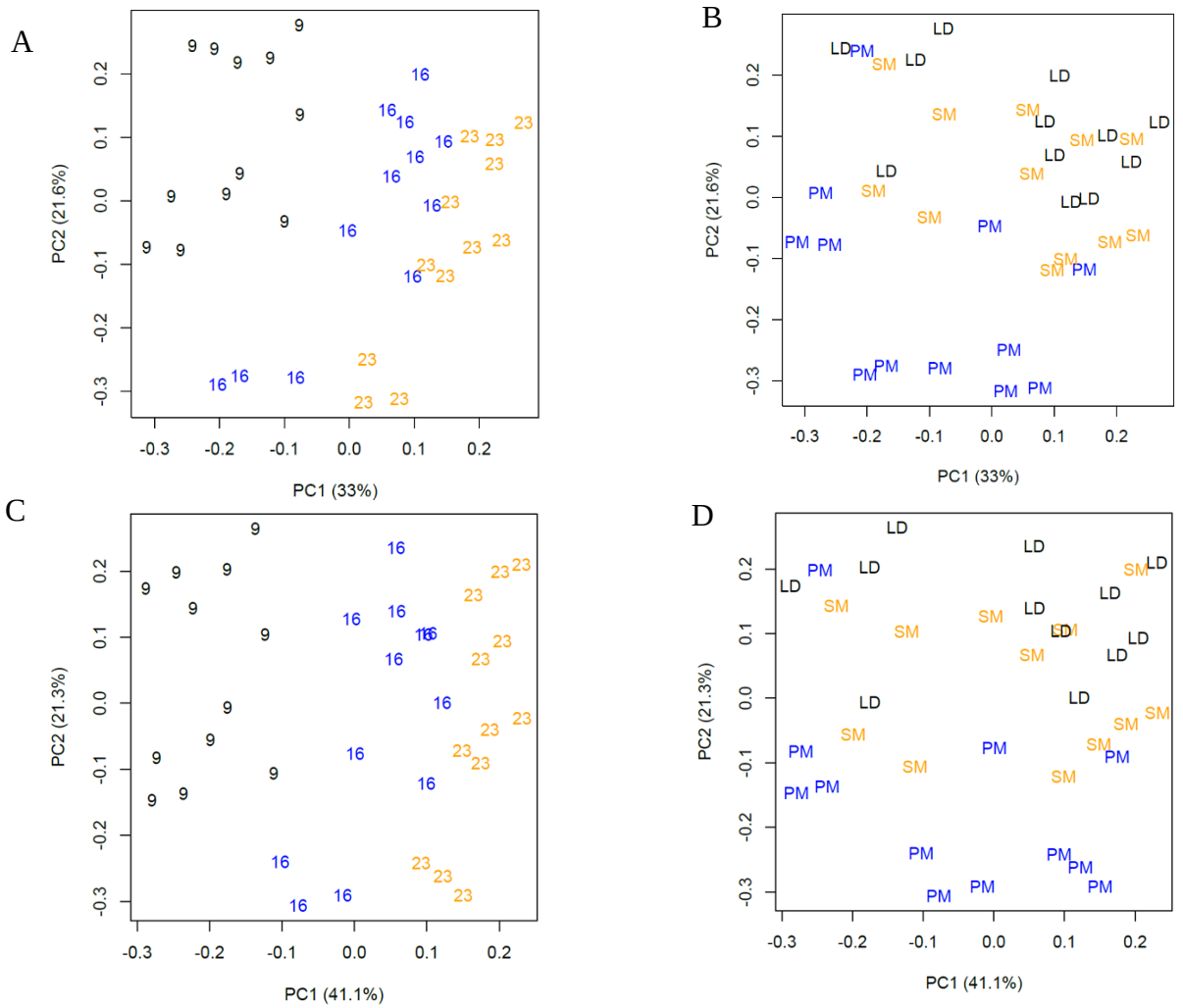

Figure 3.2 Correlation heatmaps between metabolites and color (A) or chemical oxidation (B) characteristics.

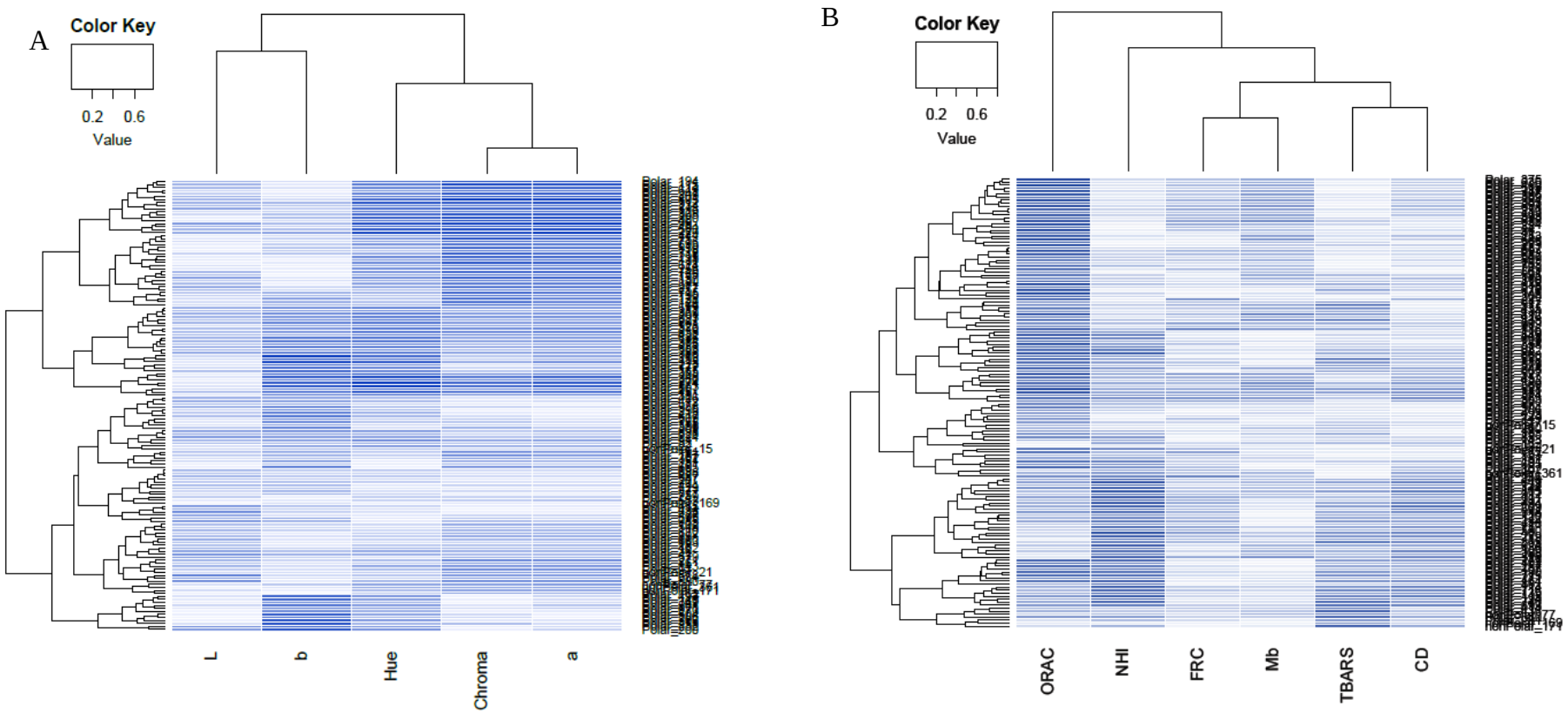

Table 3.1 Metabolites significantly affected by aging time, tested by ANOVA

Mass_Input	Putative compound	Formula	Mass_Database	Δppm	More Abundant*	P-value	FDR
Carnitines							
203.1146	Acetylcarnitine	C9H17NO4	203.1158	5	9d	0.011	0.052
231.1461	Butyryl-L-carnitine	C11H21NO4	231.1471	4	9d	0.005	0.026
245.1629	Pivaloylcarnitine	C12H23NO4	245.1627	0	9d	0.016	0.067
259.1786	L-Hexanoylcarnitine	C13H25NO4	259.1784	0	9d	0.007	0.034
261.1586	3-Hydroxyisovalerylcarnitine	C12H23O5N1	261.1576	3	9d	0.041	0.132
287.2085	L-Octanoylcarnitine	C15H29NO4	287.2097	4	9d	0.008	0.039
315.2398	Decanoyl-L-carnitine	C17H33NO4	315.2410	3	16d	0.035	0.118
Free Amino Acids							
165.0782	L-Phenylalanine	C9H11NO2	165.0790	0	16d	0.005	0.030
181.0736	L-Tyrosine	C9H11NO3	181.0739	0	23d	<0.001	0.004
204.0902	L-Tryptophan	C11H12N2O2	204.0899	0	23d	0.027	0.100
Glucuronides							
297.119	Phenethylamine glucuronide	C18H19NOS	297.1187	0	23d	<0.001	0.000
313.1168	Tyramine glucuronide	C14H19NO7	313.1162	2	23d	<0.001	0.000
Nucleotides							
268.0812	Inosine	C10H12N4O5	268.0808	6	16d	0.032	0.112
348.047	IMP	C10H13N4O8P	348.0471	3	16d	0.001	0.006
Vitamins and Coenzymes							
122.0471	Niacinamide	C6H6N2O	122.0480	7	9d	0.003	0.017
663.1092	NAD	C21H27N7O14P2	663.1091	0	9d	<0.001	0.000
665.1249	NADH	C21H29N7O14P2	665.1248	0	9d	<0.001	0.000

* “More Abundant” was determined by least square means comparison

Table 3.2 Metabolites significantly affected by muscle type, tested by ANOVA

Mass_Input	Putative compound	Formula	Mass_Database	Δppm	More Abundant*	P-value	FDR
Carnitine							
231.1467	Butyryl-L-carnitine	C11H21NO4	231.1471	1	LD	0.021	0.853
259.1781	L-Hexanoylcarnitine	C13H25NO4	259.1784	0	LD	0.010	0.666
217.1309	Propionyl-L-carnitine	C10H19NO4	217.1314	2	LD	0.010	0.666
245.1629	2-Methylbutyroylcarnitine	C12H23NO4	245.1627	0	PM	0.049	0.853
Functional Peptides							
226.1062	Carnosine	C9H14N4O3	226.1066	1	LD	0.036	0.853
240.1214	L-Anserine	C10H16N4O3	240.1222	3	LD	0.034	0.853
Major Non-peptide Metabolites							
122.0478	Niacinamide	C6H6N2O	122.048	1	LD	0.004	0.604
145.1092	Acetylcholine	C7H15NO2	145.1103	7	LD	0.028	0.853
165.0782	L-Phenylalanine	C9H11NO2	165.079	4	LD	0.005	0.604

* “More Abundant” was determined by least square means comparison

Table 3.3 Metabolites exhibit significance in Kruskal-Wallis test.

Mass	**Putative ID**	**Fomular**	**Mass database**	**Δppm**	**Aging ***	**Muscle ***	**P-value**	**FDR**
Carnitines								
161.1039	L-Carnitine	C7H15NO3	161.1052	8	——	——	0.006	0.054
343.2704	Dodecanoylcarnitine	C19H37NO4	343.2722	5	9/16d	PM	0.007	0.054
425.3516	Vaccenyl carnitine	C25H47NO4	425.3505	2	——	——	0.002	0.054
Nucleotides								
136.0389	Hypoxanthine	C5H4N4O	136.0385	2	——	——	0.051	0.130
152.0333	Xanthine	C5H4N4O2	152.0334	0	9d16d	PM	0.003	0.054

* “More Abundant” was determined if fold change was 2× or higher

Table 3.4 Correlation coefficients (R-values) between major metabolites and color characteristics

Mass	Putative ID	Lean	Discoloration	CIE a	Chroma	Hue
Carnitines						
161.1039	L-Carnitine	-0.604*	0.576*	-0.573*	-0.551*	0.589*
203.1146	Acetylcarnitine	-0.372*	0.355*	-0.318	-0.300	0.421*
217.1309	Propionyl-L-carnitine	-0.435*	0.430*	-0.466*	-0.429*	0.483*
231.1461	Butyryl-L-carnitine	-0.429*	0.374*	-0.365*	-0.328	0.447*
245.1629	Pivaloylcarnitine	-0.348	0.399*	-0.383*	-0.328	0.437*
259.1786	L-Hexanoylcarnitine	-0.741*	0.700*	-0.644*	-0.578*	0.782*
287.2085	L-Octanoylcarnitine	-0.670*	0.635*	-0.563*	-0.487*	0.721*
315.2398	Decanoyl-L-carnitine	-0.603*	0.637*	-0.563*	-0.514*	0.664*
343.2704	Dodecanoylcarnitine	-0.538*	0.535*	-0.433*	-0.384	0.546*
425.3516	Vaccenyl carnitine	0.490*	-0.525*	0.598*	0.566*	-0.465*
Vitamins and Coenzymes						
122.0471	Niacinamide	-0.114	0.095	-0.099	-0.103	0.157
663.1092	NAD	0.584*	-0.553*	0.647*	0.634*	-0.494*
665.1249	NADH	-0.126	0.086	-0.024	0.025	0.256
Free Amino Acids						
165.0781	L-Phenylalanine	-0.769*	0.753*	-0.730*	-0.675*	0.732*
181.0736	L-Tyrosine	-0.265	0.326	-0.424*	-0.510*	0.165
204.0902	L-Tryptophan	-0.585*	0.613*	-0.641*	-0.648*	0.547*
Glucuronide						
297.119	Phenethylamine glucuronide	-0.498*	0.564*	-0.612*	-0.629*	0.437*
313.1168	Tyramine glucuronide	-0.427*	0.513*	-0.552*	-0.600*	0.348
Nucleotides						
136.0383	Hypoxanthine	-0.686*	0.723*	-0.731*	-0.722*	0.663*
152.0333	Xanthine	-0.633*	0.632*	-0.574*	-0.511*	0.686*
268.0812	Inosine	-0.372*	0.345*	-0.316*	-0.287	0.392*
348.0469	IMP	0.175	-0.321	0.307*	0.337*	-0.151

* P<0.05

Table 3.5 Correlation coefficients (R-values) between major metabolites and chemical/oxidation attributes

Mass	Putative ID	NHI	Mb	TBARS
Carnitines				
161.1039	L-Carnitine	0.373*	0.333	0.189
203.1146	Acetylcarnitine	0.122	0.430*	0.002
217.1309	Propionyl-L-carnitine	0.336*	0.296	-0.073
231.1461	Butyryl-L-carnitine	0.095	0.309	-0.003
245.1629	Pivaloylcarnitine	0.159	0.435*	0.103
259.1786	L-Hexanoylcarnitine	0.445*	0.376	0.225
261.1586	3-Hydroxyisovalerylcarnitine	0.108	0.130	0.235
287.2085	L-Octanoylcarnitine	0.371*	0.367*	0.112
315.2398	Decanoyl-L-carnitine	0.443*	0.408*	0.111
343.2704	Dodecanoylcarnitine	0.323	0.417*	-0.040
425.3516	Vaccenyl carnitine	-0.469*	-0.319	-0.240
Vitamins and Coenzymes				
122.0471	Niacinamide	-0.125	0.241	-0.061
663.1092	NAD	-0.636*	-0.094	-0.557*
665.1249	NADH	-0.134	0.351*	-0.079
Free Amino Acids				
165.0781	L-Phenylalanine	0.628*	0.220	0.289
181.0736	L-Tyrosine	0.427*	-0.043	0.294
204.0902	L-Tryptophan	0.477*	0.231	0.468*
Glucuronides				
297.119	Phenethylamine glucuronide	0.654*	0.229	0.320
313.1168	Tyramine glucuronide	0.620*	0.209	0.224
Nucleotides				
136.0383	Hypoxanthine	0.617*	0.473*	0.332
152.0333	Xanthine	0.310*	0.415*	0.175
268.0812	Inosine	0.095	0.346*	-0.024
348.0469	IMP	-0.437*	-0.029	0.025

* $P<0.05$

CHAPTER 4. CONCLUSION

The current research project investigated oxidation stabilities in the three bovine muscles that have distinct metabolic properties with up to 23 days postmortem aging. The results from the Chapter Two suggest that oxidation stability could be differently influenced by muscle type and aging time. Particularly, psoas major muscles exhibit intensified lipid oxidation and more rapid discoloration, coupled with lower free radical scavenging activity and higher non-heme iron content, compared with longissimus and semimembranosus muscles. In the Chapter Three, The HPLC-MS metabolomics profiling effectively identified the metabolic features that associated with oxidation stabilities in three bovine muscles with postmortem aging. The results confirmed the relevance of NAD and NADH in myoglobin redox stability. Other metabolites showed high association with oxidation characteristics include carnitines, nucleotides and glucuronides, which could be related to color and/or lipid oxidation stabilities of aged beef muscles.

In conclusion, the results of the current study indicate that color and lipid oxidation stabilities could be negatively associated with aging, and different muscles have different susceptibilities to oxidation. This observation suggests that developing a post-harvest aging strategy to track optimal aging times for different muscles would be beneficial for the meat industry to minimize the oxidation-related quality defects while

maintain consistent eating attributes. Further, the results from the current study suggest that metabolomics can be effectively used for profiling compounds associated with oxidation stabilities of beef muscles with aging. The identified key metabolites should be further investigated to reveal the chemical basis of oxidation stability of beef muscle, and could be validated into biomarkers for the industry to track the quality development of beef during aging.

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