METABOLOMICS OF MUSCLE-SPECIFIC BEEF COLOR STABILITY

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METABOLOMICS OF MUSCLE-SPECIFIC BEEF

COLOR STABILITY

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Abstract:

Inherent metabolite differences in muscles can significantly affect biochemical properties and meat color. The objective was to compare metabolite profile differences between beef *longissimus* and *psoas* muscles during display. Beef short loins were collected 3 d postmortem (n = 10). Steaks were cut from each *longissimus lumborum* (LL) and *psoas major* (PM) muscles and displayed under retail conditions for 7 d. Surface color, biochemical properties, and metabolomics were analyzed during display. *Psoas major* discolored ($P < 0.05$) by d 3 compared with *longissimus lumborum*. There were significant differences in metabolite concentrations ($P < 0.05$) for each muscle type at each time point. Sugars, amino acids, Tricarboxylic acid cycle substrates and glycolytic substrates were detected in both muscles. Glycolytic metabolites like pyruvic acid, glucose–6–phosphate and, fructose were greater in LL at all display times. TCA metabolites, citric acid, and succinic acid, which were lower in concentration on d0, in LL, became overabundant in LL by d 7. The amino acid carnitine was lower in LL at all display times. The results suggest that in addition to muscle - specific differences in mitochondrial and enzyme activities, inherent metabolite differences also can contribute to muscle color stability.

Key words: Metabolomics, beef color, mass spectrometry, longissimus and psoas

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CHAPTER I

INTRODUCTION

Myoglobin is a sarcoplasmic protein that is primarily responsible for meat color. In fresh meat, myoglobin can exist in three different redox forms, namely deoxymyoglobin, oxymyoglobin, and metmyoglobin. Deoxymyoglobin imparts purple color while predominant oxymyoglobin gives consumer preferred bright-red color to beef. Oxidation of both oxy- and deoxymyoglobin result in the formation of metmyoglobin and discoloration of meat (Faustman and Cassens, 1990; Smith et al., 2000). Although various pre- and post-harvest factors can increase myoglobin oxidation, meat has an inherent capacity to delay metmyoglobin accumulation by a process called metmyoglobin reducing activity (Ledward, 1985a). The concentration of reduced nicotinamide adenine dinucleotide (NADH) and mitochondrial activity play a significant role in metmyoglobin reduction (Tang et al., 2005; Ramanathan and Mancini, 2010). In postmortem muscle, enzymes involved in the glycolytic and tricarboxylic acid cycle (TCA) retain activity. However, metabolite concentration decreases with postmortem time. Various studies have shown that the addition of metabolites such as lactate, pyruvate, and succinate to meat or isolated mitochondria can regenerate NADH and can influence beef color (Giddings and Hultin, 1974; Ramanathan et al., 2011). Therefore, characterizing tricarboxylic and glycolytic substrate changes in postmortem muscle is critical to understand the fundamental basis for meat discoloration.

Muscles in a beef carcass can be classified as color-stable and color-labile depending on the proportion of red and white fibers. The concentration of muscle fibers can influence the capillary network and metabolite use for cellular metabolism. *Longissimus lumborum* (LL) which is commonly merchandised as New York strip steak is a color-stable muscle while tenderloin (*Psoas* major; PM*)* is a color-labile muscle (O'Keeffe and Hood, 1982b; McKenna et al., 2005; Seyfert et al., 2006). Various researchers have shown that biochemical properties vary between LL and PM. A recent study suggests that the sarcoplasmic proteome differs significantly between LL and PM (Joseph et al., 2012). More specifically, an overabundance and positive correlation of enzymes involved with color stability such as aldose reductase, pyruvate dehydrogenase, β-

enolase, and triose phosphate isomerase involved in the glycolytic pathway were reported in LL muscle. Red muscle (PM) will have more mitochondria and capillary density than white muscle (LL). Hence TCA and glycolytic substrates will be utilized at different rates (Kushmerick et al., 1992; Glancy and Balaban, 2011). Thus, investigating muscle metabolomic profile differences between the LL (color-stable) and PM (color-labile) muscles will provide valuable information about the interrelationship between metabolomic profile changes and meat color.

Metabolomics technique allows simultaneous measurement of hundreds of low molecular weight metabolites such as sugars, amino acids, nucleosides, fatty acids and other compounds such as nucleotides in a biological system (Kanani et al., 2008). The application of metabolomics has been used in diverse research areas like human medicine, drug discovery, plant science, human nutrition and food science (Kaddurah-Daouk and Krishnan, 2008; Wishart, 2008a; Cevallos-Cevallos et al., 2009). Few studies have utilized metabolomics techniques to characterize the role of metabolites in tenderness and water-holding capacity (Bertram et al., 2010; Graham et al., 2010; D´Alessandro et al., 2011; Warner et al., 2015). Recently metabolomics was used to study color stability in ovine meat (Subbaraj et al., 2016). However, limited studies have utilized metabolomic techniques to characterize beef color. Therefore, the objective of this study was to determine the metabolomic profile differences between LL and PM muscles using GC-MS-based metabolomics approach to gain more insights into muscle-specific differences in color stability.

CHAPTER II

REVIEW OF LITERATURE

Meat color.

Meat color is one of the defining qualities, which has a great impact on consumer purchasing decisions. Consumers prefer bright cherry-red beef, over brown discolored beef (Hood and Riordan, 1973; Bekhit and Faustman, 2005; Joseph et al., 2015). Discoloration leads to annual loss of $1 billion to the meat industry (Smith et al., 2000). The red color of beef is mainly due to myoglobin, a heme-containing protein pigment. In a live animal, myoglobin carries oxygen to mitochondria for cellular respiration. However, in postmortem muscle, myoglobin is the main protein responsible for meat color. Hemoglobin and cytochrome C; also contribute to meat color. However, their role is relatively insignificant. A majority of hemoglobin will be removed during proper bleeding. The color of meat depends on various factors like age, sex, rate of decline of pH, and ultimate pH (Seideman et al., 1984). Further, color also differs between species and is dependent on myoglobin concentration. Among the domestic animals, beef has the highest concentration of myoglobin (Seideman et al., 1984). Older animals will have a higher concentration of myoglobin (Morita et al., 1970). The pH is another factor which can play an important role in determining color. Both ante-mortem and post-mortem conditions have an effect on the rate of decline and ultimate pH. Among the various intrinsic factors that affect color stability, muscle type also has an effect.

I. Myoglobin

Myoglobin is an iron-containing globular protein, present in skeletal and cardiac muscles (Giddings and Solberg, 1977) and it carries oxygen from the blood to the muscles. Myoglobin (Mb) is a heme containing protein present in the sarcoplasm of myocytes responsible for the color of meat. Myoglobin is a monomeric protein composed of 153 amino acids. It has a heme prosthetic group and a globin moiety with a molecular weight of 17.5 kilodaltons. The heme is entrapped within the globin chain (Suman and Joseph, 2013). The globin chain is helically coiled with eight segments. Heme gives the ability to carry oxygen through reversible binding. The

prosthetic group of the myoglobin has an iron atom at the center and has six sites for coordination (Mancini and Hunt, 2005). The color of meat depends on the ionic state of heme and the ligand attached to the sixth coordination site of the myoglobin. The heme group contains a porphyrin ring with an iron atom at the center that can exist in reduced (ferrous/Fe ++) or in an oxidized form (ferric/Fe+++). Of the six coordination sites, four are occupied by pyrrole groups, of the rest two, one is bonded with proximal histidine (HIS residue 93), and the 6^{th} position is available to bind with a ligand. In reduced state (Fe++), myoglobin has a greater affinity for oxygen and so the 6^{th} position will be bound by oxygen, whereas in the oxidized state, the ligand is a water molecule. In the reduced state, myoglobin can also bind to other small ligands such as NO and CO (Mancini and Hunt, 2005). Myoglobin exists in three different redox forms, oxymyoglobin (OxyMb), deoxymyoglobin (DeoxyMb) and metmyoglobin (MetMb) depending on the redox state of the heme iron. The OxyMb form imparts bright cherry red color to the meat. Deoxygenation of OxyMb will lead to the formation of deoxymyoglobin. Deoxymyoglobin can be found in freshly cut meat surface, and it will be dark purple in color. Metmyoglobin imparts brown color and is formed when OxyMb or DeoxyMb is oxidized. When DeoxyMb is exposed to carbon monoxide, carboxymyoglobin (COMb) is formed and it is more stable than OMb due to structural orientation. Carboxymyoglobin also has a bright cherry-red color and is often used in modified atmosphere packaging of beef (Mancini and Hunt, 2005).The color of the surface of the meat is determined by the proportion of three forms of myoglobin. In the visible spectrum of light, these three forms of myoglobin exhibit characteristic absorption properties, which can be used to quantify the relative proportions of the three forms in meat (Tang et al., 2005).

II. Metmyoglobin reduction activity and oxygen consumption rate.

Meat has an inherent capacity to reduce the iron atom in metmyoglobin to the ferrous state by a process called metmyoglobin reducing activity (MRA). This can occur by both enzymatic and non-enzymatic pathways (Bekhit and Faustman, 2005). Metmyoglobin reducing activity is

considered as an important biochemical process that can influence beef color stability (Ledward, 1985b). Reduced nicotinamide adenine di nucleotide (NADH), plays a very important role in enzymatic and non-enzymatic metmyoglobin reduction (Echevarne et al., 1990). Of the different reducing systems, NADH-dependent cytochrome b5 reductase has received attention and several studies have determined its role in color stability. Hence, regeneration of NADH might be a practical approach to retard myoglobin oxidation and increase fresh meat color stability. The loss of metmyoglobin reducing the activity of meat during storage is due to change in substrates and cofactors for reduction, as well as the damage to the structural and functional integrity of mitochondria (Renerre and Labas, 1987).

Oxygen consumption is the ability of postmortem muscle to consume oxygen. In postmortem muscle various processes can consume oxygen. However, mitochondria are the main organelles responsible for oxygen consumption. Hence, processes that can influence mitochondrial activity can influence beef color. More specifically, mitochondria are involved in metmyoglobin reduction and bloom development. If mitochondria are very active, myoglobin will have less oxygen and results in darker meat. Mitochondria can limit metmyoglobin formation by electron-transport mediated metmyoglobin reduction and reductase activity. Hence, when mitochondria become less activate, it can promote metmyoglobin formation. Conversely, when mitochondria are very active, it can have detrimental effects on color stability. For example, if there is more tissue oxygen consumption rate (OCR), more metmyoglobin will be formed leading to lower color shelf life (O'Keeffe and Hood, 1982b; Renerre and Labas, 1987). In a study conducted by Lanari and Cassens (1991), muscles with low color stability showed higher metmyoglobin reducing activity. O'Keeffe and Hood (1982b) reported that psoas major had a higher tissue oxygen consumption rate, lower oxygen penetration, higher enzyme activity (metmyoglobin reductase activity) and, thereby, higher metmyoglobin formation. In the postmortem skeletal muscle, mitochondria will still be active and utilize oxygen (Tang et al., 2005). Oxygen

consumption by mitochondria can lead to reduced oxygen availability for myoglobin, due to low partial pressure of oxygen, thereby leading to the formation of DMb which is darker in color (Ramanathan et al., 2009). So, the OCR hence becomes a major color determining biochemical property.

III. Muscle-specific beef color

Depending on the anatomical location, physiological function of individual muscles varies. This is reflected in their metabolism, and hence the muscle color chemistry also varies (McKenna et al., 2005). The surface discoloration of beef due to metmyoglobin formation also depends on muscle type (McKenna et al., 2005; Seyfert et al., 2006). Depending on the color stability of beef when kept in retail display, beef muscles are classified as color-stable and color-labile. Color-labile muscles have greater OCR (O'Keeffe and Hood, 1982b) and a lower metmyoglobin reducing activity (Ledward, 1985a), whereas color-stable muscles have a greater reducing ability (Reddy and Carpenter, 1991). McKenna et al. (2005) classified the muscles based on color stability as high color stability, intermediate stability, low and very low color stability (Table 2.1).

Longissimus lumborum (LL) which is commonly merchandized as New York strip steak, is a color-stable muscle whereas tenderloin or Fillet Mignon, which is *psoas majo*r (PM) is a color-labile muscle. Compared to the color-stable LL the PM had higher lipid oxidation, and lower MRA (McKenna et al., 2005; Seyfert et al., 2006; Joseph et al., 2012). In LL, heat shock proteins and antioxidant proteins were greater in concentration than in PM (Joseph et al., 2012). They (Joseph et al., 2012) also reported that the concentrations of β-enolase and triose phosphate isomerase (glycolytic enzymes) were greater in LL than in PM. *Longissimus lumborum* had a greater NADH concentration compared with psoas major over a period of seven days in retail display, in a study conducted by (Kim et al., 2009). The color-labile muscle *gluteus medius* had a higher mitochondrial content and thereby more OCR compared to color-stable *longissimus dorsi* (McKenna et al., 2005). These studies suggest that there is a difference in postmortem muscle

metabolism, which can lead to a difference in muscle biochemistry and color stability. The color-labile muscle *gluteus medius* had a higher mitochondrial content and thereby more OCR compared to color-stable *longissimus dorsi*.

IV. Metabolomics.

Metabolomics is an approach that allows identification and quantification of a large number of metabolites in a biological sample. It is a large-scale study of all low molecular weight compounds such as sugars, amino acids, lipids and other small metabolites (Fiehn, 2002; Julian, 2004). Metabolites play a role in macromolecular reactions through signal pathways or feedback inhibition. The analysis of the metabolome, which is the entire set of metabolites, helps to identify the ultimate phenotypical changes which happen to cells or tissue due to the changes in environment or gene expression. A variety of conditions such as diseases or stress can change the normal functioning of the cell, which will be reflected in the biological pathways. There are two type of approaches in metabolomics: global metabolomics and targeted metabolomics (Kaddurah-Daouk et al., 2008). In the global approach, the analyst tries to identify and characterize all the metabolites present in the biological sample; whereas, in the targeted approach, only a specific number or class of metabolites are studied (Kaddurah-Daouk et al., 2008). The complete metabolome of an organism or tissue contains a large number of metabolites. So, it is difficult to do a comprehensive analysis using a single platform (Villas-Bôas et al., 2005). The metabolomic analysis involves usage of either a single technique or a combination of techniques. The popular analytical tools are Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography – Mass Spectrometry (LC-MS), Capillary Electrophoresis – Mass Spectrometry (CE-MS) and Nuclear Magnetic Resonance (NMR) (Bino et al., 2004; Sato et al., 2004; Dunn and Ellis, 2005; Baker et al., 2006). Gas chromatography-mass spectrometry is highly useful to analyze low molecular weight metabolites (Garcia and Barbas, 2011). Each method will have a particular extraction step followed by processing steps prior to analysis. Along with these analytical techniques, various bioinformatics tools for data mining will be used to interpret the results.

V. Metabolomics in meat and food science

Apart from human medicine, nutrition, and plant sciences, metabolomics has application in the field of food and agriculture. Metabolomics were included as one of the approaches used to assess the safety of genetically modified (GM) crops/food by the European Thematic Network on the safety assessment of Genetically Modified Foods (ENTRANSFOODS; Dixon et al., 2006). It can also be employed to study the effects of environment on crops, effects of hazardous chemicals, and stress on organisms (Dixon et al., 2006). Targeted metabolomics can be used to assess the quality of agricultural produce and also to identify specific biomarkers in the case of diseases (Wishart, 2008b). Metabolite predictive models have been developed to estimate sensory attributes of green tea, watermelon, and mushrooms. With the help of multiple sensory evaluations, the particular compound /metabolite which can improve consumer acceptance can be determined (Wishart, 2008b).

Metabolomics can be used as a powerful tool to unearth the inherent biochemical profile which can play a crucial role in meat quality. Proteomic tools have been used previously to study the differences between the sarcoplasmic proteomes of color-stable *longissimus lumborum* and color-labile *psoas major* muscles (Joseph et al., 2012). Proteomics was also used earlier to study the biochemical mechanisms involving muscle to meat conversion (Sayd et al., 2006; Jia et al., 2007) and in tenderness studies (Morzel et al., 2008; Jia et al., 2009; Laville et al., 2009). The recent advancements in technology in this field have increased the interest of researchers to metabolomics. Improved MS instruments using Fourier transform ion cyclotron resonance and orbitrap has increased the quantity and accuracy of metabolomic spectra (D'Alessandro et al., 2012). The emergence of new software and bioinformatics tools to decipher data like XCMS, Metlin, Metabosearch, Human Metabolome Database and KEGG has also helped in increased use of metabolomic tools for research (D'Alessandro et al., 2012).

Postmortem meat metabolism plays an important role in meat quality because it can affect various parameters such as pH, water holding capacity, tenderness, color, and flavor. So metabolomics can be a potential tool to study postmortem muscle metabolism and thereby help in developing strategies to improve meat quality. For example, metabolomics technique has been used to analyze pork quality traits of Casertana and Large White breeds (D´Alessandro et al., 2011), to predict postmortem age of the beef using NMR (Castejón et al., 2015) and, to identify food products that incorporated mechanically recovered meat (Surowiec et al., 2011).

Gas Chromatography–Mass Spectrometry

Gas chromatography-mass spectrometry is a widely used metabolomic technique in most of the metabolomic studies (Kanani et al., 2008; Koek et al., 2011). In GC-MS, metabolites are separated by gas chromatography, and the compounds are identified using mass spectrometry. There are several advantages that make GC-MS a preferred choice for researchers. Gas chromatography has a high separation efficiency and is comparatively easy to operate and has a high sensitivity which greatly reduces the amount of the sample required for measurement. The compounds are better separated in gas phase than in liquid phase (Kanani et al., 2008). Among all the metabolomic techniques, GC-MS has the lowest operational and maintenance costs and is more user-friendly (Kanani et al., 2008). Gas chromatography has been widely used in biotechnology, clinical, and forensic studies. The availability of mass spectral libraries for GC-MS helps in identification of compounds as compared to other methods (Koek et al., 2011). But the metabolites must be volatile and heat stable to undergo GC –MS analysis. Following the GC-MS analysis, the data should be processed. There are various steps involved in GC-MS analysis, and it can be summarized as shown in Figure.2.1.

Since the eluted compounds from a GC column are in gas phase, they are directly compatible with mass spectrometry and can be easily subjected to ionization by electron impact or by chemical means. In general, fixed electron voltages are used to ionize molecules to produce databases containing mass spectra and retention time. These two-dimensional databases can be

used for other analytical platforms (lipidomics, proteomics). One of the leading metabolite database libraries for GC-MS is FeihnLib (Kind et al., 2009).

Sample preparation is an important step in the metabolomics analysis. The samples should be free of exogenous compounds/metabolites which otherwise can interfere with analysis and produce false results. The extraction process is very critical in metabolomics analysis, especially in global metabolomics approaches. Since the goal is to extract and quantify as much of metabolites possible, the usage of right extraction method is very essential for the success of the analysis. The extraction protocol also should be targeted towards the metabolite of interest. The solvent for extraction depends on the type of metabolite of interest. For polar metabolites, methanol, acetonitrile, or ethanol are commonly used (Jiye et al., 2005). In the case of lipophilic metabolites, chloroform is used as a solvent of choice for extraction.

Since a majority of the metabolites is polar, they have to be derivatized, so that they are thermally stable and volatile at temperatures for the GC analysis (Koek et al., 2011).The majority of GC methods rely on oximation followed by silylation or silylation alone as the derivatization procedure (Koek et al., 2011). Silylation is a widely used procedure where in the active proton groups like hydroxyl (OH), will be displaced by an alkyl silyl group (-R-Si) (Segura et al., 1998). Through silylation, almost all protic functional groups can be preserved for GC analysis. Trimethyl silylation (TMS) is the common approach for silylating, where in trimethyl silyl amides *like N, O-bis-trimethylsilyl-triflouroacetamide* (BSTFA) or *N, O–bis–N–methyl–N–trimethylsilyl–trifluoroacetamide* (MSTFA) are used to prepare trimthylsilyl (TMS) derivatives of the metabolites (Segura et al., 1998). Trimethylsilyl derivatives exhibit a high volatility, thermal and chemical stability which is preferred for GC analysis (Segura et al., 1998). The silylating power of these amides can be increased by adding a catalyst such as trimethylchlorosilane (TMCS). A combination of BSTFA +1%TMCS has been widely used in drug testing analysis (Segura et al., 1998).

Following data acquisition, the data have to be processed prior to statistical analysis (Koek et al., 2011). This is done to get an unbiased result since there will be many metabolites or chromatographic peaks from a single sample. Also, there is a need to obtain only one entry per metabolite and correctly identify it since some compounds can co-elute thereby making the identification process difficult. Koek et al. (2011) pointed out that deconvolution is a good method to process GC-MS data, since it will help in aligning the peaks thereby finding out co-eluted compounds, and is automated. Deconvolution makes use of the mass spectral data to distinguish between overlapping peaks (co-eluting compounds). After data processing, the data will be analyzed statistically with the help of multivariate techniques such as principal component analysis (PCA) to draw relevant conclusions (Koek et al., 2011).

Post-mortem muscle biochemistry plays an important role in determining the meat color and color stability. There are possible differences in metabolite utilization between color-stable LL and color-labile PM. Therefore, a better understanding of the post-mortem metabolite profile might be helpful to gain more insights into post-mortem muscle metabolism and meat color. Metabolomic analysis can be used as a powerful tool to characterize metabolites and, better understand the mechanisms involved in the metabolism, thereby gaining more knowledge on meat color stability. The advancements in this field can be used to predict the color stability or to find ways to improve the shelf-life of beef.

Table 2.1. Beef muscles classified based on their color stability Adapted from McKenna et al., (2005).

Muscle	**Color stability**
Longissimus lumborum, *Longissimus thoracis,* *Semitendinosus,* *Tensor fasciae latae*	High
Semimembranosus *Rectus femoris* *Vastus lateralis* *Trapezius* *Gluteus medius* *Latissimus dorsi*	Intermediate
Triceps brachii – long head *Biceps femoris* *Triceps brachii – lateral head* *Pectoralis profundus* *Adductor* *Serratus ventralis*	Low
Supraspinatus *Infraspinatus* *Psoas major*	Very low

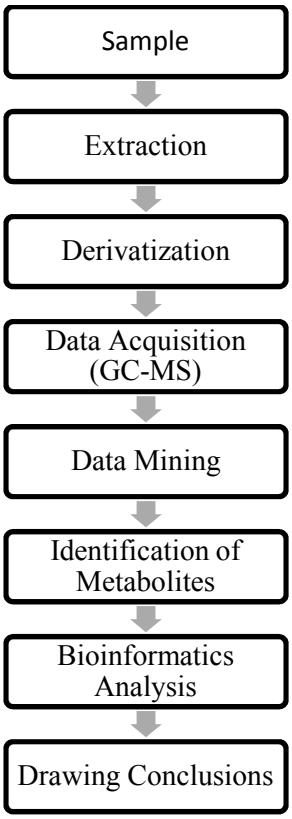

Figure 2.1. Flow chart of GC-MS analytical steps (Kanani et al., 2008).

CHAPTER III

MATERIALS AND METHODS

Raw materials and processing

Ten USDA Choice short loins were purchased from a major packing facility 3-d postmortem. Vacuum packaged loins were transported on ice to the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University. Ten beef strip loins (*longissimus lumborum;* IMPS #180, NAMP, 2002), ten tenderloins (psoas major; IMPS #190A, NAMP, 2002) were separated from each short loin, and each was cut into five 2.5-cm-thick steaks using a meat slicer (Bizerba USA Inc, Piscataway, NJ). The steaks were placed onto foam trays with absorbent pads, over-wrapped with a PVC film (oxygen-permeable polyvinyl chloride fresh meat film; 15,500–16,275 cm^3 O_2/m_2/24 h at 23 °C, E-Z Wrap Crystal Clear Polyvinyl Chloride Wrapping Film, Koch Supplies, Kansas City, MO) and kept in retail display under fluorescent lighting for 7 d. Following surface color measurements, steaks were cut in half. First, half was used to measure metmyoglobin reducing activity (MRA) and oxygen consumption (OC), and the second half was used to measure metabolite profile and pH. The steak half assigned to MRA and OC was then bisected parallel to the oxygenated surface to expose the interior of steak (resulting in two interior pieces). The first interior piece was used to measure MRA and the second interior piece was used to measure OC. From the second half, representative samples that contained both oxygenated and interior sections were taken for both metabolomics and pH measurements.

Retail display

After packaging, steaks were placed in a coffin-style open display case maintained at 2°C ± 1 under continuous lighting (1612 to 2152 lx, Philips Deluxe Warm White Fluorescent lamps; Andover, MA; color rendering index = 86; color temperature = 3000 K). All packages were rotated daily to minimize differences in light intensity or temperature caused by location.

Surface color measurement

The surface color was measured on the steaks assigned for display at two random locations on each steak using a HunterLab MiniScan XE Plus spectrophotometer (HunterLab

Associates, Reston, VA, USA) with a 2.5-cm diameter aperture, Illuminant A, and 10° standard observers. Reflectance (R) at isobestic wavelengths from 400 – 700 nm was used to quantify myoglobin redox forms on the surface of steaks. Reflectance at 474, 525, 572, and 610 nm were converted to K/S values using the following equation: $K/S = (1-R)^2 / 2R$. These values were then substituted into the appropriate equations (AMSA, 2012) to calculate the percentage of DeoxyMb, OxyMb, or MetMb. Percentage myoglobin form values also were used to calculate MRA and OC.

Metmyoglobin Reducing Activity (MRA)

Metmyoglobin reducing activity was determined according to the procedure described by (Sammel et al., 2002). Samples from the interior of steak halves were submerged for 20 min in a 0.3% solution of sodium nitrite (Sigma, St. Louis, MO) to facilitate metmyoglobin formation, and then removed, blotted dry, vacuum packaged (Prime Source Vacuum Pouches, 4 mil, Koch Supplies Inc., Kansas City, MO), and scanned with a HunterLab MiniScan XE Plus Spectrophotometer to determine pre-incubation metmyoglobin values (AMSA, 2012). Each sample was incubated at 30°C for 2 h to induce MetMb reduction. Upon removal from the incubator, samples were rescanned to determine the percentage of remaining surface MetMb. The following equation was used to calculate metmyoglobin reducing activity: [(% surface MetMb pre-incubation - % surface MetMb post-incubation) ÷ % surface MetMb pre-incubation] x 100. Increased MRA is associated with improved color stability.

Oxygen consumption (OC)

Oxygen consumption was determined according to a modified procedure of Madhavi and Carpenter, (1993), on the fresh-cut surface of the bottom half portion of the cube removed prior to MRA analysis. The samples were allowed to oxygenate for 30 min at 1°C, were vacuum packaged, and then were scanned twice (as described in the MRA procedure) on the bloomed surface (representing the previously unexposed interior of the original cube) to measure OxyMb. Oxygen consumption (measured by conversion of OxyMb to DeoxyMb) was induced by

incubating samples at 30°C for 30 min. Samples were rescanned immediately upon removal to determine remaining surface OxyMb as a percentage by using K/S ratios and equations (AMSA, 2012). To calculate OC, changes in OxyMb values pre- and post-incubation were used.

Metabolomic analysis

Sample preparation

The metabolites were extracted from the muscle samples, following a modified procedure of Brown et al. (2012). Briefly, 0.5 g of muscle sample was kept in 1.5 mL of methanol (GC-MS grade, J.T Baker, USA) in borosilicate glass vials with PTFE-lined caps. The vials were then vortexed, for 30 s and kept for incubation for 20 h at room temperature (22 - 26 °C). Following incubation, the samples were vortexed again for 10 s and centrifuged for 5 min at 2000 rpm. From the supernatant 200µL was transferred to amber colored vials and ribitol (2 µg) was added as an internal standard to all the samples. The samples were then dried under a gentle stream of nitrogen gas.

Metabolomic Profiling

The metabolomic profiling was done using gas chromatography–mass spectrometry (GC-MS; Agilent 6890 GC coupled with a 5973N mass selective detector (MSD); Agilent Technologies, Palo Alto, CA). Samples were derivatized prior to GC-MS analysis, using a modified procedure described by Rudell et al. (2008). The dried samples were reconstituted with 100µL methoxyamine (2% methoxyamine hydrochloride in pyridine; Pierce, IL, USA) and were incubated at 50°C for 2 hours. Silylation was done with 100µL of *N*, *O*-bis (Trimethylsilyl) trifluoroacetamide with 1% trichloromethylsilane (BSTFA+1%TMCS; ThermoScientific, PA, USA) and incubated for 30 min at 50°C. After incubation the mixture was transferred to glass vials containing deactivated polyspring glass inserts prior to analysis.

One microliter of the extract was injected using an Agilent 7683B auto sampler injector in splitless mode into an Agilent 6890 GC coupled with a 5973N mass selective detector (MSD; Agilent Technologies, Palo Alto, CA). The temperature of the inlet was kept at 250°C to vaporize the sample and a splitless glass liner with the tapered bottom were used to focus the vapors to the column which was a DB-5MS GC capillary column (Agilent Technologies; 30 m x 250 µm x 0.25 µm). Ultra-pure helium (Stillwater Steel Supplies, Stillwater, OK, USA) was used as the carrier gas at constant flow mode (1 mL/min). The oven was set to an initial temperature of 50 °C for 5 min followed by a ramp of 5°C/min to a final temperature of 315°C which was kept for 3 min. The MSD was operated in electron ionization (EI) mode with transfer line and source temperatures kept at 230°C and quadrupole temperature maintained at 106°C. Mass spectra ranging from m/z 50 to 650 were recorded in scan mode. Data were then processed by the MSD Chemstation (Agilent Technologies).

Statistical analysis

The experimental design was a split-plot with randomized block design in the whole plot (n = 10 replications). Each short loin from an animal served as a block, and each muscle type (LL or PM) was fabricated into four steaks and randomly assigned to 0, 1, 3, and 7 days. Fixed effects included muscle types, storage time, and their interactions. Random effects included animal, animal x muscle types, and residual error. Surface color, MRA, and OCR were analyzed using the MIXED PROC of SAS (9.3) and the differences among means were detected using the least significance differences (LSD) at $P < 0.05$.

Metabolomics data from the Chemstation were deconvoluted using AMDIS (Automated Mass Spectral Deconvolution and Identification System, NIST) using simple analysis method. To extract good quality data, the signal to noise ratio (S/N) was set to 20, with medium settings for resolution, sensitivity and shape requirements. A new mass spectral library was created by combining mass spectral data of compounds included in the Fiehn Metabolomics RTL library

(Agilent Technologies, Santa Clara, CA, USA) and the NIST 05 library (National Institute of Standards and Technology, MD, USA). The compounds in the samples were then identified by matching the mass spectra with those in the newly created library with the minimum match factor set to 80, to rule out false positives. Peak alignment, normalization and statistical analysis of the identified compounds were performed using Agilent Mass Profiler Professional (MPP) (Agilent Technologies).

The intensity of the identified masses was normalized based on the internal standard (ribitol) and log transformed followed by baseline transformation to the median of all samples. The compounds which were identified in only one sample were then omitted and to further increase the quality of the data, only those which were featured in at least 50% of samples in one condition were selected for statistical analysis. The compounds identified by AMDIS using the mass spectra of the libraries (Fiehn Metabolomics RTL Library and NIST 05) were analyzed to identify the compounds which are significantly different, based on the normalized intensity values in the samples using the MPP. One-way ANOVA ($P < 0.05$) was used to find the statistical significance, and to further increase the data quality, Benjamini-Hochberg multiple test corrections ($P < 0.05$) was applied. The unsupervised hierarchical clustering analysis (HCA); was performed, on all identified metabolites and also on the significant metabolites. The clustering was done both on metabolites and samples using Euclidean distance and Ward's linkage rule. The samples and entities were clustered based on normalized intensity values. An unsupervised principal component analysis (PCA) on conditions was performed on the data.

CHAPTER IV

RESULTS AND DISCUSSION

Postmortem muscle is biochemically active and enzymes involved in glycolytic, and TCA cycle remains active. However, substrates required to regenerate reducing equivalents are depleted with postmortem time. Hence, characterizing the metabolite changes will increase our understanding related to beef color. We utilized GC-MS based non-targeted metabolomics approach to study metabolome profile changes in LL and PM muscles. As expected, PM discolored quickly compared with LL. By day 3, PM had lower redness compared with LL (Figure 4.1). The changes in redness during display was greater for PM (11 a* units) than LL (3.6 a* units). Interestingly, both oxygen consumption and MRA were greater for PM on day 0 than LL (Figures 4.2 and 4.3). However, by d 3, oxygen consumption and MRA decreased rapidly for PM than LL. This suggests that oxidative changes are greater for PM than LL. Previous studies have also reported color-labile nature of PM than LL (McKenna et al., 2005; Joseph et al., 2012).

Metabolism of carbohydrate, amino acid, and fatty acids are inter-related. Nevertheless, metabolism of carbohydrates can be attributed to the generation of reducing equivalents such as NADH and FADH. Mitochondrial content is greater in PM than LL (Mohan et al., 2010;Ramanathan et al., 2014) which can greatly influence the utilization of metabolites postmortem. With the help of the libraries, 141 metabolites were identified. Twenty-nine compounds were found to be significantly different ($P < 0.05$) after Benjamini Hochberg multiple test correction. Of these 19 compounds were found to have a fold change difference greater than 2 on a logarithmic scale. In the current study, an intensity difference of 2 log fold was considered significant. The significantly different compounds are represented in Table 4.1, with the P value (after Benjamini Hochberg correction) mass and retention time.

The principle component analysis scores plot (Figure 4.4) clearly shows the separation between LD and PM samples. The component 1 explains 55.75% variation in metabolite changes between samples while the component 2 explains 22.12% of the variation. Further, PCA plot indicates LL metabolite changes among d 0, 3, and 7. However, for the PM, d 3 and 7 had a little separation compared to d 0 across component 2. From the loadings plot (Figure 4.5) the

metabolites responsible for maximum variation were identified and the absolute loading values for component 1 are given in the Table 4.2. Uracil, hypoxanthine, malic acid, carnitine, dyhydroxy acetone, had positive loadings indicating their effect on the PM samples; whereas fructose, glucose–6–phosphate, methionine, succinic acid had negative loadings indicating their effect on the LL samples. The PCA loading plot indicates how each metabolite concentration influences the variation between muscle type.

Glycolytic compounds (fructose, glucose – 6 – phosphate, pyruvic acid) are overabundant in LL (Tables 4.3, 4.4 and, 4.5). This supports previous research (O'Keeffe and Hood, 1982; Joseph et al., 2012) which also indicated that color stable muscles have predominantly glycolytic metabolism. Citric acid was greater in PM samples on days 0 and 3 compared with LL (Tables 4.3, 4.4 and, 4.5). However, on day 7 the citrate levels were greater in LL muscles than PM. This can be attributed to utilization of citrate for mitochondrial activity. A previous study indicated that pyruvate dehydrogenase (Joseph et al., 2012) was overabundant in color stable muscle. This suggests that in LL pyruvate might be entering the Krebs cycle and, thereby, increasing citrate levels. Further, PM has a higher mitochondrial aconitase (an enzyme that converts citrate to isocitrate; Joseph et al., 2012), which might be another possible reason for the lower level of citrate in PM.

Succinate is a complex II substrate for electron transport chain and also an intermediate metabolite in TCA cycle. The addition of succinate can limit metmyoglobin formation by electron transport mediated metmyoglobin reduction (Tang et al., 2005) and also by reverse electron transport (Belskie et al., 2015). On d 3 and 7, succinate levels in PM were lower than LL. Hence, rapid discoloration in PM can be attributed to lower levels of succinate. Greater levels of succinate might be due to (1) less utilization by mitochondria and (2) formation from glutamic acid or valine. In the current study, valine content in LL was greater than PM. However, further research is required to validate the conversion of valine to succinate in postmortem muscle.

The amino acid derivative carnitine is an important metabolite for the fatty acid transport into mitochondria for fatty acid oxidation. *Psoas major* had greater carnitine compared to LL. This can be attributed to greater mitochondrial content in PM than LL. In contrast to findings of Subbaraj et al. (2016), methionine levels were higher in LL. Hypotaurine, a metabolic breakdown product of cysteine and methionine metabolism, was lower in LD. So, there might be a possible difference in cysteine – methionine metabolism in the color stable muscle. Joseph et al. (2012) reported greater levels of antioxidant protein peptide methionine sulfoxide reductase (PMSR) which prevents the oxidation of methionine in the longissimus, which might also be a possible reason for the higher levels of methionine in LD.

Nucleotide degradation rate varied between the muscle types. For example, there was a difference in hypoxanthine and ribose–5–phosphate levels between LL and PM muscles. These compounds are formed from the hydrolysis of inosine -5- monophosphate due to the action of nucleosidases (Koutsidis et al., 2008). Hypoxanthine can also be formed from purine metabolism. The difference in levels of uracil is suggestive of muscle specific differences in rates of nucleic acid hydrolysis postmortem. In the current study, we extracted polar metabolites; however, some non-polar fatty acids were also detected. Differential levels of fatty acids like stearic acid, palmitoleic acid, and linoleic acid which were lower in LD compared to PM. There is a need for more research to ascertain the differences in fatty acids

In order to visualize metabolite changes, we also plotted the changes in metabolites using Heat maps (Figure 4.6) that were different ($P < 0.05$). Hierarchical clustering analysis indicated that there is a difference between LL and PM as they fell in different clusters (Figure 4.7 and 4.8). For both longissimus and PM, metabolite clusters were similar on d 0 and 3 and were different on d 7.

Table 4.1 – List of metabolites differentially abundant in two muscles with *p* values, mass, retention time, and chemical abstracts service (CAS) number.

Metabolite	**P-value**	**Mass**	**Retention time**	**CAS number**
Nucleoside metabolites				
Uracil	4.92×10^{-12}	241	28.277632	66-22-8
Hypoxanthine	0.0473757	265	39.709084	68-94-0
Carbohydrate metabolites and intermediates				
Fructose	1.04×10^{-12}	73	40.72578	57-48-7
Gluconic acid	1.32×10^{-12}	73	43.307842	526-95-4
D-malic acid	4.14×10^{-12}	73	32.104206	617-48-1
Citric acid	1.77×10^{-12}	273	39.673515	5949-29-1
D-glucose-6-phosphate	0.0010929	387	49.11201	56-73-5
Ribonic acid-gamma-lactone	0.0015542	73	36.767242	8-3-5336
1,3-dihydroxyacetone	0.003798	73	25.780489	96-26-4
Succinic acid	0.009169	147	27.61917	29915-38-6
D-ribose-5-phosphate	0.01282	315	45.262295	15673-79-7
Pyruvic acid	0.0389019	73	19.87522	127-17-3
Maltose	0.042729	361	55.512077	69-79-4
Amino acid metabolites and intermediates				
L-methionine	4.68×10^{-12}	176	33.021603	63-68-3
Hypotaurine	0.0027508	188	35.004578	300-84-5
Aspartic acid	0.003775	232	32.967148	56-84-8
L-carnitine	0.0139388	195	37.45107	541-15-1
L-valine	0.0285909	72	21.183283	72-18-4
Fatty acid metabolites and intermediates				
Glyceric acid	4.00×10^{-12}	73	27.939327	473-81-4

Palmitoleic acid	$8.50x10^{-12}$	311	44.014893	373-49-9
Linoleic acid	0.0019479	337	47.43296	60-33-3
Stearic acid	0.0023763	341	47.95428	57-11-4
Palmitic acid	0.0220514	313	44.391453	64519-82-0

Table 4.2: List of metabolites with their weighted loadings and absolute loadings for principle component 1 (PC1)

Metabolites	Component 1 (55.75%)	Absolute value
Uracil	0.24776196	0.24776196
Hypotaurine	0.24589851	0.24589851
L-carnitine	0.24571924	0.24571924
D-malic acid	0.23598695	0.23598695
Palmitoleic acid	0.23324004	0.23324004
Hypoxanthine	0.23120116	0.23120116
Fructose	-0.22991428	0.22991428
D-glucose-6-phosphate	-0.22124898	0.22124898
1,3-dihydroxyacetone	0.2190422	0.2190422
Stearic acid	0.21383685	0.21383685
Aspartic acid	0.20664789	0.20664789
D-ribose-5-phosphate	0.20287064	0.20287064
L-methionine	-0.18120633	0.18120633
L-valine	-0.16892305	0.16892305
Linoleic acid	0.1612704	0.1612704
Glyceric acid	-0.16115013	0.16115013
Palmitic acid	0.14535612	0.14535612
Succinic acid	-0.14027472	0.14027472
Citric acid	0.05239423	0.05239423
Pyruvic acid	-0.042259008	0.042259008
Ribonic acid-gamma-lactone	0.023172794	0.023172794
Maltose	-0.007306586	0.007306586

Metabolites (P value < 0.05) with a fold change of 2 on a logarithmic scale are presented

Table 4.3: Regulation of metabolites in LL compared to PM on day 0

Compound	Regulation in LL on day 0
1,3-dihydroxyacetone	down
D-malic acid	down
D-ribose-5-phosphate	down
L-carnitine	down
Aspartic acid	down
Citric acid	down
Hypotaurine	down
Linoleic acid	down
Palmitoleic acid	down
Stearic acid	down
Uracil	down
D-threitol	down
Hypoxanthine	down
L-methionine	up
Fructose	up
Glyceric acid	up
Pyruvic acid	up
D-glucose-6-phosphate	up
L-valine	up

Metabolites (P value < 0.05) with a fold change of 2 on a logarithmic scale are presented

Table 4.4: Regulation of metabolites in LL compared to PM on day 3

Compound	Regulation in LL on day 3
1,3-dihydroxyacetone	down
D-malic acid	down
D-ribose-5-phosphate	down
L-carnitine	down
Aspartic acid	down
Citric acid	down
Hypotaurine	down
Linoleic acid	down
Palmitoleic acid	down
Stearic acid	down
Uracil	down
Hypoxanthine	down
L-methionine	up
Fructose	up
Glyceric acid	up
Pyruvic acid	up
Succinic acid	up
D-glucose-6-phosphate	up

Metabolites (P value < 0.05) with a fold change of 2 on a logarithmic scale are presented

Table 4.5: Regulation of metabolites in LL compared to PM on day 7

Compound	Regulation in LL on day7
1,3-dihydroxyacetone	down
D-malic acid	down
D-ribose-5-phosphate	down
L-carnitine	down
Aspartic acid	down
Hypotaurine	down
Palmitoleic acid	down
Stearic acid	down
Uracil	down
Hypoxanthine	down
Ribonic acid-gamma-lactone	down
L-methionine	up
Citric acid	up
Fructose	up
Glyceric acid	up
Succinic acid	up
D-glucose-6-phosphate	up
L-valine	up

Metabolites (P value < 0.05) with a fold change of 2 on a logarithmic scale are presented

Table 4.6: List of some metabolites with their intensity values and *p* values after Benjamini Hochberg correction

Metabolites	Intensity values						
	LL0	PM0	LL3	PM3	LL7	PM7	P value
Nucleoside metabolites							
Hypoxanthine	142	9.49×10^5	4.02×10^3	1.06×10^6	1.77×10^3	7.57×10^6	0.09
Inosine	2.14×10^7	4.03×10^7	5.66×10^7	6.28×10^6	8.51×10^5	3.32×10^6	0.31
Uracil	2.63	9.06×10^5	3.24	4.43×10^4	10.6	1.08×10^6	9.14×10^{-12}
Xanthine	179	1.91×10^3	9.41×10^3	2.24×10^3	1.04×10^3	1.45×10^4	0.68
Myo-inositol	2.66×10^7	4.90×10^7	2.78×10^7	3.22×10^7	3.02×10^7	4.78×10^7	0.89
Inosine 5'-monophosphate	3.82	1*	16.3	1*	1*	1*	0.20
Amino acid metabolites							
Hypotaurine	1*	3.42×10^3	1*	2.13×10^2	1*	1.24×10^3	0.01
Aspartic acid	1*	7.78×10^2	1*	3.67	4.48	3.59×10^3	0.01
L-alanine	1.71×10^7	3.37×10^7	2.36×10^7	3.66×10^6	2.27×10^7	4.03×10^7	0.58
L-carnitine	3.81	2.85×10^3	3.56	7.96×10^2	4.19	1.35×10^4	0.03
L-cysteine	3.55	3.93	1.46×10^2	474	17.3	3.07×10^3	0.30
L-glutamic acid	1.20×10^4	1.98×10^6	3.06×10^5	2.70×10^4	1.01×10^6	2.55×10^4	0.34
L-methionine	12.7	1*	8.00×10^5	5.45×10^2	9.18×10^5	4.03	8.68×10^{-10}

L-ornithine 2	3.82	4.07	1*	1*	1*	1*	0.79
L-proline	$1.91x10^{6}$	$2.16x10^{6}$	$2.47x10^{6}$	$3.83x10^{5}$	$2.16x10^{6}$	$2.09x10^{6}$	0.75
L-valine	3.5	1*	729	60.1	19	1*	0.04
Carbohydrate metabolites							
Citric acid	56	$1.82x10^{2}$	21.1	$8.62x10^{5}$	$9.95x10^{5}$	$1.68x10^{4}$	$3.28x10^{-4}$
Creatinine	$2.63x10^{7}$	$1.28x10^{8}$	$4.31x10^{6}$	$8.32x10^{7}$	$1.83x10^{8}$	$1.52x10^{8}$	0.70
D-glucose	$6.73x10^{7}$	$5.32x10^{7}$	$7.85x10^{7}$	$6.65x10^{7}$	$8.27x10^{7}$	$2.06x10^{7}$	0.29
D-erythrose-4-phosphate	$9.98x10^{3}$	$3.29x10^{3}$	$7.93x10^{2}$	$8.32x10^{2}$	$2.98x10^{2}$	31.4	0.37
D-glucose-6-phosphate	$1.21x10^{7}$	$1.02x10^{3}$	$6.67x10^{7}$	$2.38x10^{6}$	$7.73x10^{6}$	$2.73x10^{3}$	0.002
D-malic acid	$1.91x10^{6}$	$1.73x10^{7}$	$1.32x10^{6}$	$7.36x10^{6}$	$8.94x10^{5}$	$1.11x10^{7}$	$7.68x10^{-6}$
D-ribose-5-phosphate	$1.08x10^{5}$	$2.22x10^{6}$	$7.64x10^{3}$	$2.54x10^{5}$	$9.99x10^{2}$	$1.96x10^{6}$	0.02
Fructose	$6.01x10^{7}$	$3.50x10^{7}$	$9.95x10^{7}$	$3.33x10^{7}$	$1.12x10^{8}$	$5.40x10^{7}$	$1.93x10^{-6}$
Fumaric acid	$2.26x10^{4}$	$3.36x10^{6}$	$1.49x10^{4}$	$9.19x10^{5}$	$7.10x10^{4}$	$1.96x10^{6}$	0.13
L-(+) lactic acid	$1.08x10^{5}$	$3.86x10^{6}$	$2.13x10^{8}$	$2.84x10^{7}$	$3.23x10^{7}$	$4.74x10^{6}$	0.40
Malonic acid	$2.15x10^{5}$	$7.05x10^{4}$	$4.93x10^{2}$	$1.32x10^{2}$	$3.32x10^{3}$	$6.93x10^{3}$	0.17
Maltose	$9.60x10^{5}$	$2.86x10^{6}$	$3.23x10^{6}$	$2.78x10^{6}$	$3.06x10^{6}$	$5.28x10^{6}$	0.08
Pyruvic acid	$1.42x10^{2}$	41.7	$1.45x10^{2}$	3.51	$4.48x10^{3}$	$2.93x10^{4}$	0.07

Succinic acid	$1.38x10^{6}$	$1.19x10^{6}$	$1.98x10^{6}$	$4.70x10^{4}$	$2.73x10^{6}$	$2.05x10^{6}$	0.02
Other metabolites							
Urea	$2.74x10^{7}$	$2.52x10^{7}$	$2.76x10^{7}$	$2.07x10^{7}$	$2.52x10^{7}$	$2.42x10^{7}$	0.10
Pyrophosphate	$7.24x10^{3}$	$3.74x10^{4}$	$1.65x10^{3}$	$2.51x10^{3}$	$3.42x10^{3}$	$3.72x10^{4}$	0.92

1* Indicates the intensity value is less than 10000 counts. The cutoff level for the signal intensity to be considered for analysis was 10000, below 10000 counts, the signal was considered as noise. LL indicates *longissimus lumborum* and PM indicates *psoas major*. LL0, PM0, LL3, PM3, LL7 and PM7 indicates the muscle type at their display d, 0, 3 and, 7 respectively.

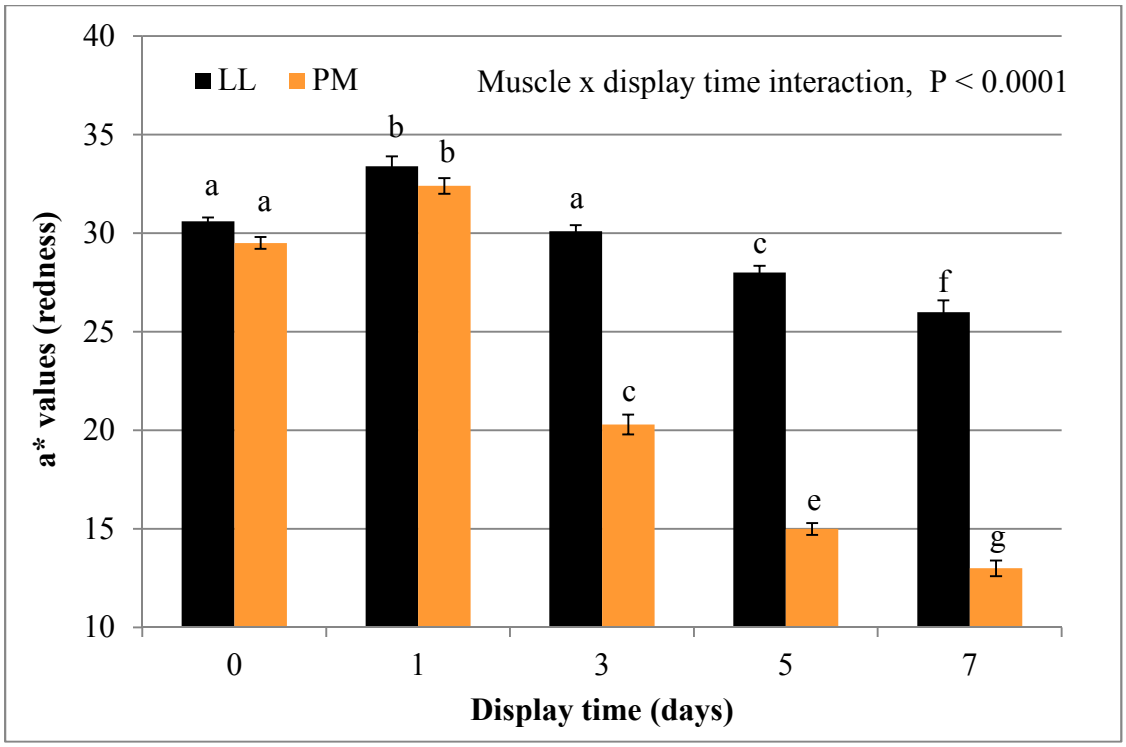

Figure 4.1: Figure 1: Least squares means for surface redness (a* values; muscle x display time interaction) of beef steaks packaged in PVC and displayed 7 d

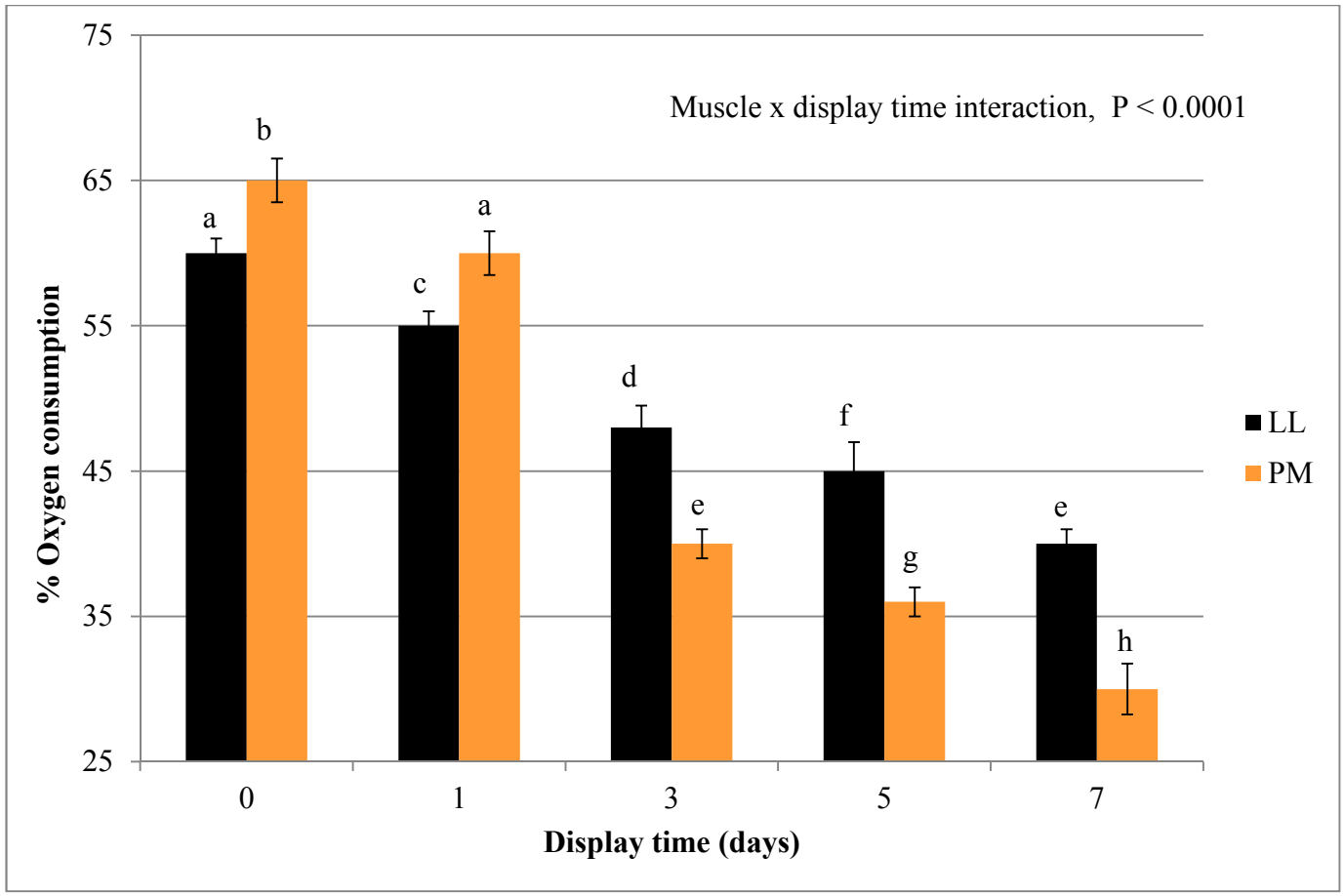

Figure 4.2: Least squares means for oxygen consumption (muscle x display time interaction) of beef steaks packaged in PVC and displayed 7 d

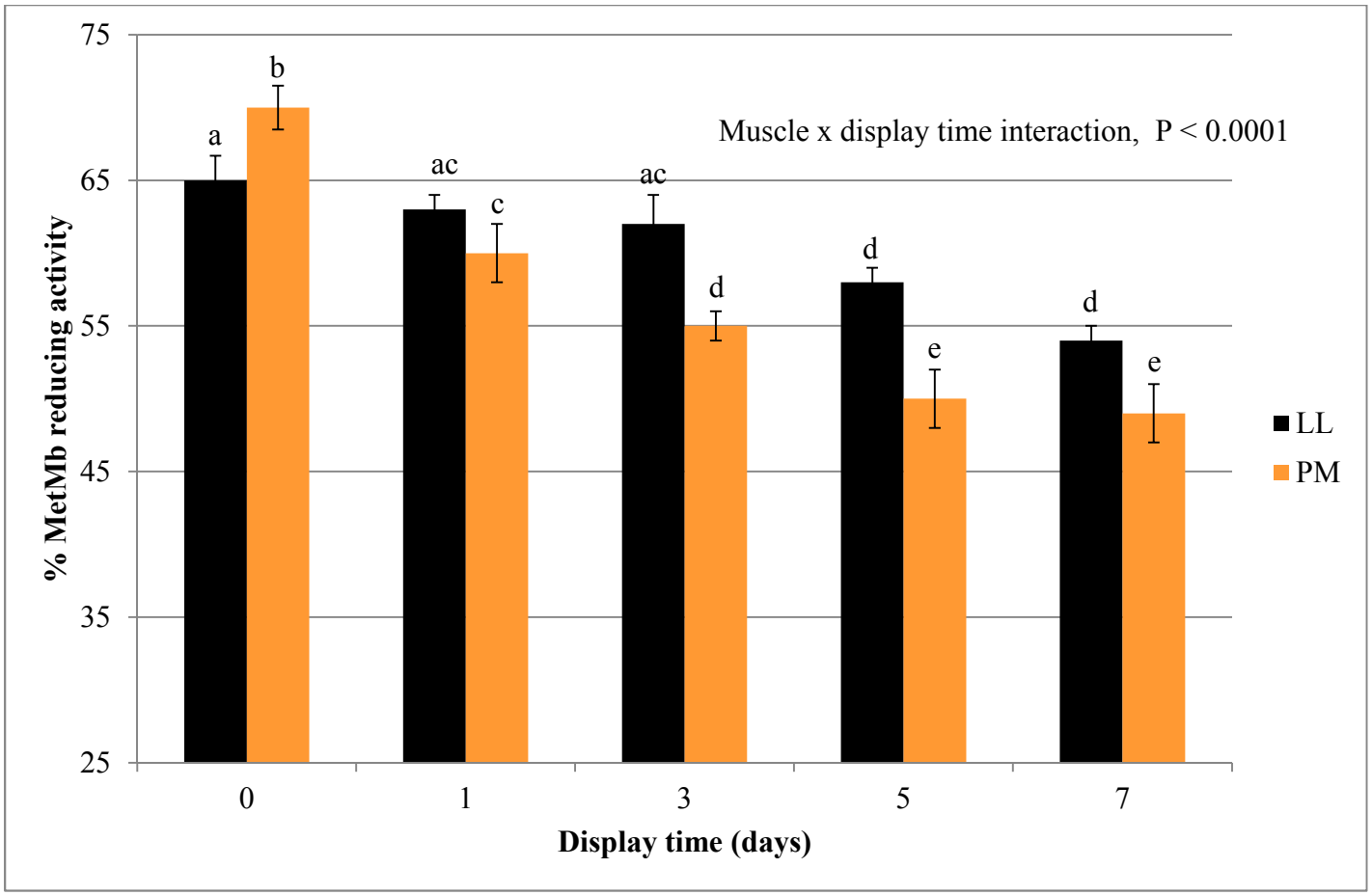

Figure 4.3: Least squares means for metmyoglobin reducing activity (muscle x display time interaction) of beef steaks packaged in PVC and displayed 7 d

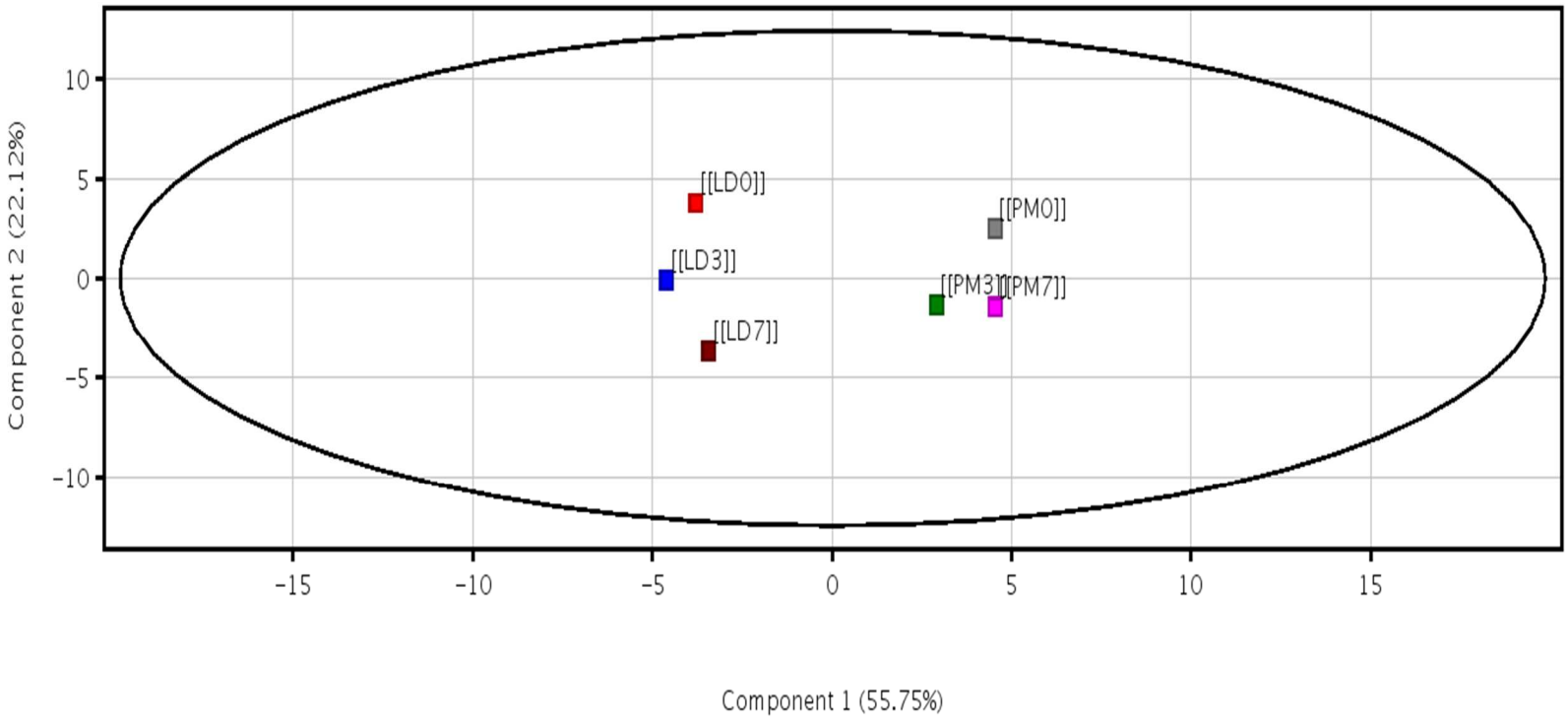

Figure 4.4: The PCA score plots with Hotelling's T2 ellipse (95% confidence interval) of metabolites in longissimus and psoas muscle during display time. X-axis represents PC1, which accounts for 55.75% variation, and PC2 is represented in the Y-axis, which accounts for 22.12% variation.

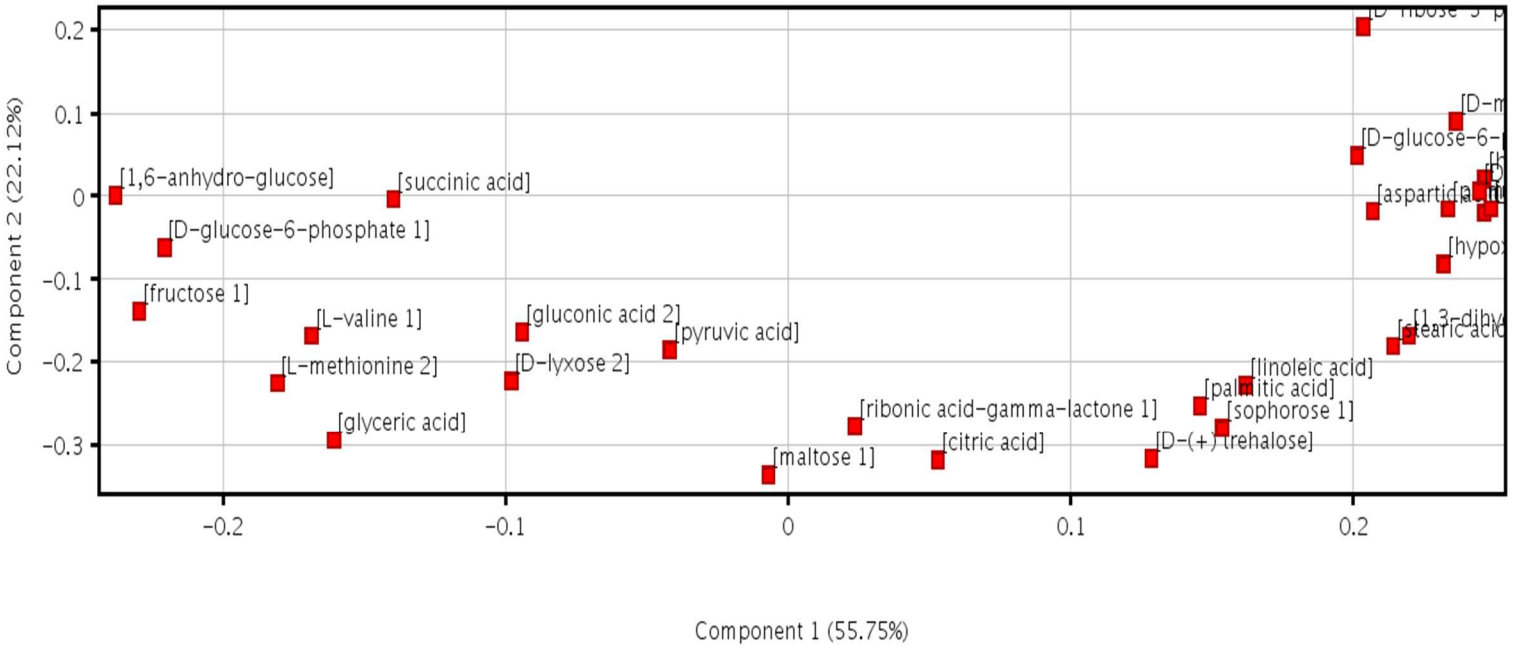

Figure 4.5: PCA loadings plot. X-axis is represented by PC1 (55.75% variation) and PC2 (22.12%) represents Y-axis.

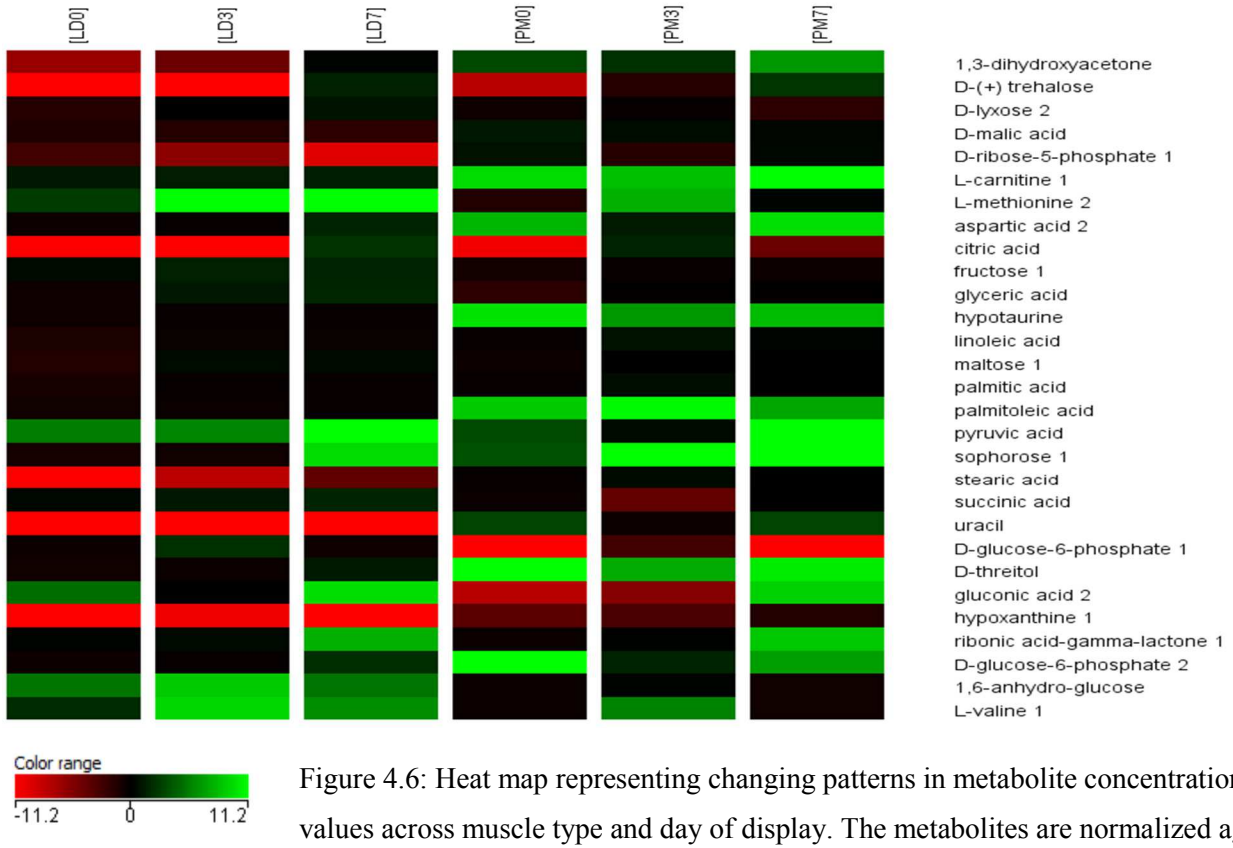

Figure 4.6: Heat map representing changing patterns in metabolite concentrations based on normalized intensity values across muscle type and day of display. The metabolites are normalized against the abundance of internal standard ribitol (2 ng per injected sample). The red color indicates lesser abundance and green color indicates a higher abundance. LD0 – *longissimus lumborum* on d 0, LD3 – *longissimus lumborum* on d 3, LD7 – *longissimus lumborum* on d 7, PM0 – *psoas major* on d 0, PM3 – *psoas major* on d 3, PM7 – *psoas major* on d 7

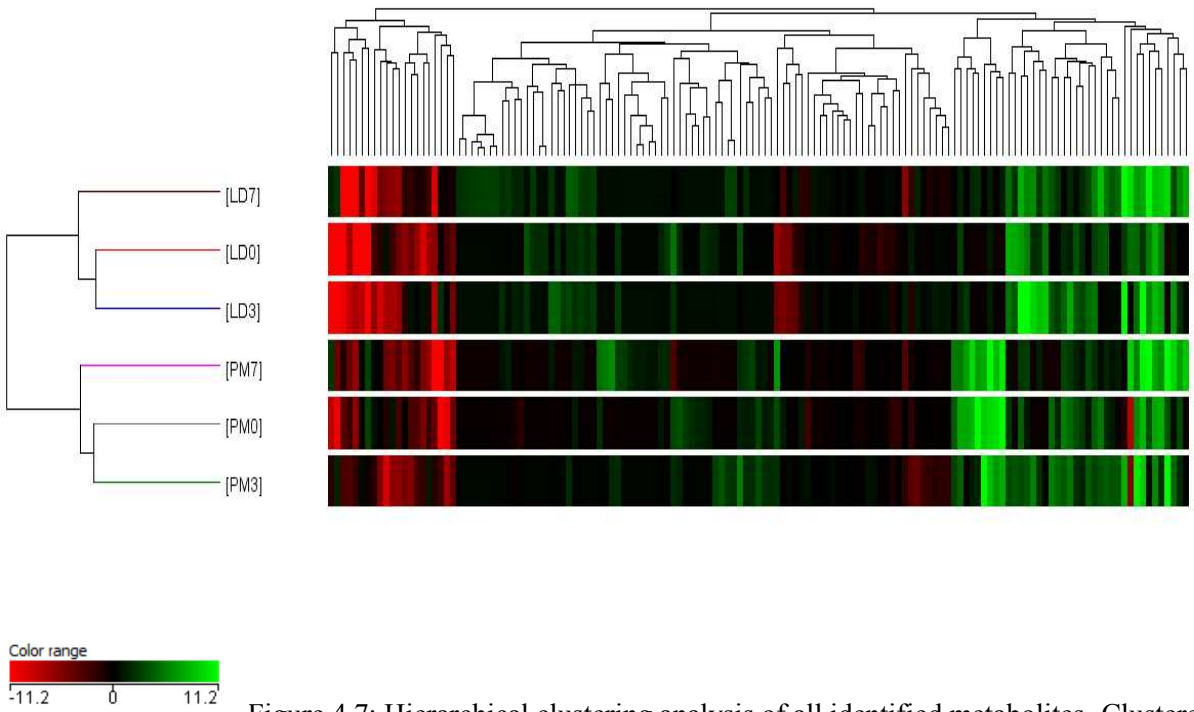

Figure 4.7: Hierarchical clustering analysis of all identified metabolites- Clustered on both metabolites and muscle type. Rows indicate muscle type and day of display, and columns indicate metabolites. Clustered based on normalized intensity values. The metabolites are normalized against the abundance of internal standard ribitol (2 ng per injected sample). The red color indicates lesser abundance and green color indicates a higher abundance. LD0 – *longissimus lumborum* on day 0, LD3 – *longissimus lumborum* on d 3, LD7 – *longissimus lumborum* on d 7, PM0 – *psoas major* on d 0, PM3 – *psoas major* on d 3, PM7 – *psoas major* on d 7

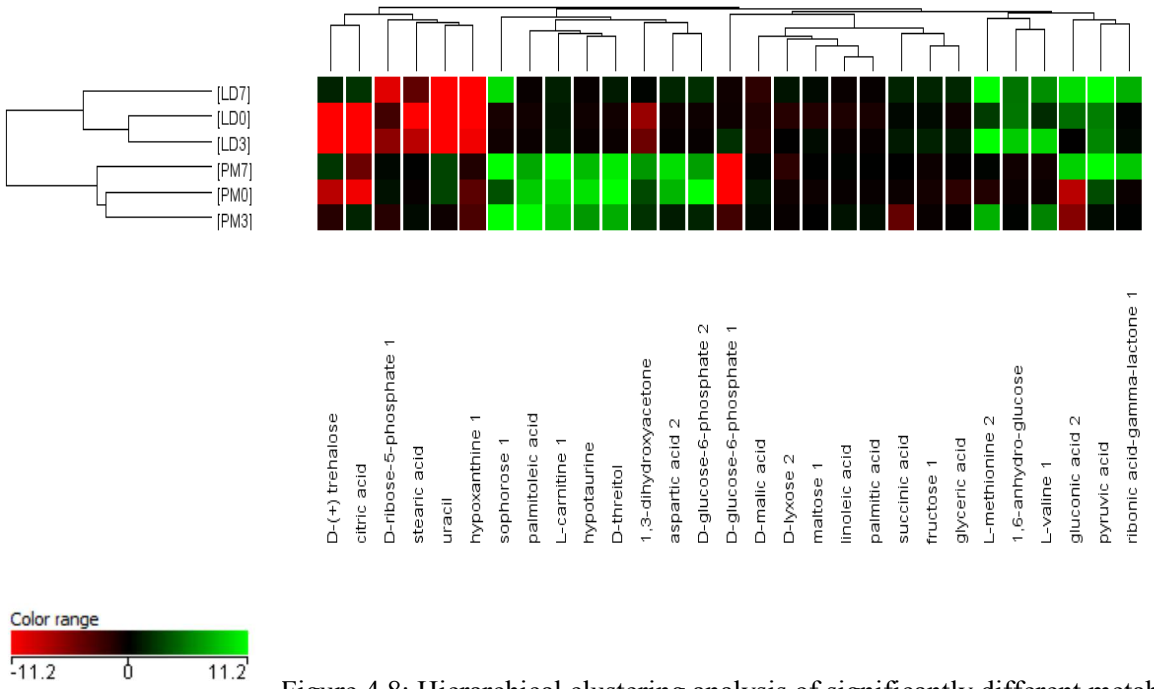

Figure 4.8: Hierarchical clustering analysis of significantly different metabolites ($P < 0.05$) - Clustered on both metabolites and muscle type. Rows indicate muscle type and day of display, and columns indicate metabolites. Clustered based on normalized intensity values. The metabolites are normalized against the abundance of internal standard ribitol (2 ng per injected sample). The red color indicates lesser abundance and green color indicates a higher abundance. LD0 – longissimus lumborum on day 0, LD3 – longissimus lumborum on d 3, LD7 – longissimus lumborum on d 7, PM0 – psoas major on d 0, PM3 – psoas major on d 3, PM7 – psoas major on d 7

CHAPTER V

CONCLUSION

Gas chromatography-mass spectrometry based metabolomic approach was utilized to elucidate the metabolite profile differences between LL and PM. Differences in color stability between muscles can be partly due to changes in metabolite profiles between muscles. Glycolytic metabolites, citric acid, and succinate were greater in LL than PM. An increase in citrate and succinate might be helping in replenishing the reducing equivalents required for metmyoglobin reducing activity. Carnitine was abundant in PM, suggestive of higher lipid oxidation which may lead to increased protein oxidation due to higher levels of reactive oxygen species. The differences in amino acids like valine, methionine, glutamic acid, suggest the utilization of amino acids are different between muscles post-mortem. Therefore, measures to increase antioxidant levels and reducing equivalents might be helpful in maintaining the color during retail display.

Heavier metabolites were not analyzed because of the inability of GC-MS technique to identify heavier molecules. Within the cell there are various pathways through which, metabolites are utilized and formed. So it will be better to include other techniques such as LC-MS so as to analyze metabolites such as ATP, ADP, NADH, etc. This will help to better understand the fate of the metabolites post-mortem.

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