THE EFFECTS OF LIPID PEROXIDATION PRODUCTS 4-HNE AND 4-ONE ON LACTATE DEHYDROGENASE ACTIVITY, MYOGLOBIN REDOX STABILITY, AND MITOCHONDRIAL STRUCTURAL INTEGRITY

by

SAMUEL ARMANDO GONZALEZ

(Under the Direction of ANAND MOHAN)

ABSTRACT

Meat quality is affected by many components, such as sarcoplasmic enzymes, mitochondria, and myoglobin. Rancidity of lipids and hemoprotein oxidation are interlinked. Both of these constituents' reactions are important in muscle as well as meat. In a living system, pathways exist to prevent harmful oxidation, detoxify the compounds resulting from lipid peroxidation, and reduce myoglobin to its functional redox state. In post mortem skeletal muscle, however, these systems rapidly deplete cofactors and substrates that are necessary to carry out enzymatic functions. As a result, membrane lipids begin to oxidize, leading to rancidity. Myoglobin, the primary pigment in meat, begins to oxidize to metmyoglobin, leading to meat quality loss. This research study seeks to determine how two lipid peroxidation products, 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal, accelerate quality loss. Results suggest that 4-ONE inhibits lactate dehydrogenase activity, myoglobin stability, and mitochondrial integrity.

INDEX WORDS: lipid peroxidation, meat biochemistry, myoglobin, lactate dehydrogenase

THE EFFECTS OF LIPID PEROXIDATION PRODUCTS 4-HNE AND 4-ONE ON LACTATE DEHYDROGENASE ACTIVITY, MYOGLOBIN REDOX STABILITY, AND MITOCHONDRIAL STRUCTURAL INTEGRITY.

by

SAM GONZALEZ

B.S., University of Georgia, 2013

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA,

2016

THE EFFECTS OF LIPID PEROXIDATION PRODUCTS 4-HNE AND 4-ONE ON LACTATE DEHYDROGENASE ACTIVITY, MYOGLOBIN STABILITY, AND MITOCHONDRIAL ULTRASTRUCTURE

by

SAM GONZALEZ

Major Professor:	Anand Mohan
Committee:	Rakesh Singh
	Ronald Pegg

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2016

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Meat is a muscle food consisting primarily of water, proteins, vitamins, minerals and lipids. The interactions between these components in postmortem beef skeletal muscle can lead to meat quality degradation. The color of meat is an important quality characteristic. Color is imparted by water soluble hemoprotein, myoglobin (A.L. Alderton 2003). The redox reactions of myoglobin are a determining factor of the color stability of meat. Color is the most significant trait that consumers evaluate before purchasing (D.J. Troy 2010). After animal slaughter, the pH of skeletal muscle tissue rapidly declines to around from pH 7.4 to 5.8. The biochemical events that occur after animal slaughter are summarized in figure 1.1. In beef, rigor mortis starts to set in 24 hours after slaughter. At this point, the muscle glycogen starts to be hydrolyzed to glucose. This produces pyruvate for lactate dehydrogenase to enzymatically convert to lactic acid leading to rapid decline in postmortem pH. The lack of circulatory blood flow and oxygen postmortem makes the LDH reaction predominate over aerobic metabolism (Yuan H. Kim 2006). Lower pH facilitates the activity of enzymes, primarily calpains, that degrade proteins, leading to the texture associated with aged meat (D.J. Troy 2010). This postmortem decline in pH also causes the oxidation of myoglobin to metmyoglobin (Andersen 2002). This is due to the protonation of the distal histidine that typically stabilizes coordinated molecular oxygen. This reaction produces reduced molecular oxygen, or the superoxide anion (T Gotoh 1976). This product is then able to degrade polyunsaturated fatty acids, leading to the formation of compounds such as 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal (4-ONE). Mechanisms for the generation of lipid

aldehydes from fatty acids are shown in figure 1.2. These unsaturated aldehydes have considerable reactivity towards proteins, lipids, and nucleic acids (Jonathan A. Doorn 2002).

Reactive aldehydes such as 4-HNE and 4-ONE are stable enough to diffuse from the site of generation (Luke I. Swezda 1992). This allows them to alter biochemical constituents of meat other than lipids, including myoglobin, enzymes, and mitochondria. Myoglobin, when covalently adducted with aldehydes, becomes prone to oxidation. Besides covalently modification of myoglobin, the products of lipid oxidation also react with enzymes present in meat. The glycolytic enzymes present in postmortem beef skeletal muscle continue to function, utilizing remaining substrate for their enzymatic functions. These functions include the regeneration of NADH during enzymatic functions of lactate dehydrogenase (LDH). Previous research (Y.H. Kim 2009) suggests that LDH enzymatic functions facilitates regeneration of postmortem pool of NADH that reduces metmyoglobin. Furthermore, functions of subcellular structures, such as mitochondria, are compromised by the products of lipid oxidation. This leads to decreased mitochondrial oxygen consumption and biochemical activity, as well as structural damage. When mitochondrial structure is compromised, it leads to release of redox active prosthetic groups, as well as polyunsaturated membrane phospholipids, known to be susceptible to peroxidation (Murphy 2009).

Evidence exists for the reciprocal, positive feedback nature of lipid and protein degradation in meat. While 4-HNE has been studied extensively in model food systems for 4-HNE induced damage to food quality, research documenting influence of 4-ONE is largely limited to toxicology in only living organisms. The research in chapter 3 presents a comparison of the effects of 4-HNE and 4-ONE in *in-vitro* meat systems. Lactate dehydrogenase activity was determined for the forward and backward reaction after incubation with 4-ONE and 4-HNE at

pH 5.6 and 7.4. Covalent modification of myoglobin by 4-ONE and 4-HNE at pH 5.6 and 7.4 was determined by mass spectrometry. Oxymyoglobin stability was assessed in the presence of 4-HNE and 4-ONE at pH 5.6 and 7.4. Mitochondrial membranes after incubation with 4-HNE and 4-ONE at pH 5.6 and 7.4 were visualized using electron microscopy.

References

A.L. Alderton, C. F., D.C. Liebler, D.W. Hill (2003). "Induction of Redox Instability of Bovine Myoglobin by Adduction with 4-Hydroxy-2-nonenal " Biochemistry **42**: 4398-4405.

D.J. Troy, J. P. K. (2010). "Consumer perception and the role of science in the meat industry." Meat Science **86**(1): 214-226.

Jonathan A. Doorn, D. R. P. (2002). "Covalent Modification of Amino Acid Nucleophiles by the Lipid Peroxidation Products 4-Hydroxy-2-nonenal and 4-Oxo-2-nonenal." Chem. Res. Toxicol. **15**: 1445-1450.

Luke I. Swezda, K. U., Lin Tsai, Earl R. Stadtman (1992). "Inactivation of Glucose-6-phosphate Dehydrogenase by 4-Hydroxy-2-nonenal." The Journal of Biological Chemistry **268**(5): 3342-3347.

Murphy, M. P. (2009). "How mitochondria produce reactive oxygen species." Biochemistry Journal **417**: 1-13.

R. Ramanathan, R. A. M., S.P. Suman, M.E. Cantino (2012). "Effects of 4-hydroxy-2-nonenal on beef heart mitochondrial ultrastructure, oxygen consumption, and metmyoglobin reduction." Meat Science **90**: 564-571.

T Gotoh, K. S. (1976). "Generation of the superoxide radical during autoxidation of myoglobin." Journal of Biochemistry **80**(2): 397-399.

Y.H. Kim, J. T. K., S.B. Smith, L.R. Berghman, J.W. Savell (2009). "Role of lactate dehydrogenase in metmyoglobin reduction and color stability of different bovine muscles." Meat Science **83**: 376-382.

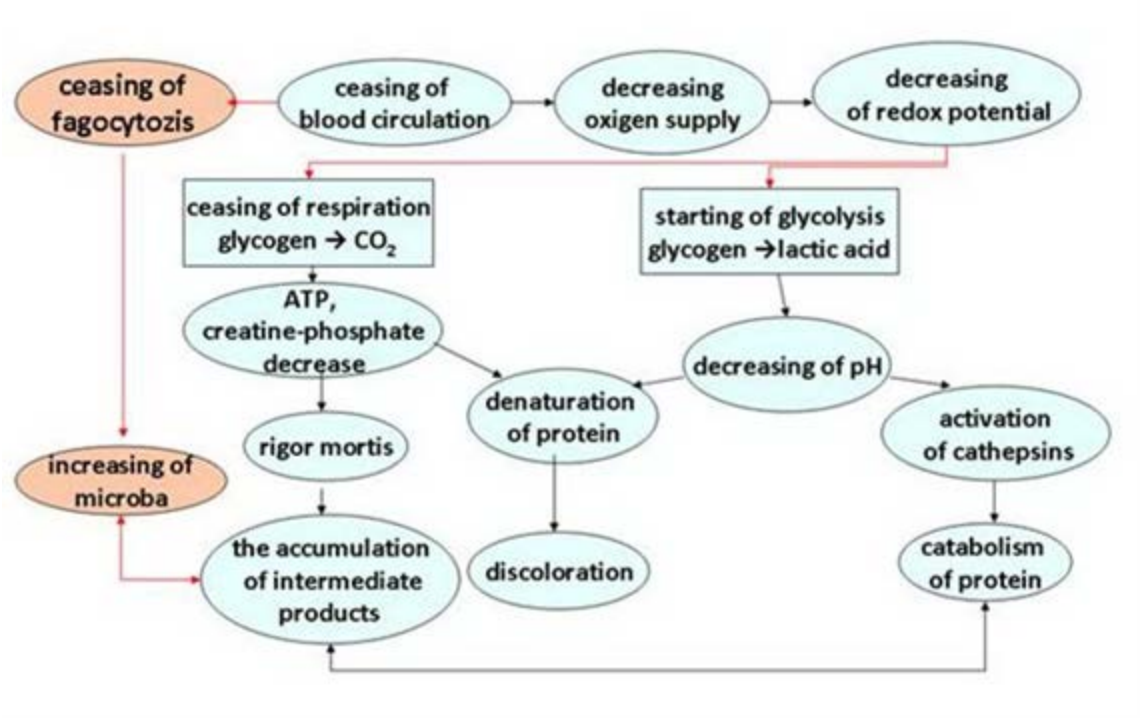

Figure 1.1 The biochemical events that occur postmortem during conversion of muscle into meat (Kincses 2013)

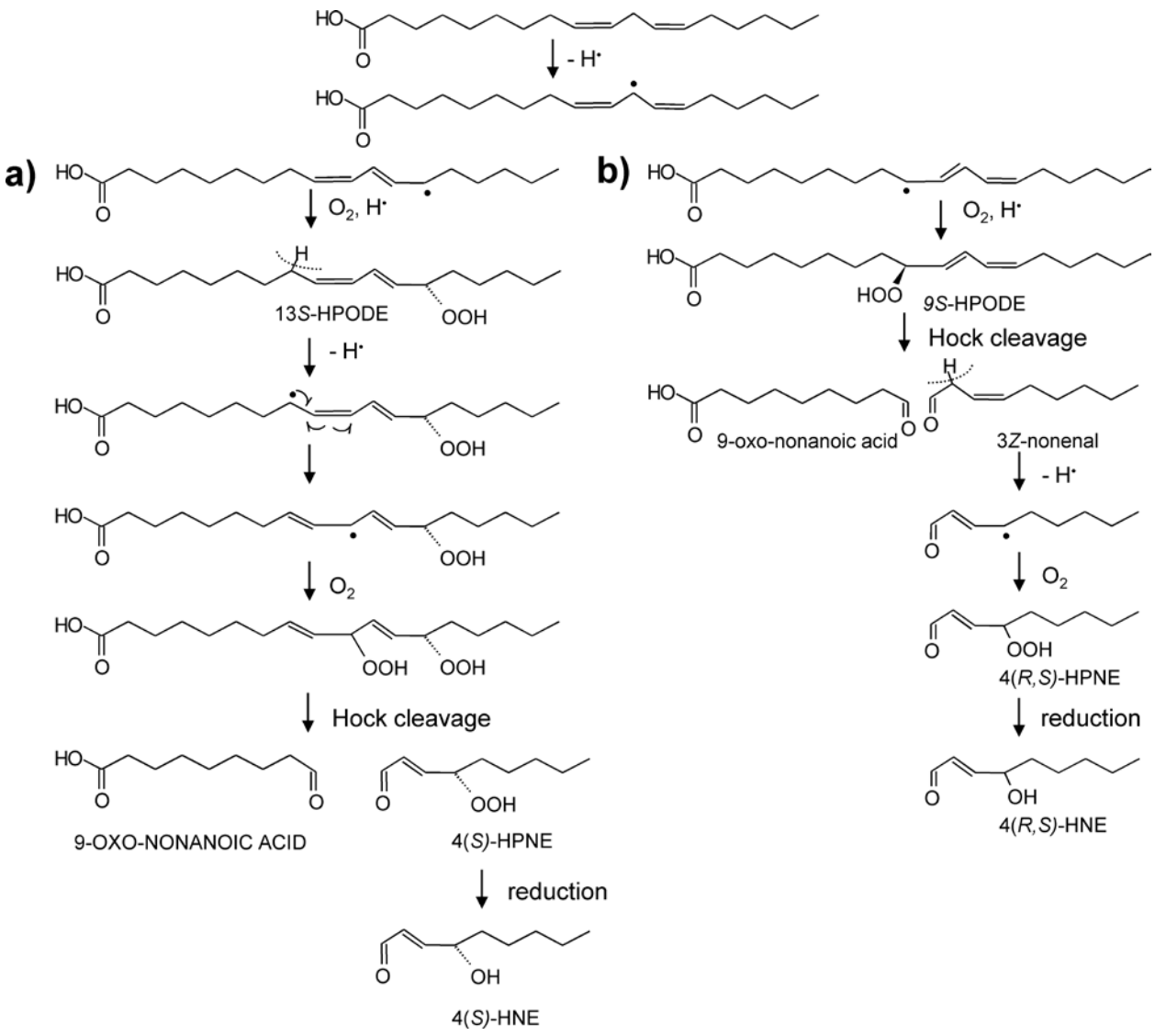

Figure 1.2 The nonenzymatic generation of 4-HNE from *n*-6 fatty acids (Schneider 2001).

CHAPTER 2

LITERATURE REVIEW

Myoglobin and Meat Quality

Meat quality refers to all the characteristics that arise from and affect production, processing, and consumption. Quality is determined by the genetics of the animal, the rearing techniques used, perimortem handling, storage, and processes the meat undergoes. Meat processors continuously demand more rapid, accurate, and affordable methods of assessing meat quality. This demand has driven research in the fields of food science and meat biochemistry for many decades. An overview of the recent work in this field, as it pertains to lipid peroxidation and protein denaturation, is presented in table 1. Much of this research in table 1 has focused on the chemistry of myoglobin. Of particular importance is the valence state of the iron ion contained within the heme cofactor. This iron ion may be in a 2+ or ferrous state, 3+ or ferric, or 4+ or ferryl state (Richards 2012). When iron within myoglobin is in the ferrous state, oxymyoglobin may be formed by cooridinating with molecular oxygen. Oxymyoglobin possesses the bright red cherry color associated with fresh meat . When the iron ion is oxidized to the ferric state, metmyoglobin is formed. This redox state of myoglobin is brown and problematic from a meat quality perspective. Mechanisms exist to reduce the ferric iron back to the ferrous state, allowing the molecule to bind oxygen. These myoglobin reduction mechanisms continue to function to the best of their ability after slaughter (Anand Mohan 2010). These states of Mb are shown in Figure 2.1.

Metmyoglobin reduction depends on reduced nicotinamide adenine dinucleotide (NADH). NADH can be generated after slaughter by various glycolytic enzymes. Some of these enzymes that have been studied include Lactate Dehydrogenase and Malate Dehydorgenase. Meat color has been frequently cited as the most important characteristic consumers can related to freshness when purchasing meat (M.N. Nair 2013). The bright red color associated with oxymyoglobin is preferred over the brown color of metmyoglobin, which is associated with spoilage. Metmyoglobin forms continuously in living tissue but is reversed by the presence of myoglobin-reducing enzyme systems. Post-mortem, these reducing systems begin to become depleted and a rapid fall in pH catalyzes the oxidation of the heme iron.(Andersen 2002) Figure 2 shows the mechanism by which low pH facilitates oxymyoglobin oxidation through protonation of the distal histidine and subsequent protonation of the bound molecular oxygen (Richards 2012).

The presence of oxidized myoglobin may suggest that lipids have also begun to oxidize, causing off-flavors and overall quality deterioration (Cameron Faustman 2010). Thus, stabilization of myoglobin and slowing down protein and lipid oxidation are important goals for meat processors. This has been achieved through refrigeration and freezing, as well as through the use of additives such as antioxidants, phosphates, and lactate. These compounds serve to quench reactive oxygen species, sequester metal ions, and alter pH and post-mortem NADH regeneration. All of these functions serve to stabilize myoglobin and enhance meat quality and shelf life, leading to savings for meat processors and retailers as well as consumers.

Color stability is not the only biochemical attribute considered important when assessing quality. Water holding capacity, lipid stability, and nutritional content have all been topics of study as well. Like myoglobin stability, water holding capacity seems to be influenced by the

post-mortem pH, both in terms of speed and magnitude of the drop (Elisabeth Huff-Lonergan 2005). Many recent studies have focused on how feed and supplements can affect the performance of livestock. Adeyemi in 2015 conducted a study to determine the effects of canola oil and palm oil on goats and their meat. Fatty acid profile, texture, water holding capacity, and antioxidant capacity of the meat were all determined. The study found that the oil supplement could reduce saturated fat content while increasing vitamin E concentration. The texture and water holding capacity were unaffected. Free sulfhydryl groups and carbonyl groups were also unaffected, suggesting that the increased unsaturated fats did not come along with an increase in protein and lipid oxidation. Research like this can enhance the animal husbandry and meat processing industry's ability to optimize their techniques while providing higher quality products.

The relation between Lipid Peroxidation and Meat Quality

The processes of lipid peroxidation and protein denaturation have been shown to be linked (Cameron Faustman 2010). Compounds derived from oxidized fatty acids have the ability to covalently alter enzymes and other proteins. Typically, this will result in a loss of the structure that gives the polypeptide its function. This also can lead to the release of prosthetic groups such as metal ions. An important example of this is the heme iron within myoglobin. These released components may then catalyze more peroxidation reactions. This causes further damage to various cellular targets. This degradative interaction between lipids and proteins is an important source of quality loss in muscle foods. These effects are second in importance only to microbial spoilage (G.H. Zhou 2010). Of particular importance is the relationship between myoglobin and oxidation compounds derived from polyunsaturated fatty acids (PUFA) such as linoleic acid. Linoleic acid is a common fatty acid in the membranes of mitochondria, the site of reactive

oxygen species (ROS) generation (Murphy 2009). The prevalence of PUFA in mitochondrial membranes, coupled with the fact that oxygen is three times more soluble in membranes than in cytoplasm,(Sruti Shiva 2001) provide the conditions necessary for the production of chemicals like 4-HNE and 4-ONE. Discovered in the early 1990's, entire volumes of research journals have been devoted to 4-HNE biochemistry and molecular biology, with research on 4-ONE being less common. Both these compounds can initiate harmful cascades upon reacting with cellular components. In living systems, these cascades can contribute to the development of chronic disease states such as diabetes and atherosclerosis (Jonathan A. Doorn 2002). In meat, however, these compounds can potentially contribute to processes that hasten oxidative rancidity and color loss.

The generation of ROS is a complex and variable process depending on numerous factors such as oxygen availability, ADP levels, the NAD/NADH ratio, and mitochondrial integrity.[8] These variables make it difficult for in-vitro data to be extrapolated to living systems, or even post-mortem meat systems, but it is generally understood that some degree of superoxide anion, O_2^- will be generated by intact mitochondria in most conditions. Superoxide is formed by the single-electron reduction of molecular oxygen, O_2. This reduction occurs at "redox-active prosthetic groups within [mitochondrial] proteins". This anion can then readily react with the allylic hydrogens bound to the interrupting methylene bridge found in PUFA. This reaction forms highly unstable lipid hydroperoxides, which may then undergo cyclization and scission reactions to eventually produce either 9 or 6 carbon unsaturated aldehydes, depending on the starting fatty acid.(Porter 1990) These aldehydes are longer lived than the lipid hydroperoxides, allowing them to travel far from the sight of generation and causes widespread damage to

cells.(Uchida 2003) The lower level of reactivity allows these compounds to be more deleterious than the less stable, more reactive parent compounds.

Sacroplasmic Enzymes or Mitochondria

Living tissue possesses the ability to reduce metmyoglobin through metmyoglobin reducing activity (MRA). Meat also possesses MRA but to a lesser degree. Metmyoglobin reduction requires the presence of reducing power. This is in the form of NADH. Reduced myoglobin and NAD+ are the products of this reaction. This can occur with or without catalysis by an enzyme. Many enzymes have been studied for their role in post-mortem NADH formation in meat, such as malate dehydrogenase (Anand Mohan 2010) and lactate dehydrogenase (Ranjith Ramanthan 2014). These enzymes consume what substrate and cofactors remain to drive metmyoglobin reduction. Thus, studying the fate of these enzymes in meat conditions has become an important topic of inquiry in meat science and food chemistry. It has been noted that the addition of various metabolic substrates, such as succinate, lactate, and malate, tend to increase stability of myoglobin in meat. Initially, it was unclear if this effect was due simply to a buffering of pH or if the actual consumption of these compounds by enzymes in the meat was responsible for the stabilizing effects. In the work of Ramanathan et al in 2013, antimycin A was used to reduce oxygen consumption by mitochondria by inhibiting Complex III of the electron transport chain. This resulted in less metmyoglobin, presumably due to less NADH being consumed by mitochondria, rendering the NADH available for metmyoglobin reduction (R. Ramanathan 2013). This reveals the complex interaction between sarcoplasmic enzymes, mitochondria, oxygen, and myoglobin. Oxygen consumption by active mitochondria after slaughter is an important characteristic of dark-cutting beef, in which the meat fails to achieve the desired bright red color (C.R. Ashmore 1971). This quality defect is caused by stressful pre-

slaughter conditions, as well as well-exercised animals. Both of these conditions serve to increase mitochondrial density, as well as the content of certain glycolytic enzymes. While glycolytic enzymes can be associated with this quality defect, others have reported that enhancing otherwise normal meat with lactate can result in greater color stability and metmyoglobin reducing activity (Yuan H. Kim 2006). At ultimate postmortem pH, deoxymyoglobin produced by metmyoglobin reduction can be rapidly reoxygenated to the desired oxymyoglobin state. Improvements on color stability by lactate enhancement has been pointed out by Kim et al to not be solely due to pH effects, as a control treatment with no lactate but with pH buffering ability displayed very poor color stability (Yuan H. Kim 2006).

Some research suggest that glycolytic enzymes in the sarcoplasm, and not mitochondrial activity, is the primary determinant of post-mortem MRA. Lighter colored muscles, with fewer mitochondria, tend to display greater color stability. Addition of glycolysis inhibitors has been shown to completely stop MRA, while inhibiting the citric acid cycle has no effect on myoglobin stability (Yuan H. Kim 2006). These observations are contrary, however, to studies that have pointed to mitochondrial subfractions as having the greatest ability to reduce metmyoglobin, showing the complexity of myoglobin biochemistry (Catherine Echevarne 1989). Conflicting research conclusions show the importance of details such as the conditions of slaughter and storage, additives used, and husbandry conditions of the animals. Stress before slaughter will induce great amounts of glycolysis and mitochondrial activity, depleting stored muscle glycogen. After death, however, glycolysis produces the pH drop that facilitates the development of the desired bright red color. High ultimate pH is also linked to poor flavor and more rapid microbial spoilage. The pH meat attains is also important for determining activity of proteolytic enzymes that determine meat texture, with a higher pH inhibiting the activity of

cathepsin, leading to a less tender product (J.A. Silva 1999). A higher, pH, however, facilitates greater metmyoglobin reductase activity as well as greater water holding capacity (Catherine Echevarne 1989). Like with glycolysis in meat, pH must be within a certain range to maximize quality. Oxygen should also be carefully moderated in meat processing, storage, and display. Too little oxygen will inhibit the formation of oxymyoglobin, while too much may increase oxidation of lipids. A large concentration of oxygen may also increase mitochondrial NADH consumption through the electron transport chain, rendering that NADH no longer available for metmyoglobin reduction (J.Y Jeong 2009).

While it is clear that malate and lactate addition can increase MRA by acting as enzymatic substrates, less clear is the function of succinate addition. Succinate is a 4 carbon dicarboxylic acid that plays a role in the electron transport chain, where it is converted to fumarate in order to reduce ubiquinone to ubiquinol. While it indeed produces reducing equivalents in the form of QH2, this is not a cofactor used by myoglobin reducing enzymes. Succinate will, however, strongly buffer pH and it seems that this effect does have a color stabilizing effect (R. Ramanathan 2013). This is interesting in light of results (Kim 2013) suggesting that pH buffering is an unimportant facet of lactate's functionality in meat quality stabilization. It remains to be discovered what precisely is the difference between succinate and lactate addition. Perhaps succinate addition is allowing the formation of ubiquinol, a well-known lipid soluble antioxidant. This could lead to color stability improvements by inhibiting the oxidation of lipids, which facilitates myoglobin oxidation as mentioned above. A small amount of research actually shows direct antioxidant effects of ubiquinol on myoglobin, concluding that CoQ10 can reduce activated ferrylmyoglobin back to oxymyoglobin and metmyoglobin (A.

Mordente 1993). Whether or not succinate-driven ubiquinone reduction can occur in meat has yet to be investigated but is an intriguing possibility.

Future Research

This review has sought to synthesize many different areas of research into a cohesive direction. The individual components of meat systems have been fairly well studied over the past century. Now, researchers are beginning to study how components such as enzymes and lipids behave not in isolation, but rather in association with one another. This multifaceted approach brings up more questions, such as what conditions will optimize post-mortem reduction and determining exactly where that activity is coming from. The expanded usage of additives in raw meat has been suggested by some current researchers, such as those adding lactate and succinate to steaks. It is unclear if this approach will find widespread usage in places like grocery stores, where consumers expect raw meat to be a single ingredient item. Still, work like what is described above contributes to the understanding of meat quality, even if a commercial application does not directly result.

References

A. Mordente, G. E. M., S. A. Santini, G. A. D. Miggiano, T. Petitti, B. Giardina, M. Battino, G. P. Littarru (1993). "Antioxidant effect of coenzyme Q on hydrogen peroxide-activated myoglobin." Journal of Molecular Medicine **71**(8): 92.

Anand Mohan, S. M., Melvin C. Hunt, Thomas J. Barstow, Terry A. Houser (2010). "Kinetics of Myoglobin Redox Form Stabilization by Malate Dehydrogenase." Journal of Agricultural and Food Chemistry **58**: 6994-7000.

Andersen, C. P. B. a. H. J. (2002). "Myoglobin-Induced Lipid Oxidation. A Review." Journal of Agricultural and Food Chemistry **50**: 3887-3897.

C.R. Ashmore, L. D., G. Foster, and F. Carroll (1971). "Respiration of Mitochondria Isolated from Dark-Cutting Beef." Journal of Animal Science **33**(3): 574-577.

Cameron Faustman, Q. S., Richard Mancini, Surendranath P. Suman (2010). "Myoglobin and lipid oxidation interactions: Mechanistic bases and control." Meat Science **86**: 86-94.

Catherine Echevarne, M. R., R. Labas (1989). "Metmyoglobin Reductase Activity in Bovine Muscles." Meat Science **27**: 161-172.

Elisabeth Huff-Lonergan, S. M. L. (2005). "Mechanisms of water-holding capactiy of meat: The role of postmortem biochemical and structural changes." Meat Science **71**: 194-204.

G.H. Zhou, X. L. X., Y. Liu (2010). "Preservation technologies for fresh meat - A review." Meat Science **86**: 119-128.

J.A. Silva, L. P., C. Martins (1999). "Influence of ultimate pH on bovine meat tenderness during ageing." Meat Science **52**: 453-459.

J.Y Jeong, S. J. H., H.S. Yang, S.H. Moon, Y.H. Hwang, G.B Park, ST. Joo (2009). "Discoloration Characteristics of 3 Majoy Muscles from Cattle During Cold Storage." Food Chemistry **74**(1).

Jonathan A. Doorn, D. R. P. (2002). "Covalent Modification of Amino Acid Nucleophiles by the Lipid Peroxidation Products 4-Hydroxy-2-nonenal and 4-Oxo-2-nonenal." Chem. Res. Toxicol. **15**: 1445-1450.

M.N. Nair, S. P. S., S. Li, P. Joseph, C.M. Beach (2013). "Lipid oxidation-induced oxidation in emu and ostrich myoglobins." Meat Science **96**: 984-993.

Murphy, M. P. (2009). "How mitochondria produce reactive oxygen species." Biochemistry Journal **417**: 1-13.

Porter, W. A. P. a. N. A. (1990). "Suggested Mechanisms for the Production of 4-hydroxy-2-nonenal from the Autoxidation of Polyunsaturated Fatty Acids." Free Radical Biology and Medicine **8**: 541-543.

R. Ramanathan, R. A. M., G.A. Dady, C.B. Van Buiten (2013). "Effects of succinate and pH on cooked beef color." Meat Science **93**: 888-892.

R. Ramanathan, R. A. M., P. Joseph, S.P. Suman (2013). "Bovine mitochondrial oxygen consumption effects on oxymoblin in presence of lactate as a substrate for respiration." Meat Science **93**: 893-897.

Ranjith Ramanthan, R. A. M., Surendranth P. Suman, Carol M. Beach (2014). "Covalent Binding of 4-Hydroxy-2-nonenal to Lactate Dehydrogenase Decreases NADH Formation and Metmyoglobin Reducing Activity." Journal of Agricultural and Food Chemistry **62**: 2112-2117.

Richards, M. P. (2012). "Redox Reactions of Myoglobin " Antioxidants and Redox Signalling **00**.

Sruti Shiva, P. S. B., Rakesh P. Patel, Peter G. Anderson, Victor M. Darley-Usmar (2001). "Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase." Proceedings of the National Academy of Sciences of the USA.

Uchida, K. (2003). "4-Hydroxy-2-nonenal: a product and mediator of oxidative stress." Progress in Lipid Research **42**: 318-343.

Yuan H. Kim, M. C. H., Richard A. Mancini, Mark Seyfert, Tom M. Loughin, Donald H. Kropf, J. Scott Smith (2006). "Mechanism for Lactate-Color Stabilization in Injection-Enhanced Beef." Journal of Agricultural and Food Chemistry **54**: 7856-7862.

Author and year	Findings
Ramanathan et al. (2014)	4-HNE can modify lactate dehydrogenase, inhibiting its activity and reducing its ability to form NADH.
Ramanathan et al. (2013)	Lactate acts as a substrate for respiration and increases metmyoglobin reducing activity as well as oxygen consumption by mitochondria.
Ramanathan et al. (2013)	Succinate addition to meat can increase color stability through increased pH and perhaps through utilization by enzymes.
Nair et al. (2013)	Species origin of myoglobin effects its reactivity towards electrophiles such as 4-HNE, leading to metmyoglobin formation.
Tatiyaborworntham et al. (2012)	Sperm whale myoglobin is destabilized by covalent reactions with 4-HNE, with concurrent release of the hemin prosthetic group.
Mohan et al. (2010)	Metabolic substrates such as malate can replenish NADH pools post-mortem through the action of enzymes, contributing to myoglobin stability.
Faustman et al. (2010)	There is significant evidence that lipid peroxidation and protein denaturation are linked, and cause quality deterioration in meat.
Jeong et al. (2009)	Mitochondria concentration in beef muscles is associated with decreased color stability and increased lipid peroxidation.
Yuan et al. (2006)	Lactate addition can increase color stability and metmyoglobin reducing activity in a mechanism independent of pH buffering.
Doorn et al. (2006)	4-ONE is also a potent modifier of biological chemicals, more toxic than the more well studied 4-HNE in many respects.
Suman et al. (2006)	Pork and beef myoglobins are both destabilized by 4-HNE even at refrigeration temperatures, particularly at meat pH (5.6). Bovine Mb is shown to be more effected.
Doorn et al. (2006)	Human mitochondrial enzymes, such as aldehyde dehydrogenase, are inhibited by both 4-ONE and 4-HNE. 4-ONE inhibits the detoxification of 4-HNE.

Table **2.1** Findings of recent work regarding lipid peroxidation, proteins, and quality in meat

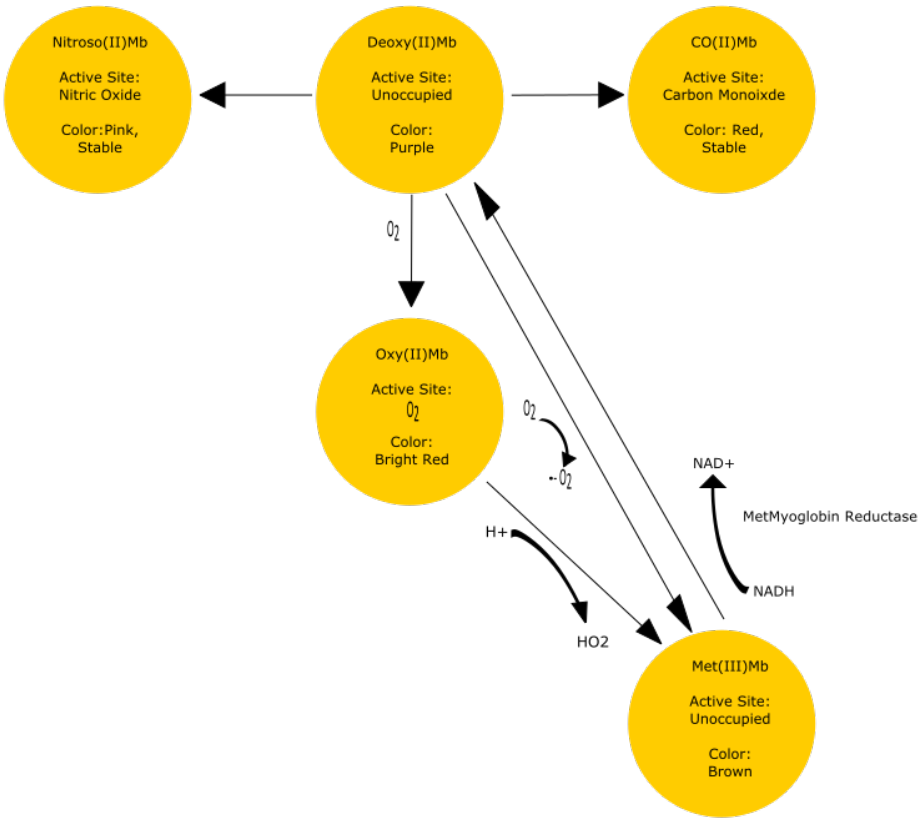

Figure **2.1** Various ligands, redox states, and reactions involving Mb

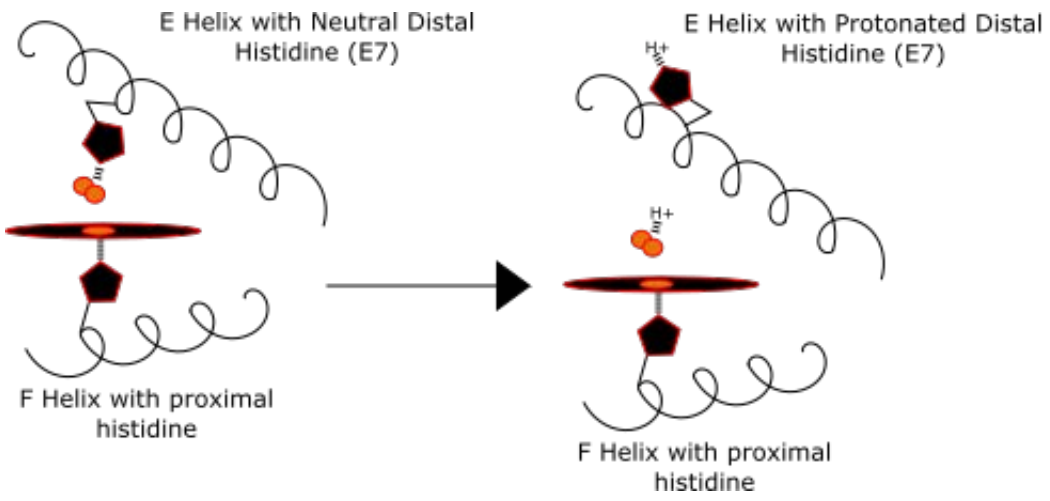

Figure **2.2** Molecular oxygen is stabilized by the distal histidine, making the oxidation of the iron ion unfavorable. Upon protonation, the distal histidine rotates out into the solvent, leaving the oxygen susceptible to be protonated. Once protonated, abstraction of an electron from iron becomes favorable, leading to metmyoglobin formation.

CHAPTER 3

THE EFFECTS OF LIPID PEROXIDATION PRODUCTS 4-HNE AND 4-ONE ON LACTATE DEHYDROGENASE ACTIVITY, MYOGLOBIN REDOX STABILITY, AND MITOCHONDRIA STRUCTURAL INTEGRITY[1]

[1] Gonzalez, S.A and Mohan, A.. To be submitted to the *Journal of Agricultural and Food Chemistry*.

Abstract

The products of lipid peroxidation, such as alpha-beta-unsaturated aldehydes, are reactive substance towards the proteins that influence meat quality. Two compounds, 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal, are prevalent end products of lipid peroxidation. These two compounds are far more stable than the lipid hydroperoxides that they form from, allowing these chemicals to diffuse from the site of generation and causing extensive cellular damage and oxidative pathology. Glycolytic enzymes involved in metmyoglobin reduction such as lactate dehydrogenase, has been demonstrated to be highly reactive with 4-HNE and become less functional. Other aldehydes, such as 4-ONE, has been shown to be more reactive than 4-HNE towards certain functional groups and moieties, such as sulfhydryl groups and arginine residues of glycolytic enzymes. In spite of this, little research has documented the influence of 4-ONE on an *in-vitro* model meat system. Findings from this research shows that 4-ONE reactivity causes significant ($P<0.05$) loss of enzymatic activity of lactate dehydrogenase activity as compared to 4-HNE at similar effective concentration. Myoglobin, when incubated with 4-ONE, exhibited a greater ($P<0.05$) loss of redox stability at pH 5.6 as compared to incubation with 4-HNE. Both 4-HNE and 4-ONE were found to be potent covalent modifiers of myoglobin, particularly at pH 7.4, as well as disruptors of functional mitochondrial morphology.

Introduction

Previous research suggests that the processes of lipid peroxidation and protein denaturation are interlinked (Cameron Faustman 2010). Evidence exists supporting the observation that addition of fat-soluble antioxidants to lipid microsomes has a stabilizing effect on water soluble proteins (C. Faustman 1999). This has an important implication on meat quality the mechanisms affecting meat quality degradation. Of particular importance to both proteins

and lipids is the redox status of the iron ion contained within the heme pocket of myoglobin, the primary pigment in muscle and meat. Meat color has been cited as the most important quality characteristic consumers evaluate when purchasing meat at retail (M.N. Nair 2013). Oxidized myoglobin, in addition to its undesirable color, has been shown to trigger lipid oxidation in lipid membranes (Andersen 2002), causing rancidity development and the generation of toxic byproducts (J. Kanner 1985) Current research in meat biochemistry has sought to understand factors effecting myoglobin, lipids, and their reactions. Of particular interest is understanding how myoglobin is oxidized to metmyoglobin, and how biochemical systems in meat function post-mortem to reduce metmyoglobin to myoglobin.

4-Hydroxy-2-nonenal (4-HNE) is an alpha beta unsaturated aldehyde that results from lipid peroxidation in foods (Martin Globisch 2015). Oxidative breakdown of omega-6 polyunsaturated fatty acids form compounds such as 4-HNE when hydrogen is abstracted from interrupting methylene groups by reactive oxygen species. The oxidative byproduct 4-HNE has been characterized as a biomarker and cause of oxidative pathogenesis in living organisms (Uchida 2003), and has been studied extensively in food systems for meat quality deterioration (Ranjith Ramanthan 2014). 4-HNE has been shown to facilitate redox instability of myoglobin, accelerating meat discoloration, and covalent modification of the protein, particularly at histidine residues (A.L. Alderton 2003). This covalent modification has been demonstrated to accelerate myoglobin oxidation and release of iron ions from the pigment, facilitating more deleterious oxidative reactions (Anuj Purohit 2014). 4-HNE is also known as a potent modifier and inhibitor of enzymes (Luke I. Swezda 1992). Some of these enzymes play a pivotal role in myoglobin redox stabilization. In postmortem conditions, enzymes and biochemical pathways continue to function depending on substrate availability. This includes the pathways involving reduction of

oxidized myoglobin (Anand Mohan 2010). Enzymes utilizing NADH as substrate have been shown to covalently modify and exhibit reduced activity when incubated with 4-HNE (Ranjith Ramanthan 2014). NADH, along with metmyoglobin reductase is necessary for metmyoglobin reduction as it provides the electron necessary to reduce ferric iron to ferrous (A.E.D Bekhit 2005). Lactate dehydrogenase has recently been reported to contribute to metmyoglobin reducing activity and for its reactivity with 4-HNE (Ranjith Ramanthan 2014). While 4-HNE has served as an important model for oxidative pathology and quality deterioration in biological systems, it is not the only product that oxidized PUFA are capable of forming, and may not be the most reactive or cytotoxic compound.

Little is known about the influence of 4-oxo-2-nonenal (4-ONE) on myoglobin redox stability, mitochondrial functions, and overall meat quality. 4-ONE has a ketone functional group at carbon 4 where 4-HNE possesses a hydroxyl group. Other than this difference, both compounds (4-HNE and 4-ONE) are similar in their structural configuration, with an aldehyde at carbon 1, a double bond between carbons 2 and 3, and an oxygen based functional group at carbon 4. The double bond between two electron withdrawing groups gives both compounds significant reactivity towards proteins, lipids, and nucleic acids. Research in human medicine has shown 4-ONE to be “both more cytotoxic and more protein reactive than 4-HNE” (De Lin 2005). While structurally similar, 4-ONE is more reactive towards thiols and amine groups than 4-HNE (Jonathan A. Doorn 2006). Both of these functional groups impart form and function to both structural and catalytic proteins.

Given the lack of data on 4-ONE in meat quality research, the objectives of this study were 1) to characterize 4-ONE’s effects on lactate dehydrogenase activity, myoglobin, and mitochondrial ultrastructure and 2) to compare these effects to the more well-known 4-HNE. The

lactate dehydrogenase reaction is reversible, thus its ability to both consume and regenerate NADH was studied. Myoglobin was studied for both its covalent reactions with 4-ONE and 4-HNE and its heme cofactor's redox stability when incubated with 4-ONE and 4-HNE. Finally, mitochondria were studied for morphological changes incurred when incubated with these lipid aldehydes. It was hypothesized that 4-ONE and 4-HNE both decrease LDH activity, covalently alter and destabilize myoglobin, and cause mitochondrial membrane degradation.

Materials and Methods

Raw materials and chemicals

Bovine heart lactate dehydrogenase (Type III), porcine glutamate-pyruvate transaminase, sodium lactate, sodium pyruvate, sodium glutamate, sodium phosphate, formic acid, acetonitrile, 10kDa Slide-a-lyzer dialysis cups, Nagarase Bacterial Protease Type XXIV, HEPES, and equine heart myoglobin were purchased from the Sigma Aldrich Chemical Co. (St. Louis, MO, USA). PD-10 Columns were obtained from GE Healthcare (Piscataway, NJ, USA). 4-Hydroxy-2-nonenal, 4-oxo-2-nonenal, NADH disodium salt, and NAD^+ free acid was obtained from Cayman Chemical Co (Ann Arbor, MI, USA). All chemicals obtained were of analytical grade.

Lactate Dehydrogenase Activity with 4-HNE or 4-ONE

Twenty Units of LDH (1 unit converts 1 μmole of pyruvate to lactate per minute at pH 7.5 and 37° C) were incubated with 4-ONE or 4-HNE or an equal volume of ethanol used to deliver the aldehyde (0, 0.25, 0.5, 1, 2 ,3, 4 mM) in a 1.5 mL Eppendorf tube (1.5 mL capacity) with a 37°C Precision incubator by GCA Corporation (Chicago, IL, USA) in either pH 5.6 or 7.4 in 50 mM phosphate buffer for 72 hours. For the forward reaction (NADH + Pyruvate → Lactate + NAD), 10 units of bovine lactate dehydrogenase were added to a quartz cuvette with a total volume of 2.6 mL. The reaction solution contained 50 mM pyruvate and 0.2 mM NADH, with

NADH added last to initiate the reaction. Reaction progress was monitored with a UV-VIS spectrophotometer (UV-1800 UV-Vis Spectrophotometer, Shimadzu, Kyoto Japan) by recording a decrease in absorbance at 340 nm, indicating NADH utilization during enzymatic reaction. For the reverse reaction, 10 units of bovine heart lactate dehydrogenase were added to a quartz cuvette with a total volume of 2.6 mL. The solution was 50 mM in lactic acid, 50 mM in glutamic acid, and 0.2 mM in NAD^+, with the NAD^+ being added last to initiate the reaction. For pyruvate trapping, 50 units of glutamate pyruvate transaminase were added to the reaction to sequester pyruvate by conversion to alanine. Completion of chemical reaction was monitored with a Shimadzu UV-Vis Spectrophotometer by recording an increase in absorbance at 340 nm, indicating NADH formation (Bergmeyer 1974).

Preparation of reduced myoglobin

Equine myoglobin was reduced by sodium dithionite (W. Duane Brown 1967). Fifteen mg of equine heart myoglobin was dissolved in 1 mL of 50 mM phosphate buffer at pH 5.6 or 7.4. Two mg of sodium dithionite was then added, and the sample diluted with 6.5 mL of the appropriate buffer. Residual sodium dithionite was removed by dialysis with Slide-a-lyzer 10kDa cups against 50 mM phosphate buffer at either pH 5.6 or 7.4 depending on what pH the myoglobin was prepared in. Absorbance spectra from 500 to 600 nm were recorded using a Spectrophotometer (UV-1800 UV-Vis Spectrophotometer, Shimadzu, Kyoto Japan) to confirm myoglobin reduction.

Incubation of myoglobin with HNE and ONE

Myoglobin prepared as described above (0.15 mM) was incubated in a 1.5 mL Eppendorf tube with either 4-ONE or 4-HNE or a volume of 95% ethanol equivalent to deliver the aldehyde (0 or 1 mM). Incubation was performed in the 37°C lab incubator.

Mass spectrometry of myoglobin

Myoglobin samples prepared according to above (0.15 mM), diluted with 50% methanol (v/v) and 0.1% (v/v) formic acid to a final concentration of 0.0375 mM, were injected directly into a Waters Micromass quadrupole Time-of-flight mass spectrometer running MassLynx software (Waters Corporation, Milford, MA, USA). Fifteen μL were manually injected at a time. The 800-3000 Dalton range was scanned for multiply charged peaks. MaxEnt 1Analysis software was used to deconvolute the spectra obtained.

Mitochondria Isolation

Mitochondria were isolated from a bovine heart according to briefly modified methods (R. Ramanathan 2012). All isolation steps were performed at 4° C. Bovine heart tissue (100 g) was trimmed of all connective tissue and fat and finely minced with stainless steel scissors. Minced heart muscle was washed twice with 250 mM sucrose. The tissue sample was suspended in 200 mL of mitochondrial isolation buffer (1 mM EGTA, 10 mM HEPES, 250 mM sucrose, pH 7.4). The suspension was stirred and treated with 50 mg Nagarase bacterial protease, while maintaining the pH at 7.4. After incubation for 20 min, 800 mL of suspension buffer were added to the 200 mL solution containing the minced heart tissue. The muscle was homogenized by three passes with a Kontes Duall Tissue grinder by Kimbal Chase (Vineland, NJ, USA) , followed by three passes with a Wheaton tissue grinder by Wheaton (Milville, NJ, USA). The resultant homogenate was centrifuged at 1200 × *g* for 20 min with a Sorval RC-6-Plus refrigerated centrifuge by Thermo Electron Corporation (Waltham, MA, USA), and the supernatant was filtered through double layered cheese cloth and centrifuged again at 26,000 × *g* for 20 min at 4°C. The pellet was washed twice with isolation buffer with further centrifugation, then suspended in mitochondria suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.4).

Mitochondria incubation with HNE and ONE

Mitochondria (protein concentration of 10 mg / mL as determined by the bicinchoninic acid assay) isolated as described above were incubated in a 1.5 mL Eppendorf tube that contained 0.4 mM 4-ONE or 0.4 mM 4-HNE or no treatment for 8 minutes in a 30°C water bath at pH 5.6 (5 mM phosphate, 120 mM KCl, 30 mM maleic acid) or 7.4 (5 mM phosphate, 120 mM KCl, 30 mM Tris-HCl).

Electron Microscopy of Mitochondria

Mitochondria, after incubation in 1.5 mL Eppendorf tubes in a 30°C water bath for 8 minutes, were processed according to briefly modified methods of R. Ramanathan 2012. Samples were fixed using glutaraldehyde (1.5%) and formaldehyde (1.5) in fixation buffer (0.1M HEPES, 0.08M NaCl, 3 mM $MgCl_2$). Samples were then suspended in 200 μL of low temperature gelling agarose (3%) and centrifuged at 12,000 × g for 5 min. The pellet was sliced into 1mm cubes, excess agarose was discarded, and samples were washed three times with buffer (0.1M HEPES, 0.08M NaC, 3 mM $MgCl_2$). Pellets were then treated with 1% osmium tetroxide 0.8% potassium ferricyanide, 0.1 M HEPES, and 0.08 M NaCl) for 1 hour at 4°C. Samples were then washed three times for 10 minutes with distilled water, and then dehydrated with 30, 50, 70, 95, and 100% ethanol and then 30, 50, 70, 95% acetone, for 15 minutes each. Embedding was performed with a mixture of Embed 812, Araldite 506, and dodecenyl succinic anhydride and polymerized for 36 h. Ultrathin slices were stained with 4% uranyl acetate (w/v) in 50% (v/v) ethanol for 10 minutes, followed by Reynold's lead citrate for 4 min.(R. Ramanathan 2012) Images were captured using a JEOL JEM1011 (JEOL, Inc. Peabody, MA) a 100 kV transmission electron microscope, with a magnification of 10,000x.

Redox Instability in Myoglobin

Myoglobin was prepared as described above in "Preparation of myoglobin". After reduction and oxygenation, Mb (0.15 mM) was incubated with 4-HNE or 4-ONE (1 mM) or buffer (50 mM phosphate buffer at pH 5.6 and 7.4). Absorbance spectra were recorded at wavelengths 500 to 600 nm using a Shimadzu UV-Vis Spectrophotometer, and the proportion of metmyoglobin was calculated according to a modified Krzywicki method (J. Tang 2004). For this method the following equation was used: $\%MetMb = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$, where $R_1 = A_{582} / A_{525}$, $R_2 = A_{557} / A_{525}$, $R_3 = A_{503} / A_{525}$.

Statistical Analysis

The One-Way ANOVA feature of VassarStats (Vassar College, Poughkeepsie, NY, USA) was used to determine statistical significance ($P < 0.05$) between means of effects of treatments observed in LDH activity and metmyoglobin formation. For LDH and myoglobin incubation, conditions and reactions were performed in triplicate. Specific LDH Activity was calculated as the mass of product formed per minute per milligram of protein and the average of three reactions for each condition was calculated. For metmyoglobin formation, incubation conditions were performed in triplicate. Metmyoglobin percentages were calculated according to a modified Kryzwicki equation and averaged.(J. Tang 2004)

Results and Discussion

Effect of 4-HNE and 4-ONE on Lactate Dehydrogenase Activity

After incubation with 4-ONE and 4-HNE, enzymatic activity of LDH was significantly inhibited ($P<0.05$), at concentrations greater than 0.5 mM. Significant differences in enzymatic activity of LDH were observed among control and treatments (0.5 to 4 mM 4-ONE, and 0.5 to 4mM 4-HNE). The backwards reaction (NAD + Lactate → NADH + Pyruvate) at pH 5.6 (Figure

1) was significantly ($P<0.05$) inhibited by all concentrations of 4-ONE tested. 4-HNE decreased activity less and showed a more linear dose dependency ($P<0.05$) as compared with the control as well as 4-ONE. These conditions, at pH 5.6 and forming NADH, reflect how LDH functions to reduce metmyoglobin in post-mortem beef skeletal muscle. Previous results showed LDH activity being reduced to about 15% of the control activity using 0.4 mM 4-HNE incubated for 5 hours and about 50% using 0.04 mM 4-HNE (Ramanathan 2014). In contrast, these finding suggest that a much higher concentration is required for a longer period of time to achieve such a high degree of inhibition. The 0.04 mM 4-HNE concentration was not found to produce a noticeable effect in this study.

At pH 7.4, the NADH forming reaction is affected less by the 4-ONE and 4-HNE treatments (0.5-4 mM). Significant differences compared to control ($P<0.05$) were not seen until 2 mM concentration for 4-ONE and 4-HNE. While the inhibitory effect of 4-ONE is greater than 4-HNE at all concentrations, the effects of the two treatments were more similar than at pH 5.6, with steady decreases in specific activity seen with 4-ONE incubation, rather than a rapid drop off (Figure 2). The differences between 4-ONE and 4-HNE were significant ($P<0.05$). 4 mM 4-HNE inhibited LDH activity to about 50% of the control, while 4 mM 4-ONE eliminates activity almost entirely. LDH functions at this pH in physiological conditions. This reaction is of importance in certain specialized living tissues such as the heart and liver, showing how these compounds play a role in pathogenesis in both humans and livestock, particularly when impaired energy metabolism and oxidative stress is present (Uchida 2003).

The forward LDH reaction, in which NADH is consumed and lactate produced, was also studied in the presence of 4-ONE and 4-HNE. As with the backward reaction, pH 5.6 showed a more pronounced inhibitory effects between 4-HNE and 4-ONE as compared to pH 7.4 (Figure

3). Significant differences ($P<0.05$) were observed between 4-ONE and 4-HNE at all concentrations except 4 mM, at which enzymatic activity reduced to non-detectable levels for both 4-ONE and 4-HNE. LDH activity towards lactate formation is an important part of the post-mortem biochemical reactions that occur following animal slaughter.

The LDH activity was assayed at pH 7.4 in the presence of NADH (Figure 4). Significant differences in activity loss ($P<0.05$) were noticed between 4-ONE and 4-HNE at concentrations 1-4 mM. This pathway is utilized by living organisms to regenerate NAD^+, and thus facilitate efficient respiration and energy metabolism. Using millimolar concentrations, which are possible in certain diseased and stressed states, it was shown that 4-ONE and 4-HNE rapidly inhibit LDH's most important biological activity in muscle, the regeneration of NAD^+ (Kim 2006).

Covalent binding of 4-ONE and 4-HNE to myoglobin

Mass spectrometry was used for confirming the covalent modification of reduced myoglobin by 4-HNE and 4-ONE. The mass of unmodified control myoglobin is shown in figures 3.3 and 3.4. While pH 5.6 facilitated inhibition of LDH by 4-ONE and 4-HNE, a reverse effect was observed for myoglobin modification, shown in figures 3.5 through 3.8. This is likely due to the electrophilic nature of the aldehydes, which act as Michael acceptors during adduction (Jonathan A. Doorn 2002). Protonation of potential Michael donors by acidic pH would reduce the likelihood of the addition reaction. The greatest extent of adduction by 4-HNE was observed at pH 7.4 (figure 3.5).

Likewise, 4-ONE's highest reactivity with myoglobin structural modification was observed at pH 7.4 (Figure 3.6). This activity was considerable less than 4-HNE, as shown by the relative heights of the peaks. This is due to the formation of products modified by species other than intact 4-ONE. This suggests that 4-ONE is being broken down into smaller compounds such

as acetaldehyde and 2-heptanone. These compounds can also covalently react with other chemical residues within myoglobin. The experiments performed at pH 5.6 for 4-HNE and for 4-ONE are shown in figures 8 and 9 respectively. A similar trend was observed at physiological pH (7.4), except with a seemingly smaller degree of structural modification, to the point of no further adduct products were visible after the 4-ONE treatment. These results are similar to previous published experiments for myoglobin reactions with 4-HNE (Alderton 2003). Myoglobin adduction with 4-HNE produced distinct significant peaks in previous research work by Alderton (2013). Peaks produced from the chemical interactions of myoglobin and 4-HNE showed addition of 3 molecules of 4-HNE, an effect that was not observed in our findings. Reaction conditions and reactant concentrations were the same in both cases. There is, however, no previous work to compare the 4-ONE+Mb mass spectra to. This is the first time adduction between 4-ONE and Mb has been shown.

Induction of Redox Instability

Spectrophotometric experiments were performed to determine the rates of myoglobin oxidation during incubation with 4-ONE or 4-HNE or control. Figure 5 shows the gradual oxidation of myoglobin at pH 7.4 and 5.6 at 4°C. Differences between the treatments and control were significant ($P<0.05$). At pH 5.6, differences between myoglobin oxidation in the presence of 4-ONE and 4-HNE were noticeable. The percentages of oxidized myoglobin were significantly different ($P<0.05$) at day 7. At pH 7.4, differences were significant ($P<0.05$) between all three conditions (4-ONE, 4-HNE, or control) all 7 days. Reaction of 4-HNE produced the highest concentration of metmyoglobin at pH 5.6, while 4-ONE produced the highest concentration at pH 7.4. This corroborates results reported previously in similar experiments (Alderton 2003). These previous results as well as the ones shown here show the

pro-oxidant effect of 4-HNE incubation, with metmyoglobin % reaching close to 60% at pH 5.6. The data reported here confirms that the redox destabilizing effect of 4-ONE is comparable to that of 4-HNE. Experiments with sperm whale myoglobin have shown that when destabilized with 4-HNE, myoglobin becomes much more likely to release its' heme subgroup (Nantawat Tatiaborworntham 2012). This contributes to the compound's ability to negatively affect redox reactions in meat.

Mitochondrial ultrastructure

This study was performed to qualitatively assess 4-ONE and 4-HNE's abilities to damage mitochondrial structural integrity, which is known to lead to the release of redox-active prosthetic groups that are capable of further damaging cellular components (Murphy 2009). Panels A and B of figure 3.6 shows control mitochondria, incubated in 50 mM phosphate buffer at pH 7.4 or 5.6 respectively, with a volume of ethanol equivalent to what was used to deliver the 4-ONE or 4-HNE. Mitochondrial membranes are mostly intact, with minimal or no outer membrane disruption. Panels C and D of figure 3.6 show mitochondria that were incubated with 0.4 mM 4-ONE, at pH 7.4 or 5.6 respectively. Significant alterations in mitochondrial morphology are observed. Panels E and F of figure 3.6 show mitochondria incubated with 0.4 mM 4-HNE at 7.4 or 5.6 respectively. These samples exhibit significant disruption of mitochondrial ultrastructure, confirming that these chemicals are capable of effecting organelles as well as enzymes and pigments in meat. The deterioration of mitochondria further contributes to the deleterious effects of the compounds investigated in this study. This effect has been observed with 4-HNE (R. Ramanathan 2012), but never before with 4-ONE. Panels C and D of figure 3.6, showing incubation with 4-ONE, clearly show much greater vacuolization than panels E and F, which show the effects of 4-HNE incubation.

Conclusions

The data shows that 4-ONE has significant deleterious effects. Particularly pronounced is the way that 4-ONE effects LDH activity at pH 5.6. This has important implications for post-mortem metmyoglobin reducing activity, color stabilizing enzymatic activity in meat and meat products. Given that 4-HNE levels have been used as a quality biomarker in meat, results from this study suggests that 4-ONE levels might be a more relevant factor influencing fresh meat quality.

Aside from effects on LDH activity, both aldehydes are found to induce myoglobin redox instability, with 4-ONE showing more significant destabilizing effect at pH 5.6. The mass spectra of incubation products of myoglobin and 4-ONE or 4-HNE confirm the covalent nature of their interactions. 4-HNE produces a straight forward series of adduction products, with clear additions of intact (156 Dalton) molecules to the myoglobin monomer. 4-ONE, however, adducts in a more complex manner. Signals were observed for the addition of intact 4-ONE (154 Dalton increases), but signals are also seen for different increases in weight, suggesting more complicated chemistry than the simple Michael addition seen with 4-HNE.

The mitochondrial ultrastructure studies show that lipid peroxidation products are capable of damaging relatively large sub-cellular structures, not just proteins like enzymes and myoglobin. All these effects documented display the damaging effects of lipid peroxidation on meat quality, and suggest that 4-ONE is a more potent source of damage than the more well-known 4-HNE. Further research is warranted to determine the importance of these differences. Both intact muscle foods and processed meat products such as ground beef and sausage are important segments of the industry that could benefit from understanding the generation, effects, and analysis of these compounds.

References

A. Mordente, G. E. M., S. A. Santini, G. A. D. Miggiano, T. Petitti, B. Giardina, M. Battino, G. P. Littarru (1993). "Antioxidant effect of coenzyme Q on hydrogen peroxide-activated myoglobin." Journal of Molecular Medicine **71**(8): 92.

A.E.D Bekhit, C. F. (2005). "Metmyoglobin reducing activity." Meat Science **71**(3): 407-439.

A.L. Alderton, C. F., D.C. Liebler, D.W. Hill (2003). "Induction of Redox Instability of Bovine Myoglobin by Adduction with 4-Hydroxy-2-nonenal " Biochemistry **42**: 4398-4405.

Anand Mohan, S. M., Melvin C. Hunt, Thomas J. Barstow, Terry A. Houser (2010). "Kinetics of Myoglobin Redox Form Stabilization by Malate Dehydrogenase." Journal of Agricultural and Food Chemistry **58**: 6994-7000.

Andersen, C. P. B. a. H. J. (2002). "Myoglobin-Induced Lipid Oxidation. A Review." Journal of Agricultural and Food Chemistry **50**: 3887-3897.

Anuj Purohit, R. S., William Kerr, Anand Mohan (2014). "Influence of Redox Reactive Iron, Lactate, and Succinate on the Myoglobin Redox Stability and Mitochondrial Respiration." Journal of Agricultural and Food Chemistry **62**: 12570-12575.

Bergmeyer, H. U. (1974). "Lactate Dehydrogenase UV-assay with pyruvate and NADH." Methods of Enzymatic Analysis **2**: 574-579.

C. Faustman, D. C. L., J.A. Burr (1999). "Alpha-Tocopherol Oxidation in Beef and in Bovine Muscle Microsomes." Journal of Agricultural and Food Chemistry **47**: 1396-1399.

C.R. Ashmore, L. D., G. Foster, and F. Carroll (1971). "Respiration of Mitochondria Isolated from Dark-Cutting Beef." Journal of Animal Science **33**(3): 574-577.

Cameron Faustman, Q. S., Richard Mancini, Surendranath P. Suman (2010). "Myoglobin and lipid oxidation interactions: Mechanistic bases and control." Meat Science **86**: 86-94.

Catherine Echevarne, M. R., R. Labas (1989). "Metmyoglobin Reductase Activity in Bovine Muscles." Meat Science **27**: 161-172.

D.J. Troy, J. P. K. (2010). "Consumer perception and the role of science in the meat industry." Meat Science **86**(1): 214-226.

De Lin, H.-g. L., Quan Liu, George Perry, Mark. A. Smith, Lawrence M. Sayre (2005). "4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal." Chemical Research in Toxicology **18**(8): 1219-1231.

Elisabeth Huff-Lonergan, S. M. L. (2005). "Mechanisms of water-holding capactiy of meat: The role of postmortem biochemical and structural changes." Meat Science **71**: 194-204.

G.H. Zhou, X. L. X., Y. Liu (2010). "Preservation technologies for fresh meat - A review." Meat Science **86**: 119-128.

J. Kanner, S. H. (1985). "Initiation of Membranal Lipid Peroxidation by Activated Metmyoglobin and Methhemoglobin." Archives of Biochemistry and Biophysics **237**(2): 314-321.

J. Tang, C. F., and T.A. Hoagland (2004). "Krzywicki Revisited: Equations for Spectrophotometric Determination of Myoglobin Redox Forms in Aqueous Meat Extracts." Food Chemistry and Toxicology **69**(9): 717-720.

J.A. Silva, L. P., C. Martins (1999). "Influence of ultimate pH on bovine meat tenderness during ageing." Meat Science **52**: 453-459.

J.Y Jeong, S. J. H., H.S. Yang, S.H. Moon, Y.H. Hwang, G.B Park, ST. Joo (2009). "Discoloration Characteristics of 3 Majoy Muscles from Cattle During Cold Storage." Food Chemistry **74**(1).

Jonathan A. Doorn, D. R. P. (2002). "Covalent Modification of Amino Acid Nucleophiles by the Lipid Peroxidation Products 4-Hydroxy-2-nonenal and 4-Oxo-2-nonenal." Chem. Res. Toxicol. **15**: 1445-1450.

Jonathan A. Doorn, T. D. H., Dennis R. Petersen (2006). "Inhibition of Human Mitochondrial Aldehyde Dehydorgenase by 4-Hydroxynon-2-enal and 4-Oxonon-2-enal " Chem. Res. Toxicol. **19**(q): 102-110.

Luke I. Swezda, K. U., Lin Tsai, Earl R. Stadtman (1992). "Inactivation of Glucose-6-phosphate Dehydrogenase by 4-Hydroxy-2-nonenal." The Journal of Biological Chemistry **268**(5): 3342-3347.

M.N. Nair, S. P. S., S. Li, P. Joseph, C.M. Beach (2013). "Lipid oxidation-induced oxidation in emu and ostrich myoglobins." Meat Science **96**: 984-993.

Martin Globisch, D. K., Thomas Henle (2015). "4-Hydroxy-2-nonenal (4-HNE) and Its Lipation Product 2-Pentylpyrrole Lysine (2-PPL) in Peanuts." Journal of Agricultural and Food Chemistry **63**: 5273-5281.

Murphy, M. P. (2009). "How mitochondria produce reactive oxygen species." Biochemistry Journal **417**: 1-13.

Nantawat Tatiaborworntham, C. F., Shuang Yin, Ranjith Ramanathan, Richard A. Mancini, Surendranath P. Suman, Carol M. Beach, Naveena B. Maheswarappa, Eric W. Grunwald, Mark P. Richards (2012). "Redox Instability and Hemin Loss of Mutant Sperm Whale Myoglobins Induced by 4-hydroxynonenal in Vitro." Journal of Agricultural and Food Chemistry **60**: 8473-8483.

Porter, W. A. P. a. N. A. (1990). "Suggested Mechanisms for the Production of 4-hydroxy-2-nonenal from the Autoxidation of Polyunsaturated Fatty Acids." Free Radical Biology and Medicine **8**: 541-543.

R. Ramanathan, R. A. M., G.A. Dady, C.B. Van Buiten (2013). "Effects of succinate and pH on cooked beef color." Meat Science **93**: 888-892.

R. Ramanathan, R. A. M., P. Joseph, S.P. Suman (2013). "Bovine mitochondrial oxygen consumption effects on oxymoblin in presence of lactate as a substrate for respiration." Meat Science **93**: 893-897.

R. Ramanathan, R. A. M., S.P. Suman, M.E. Cantino (2012). "Effects of 4-hydroxy-2-nonenal on beef heart mitochondrial ultrastructure, oxygen consumption, and metmyoglobin reduction." Meat Science **90**: 564-571.

Ranjith Ramanthan, R. A. M., Surendranth P. Suman, Carol M. Beach (2014). "Covalent Binding of 4-Hydroxy-2-nonenal to Lactate Dehydrogenase Decreases NADH Formation and Metmyoglobin Reducing Activity." Journal of Agricultural and Food Chemistry **62**: 2112-2117.

Richards, M. P. (2012). "Redox Reactions of Myoglobin " Antioxidants and Redox Signalling **00**.

Sruti Shiva, P. S. B., Rakesh P. Patel, Peter G. Anderson, Victor M. Darley-Usmar (2001). "Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase." Proceedings of the National Academy of Sciences of the USA.

T Gotoh, K. S. (1976). "Generation of the superoxide radical during autoxidation of myoglobin." Journal of Biochemistry **80**(2): 397-399.

Uchida, K. (2003). "4-Hydroxy-2-nonenal: a product and mediator of oxidative stress." Progress in Lipid Research **42**: 318-343.

W. Duane Brown, L. B. M. (1967). "Autoxidation of Oxymyoglobins." The Journal of Biological Chemistry **244**(24): 6696-6701.

Y.H. Kim, J. T. K., S.B. Smith, L.R. Berghman, J.W. Savell (2009). "Role of lactate dehydrogenase in metmyoglobin reduction and color stability of different bovine muscles." Meat Science **83**: 376-382.

Yuan H. Kim, M. C. H., Richard A. Mancini, Mark Seyfert, Tom M. Loughin, Donald H. Kropf, J. Scott Smith (2006). "Mechanism for Lactate-Color Stabilization in Injection-Enhanced Beef." Journal of Agricultural and Food Chemistry **54**: 7856-7862.

List of Figures:

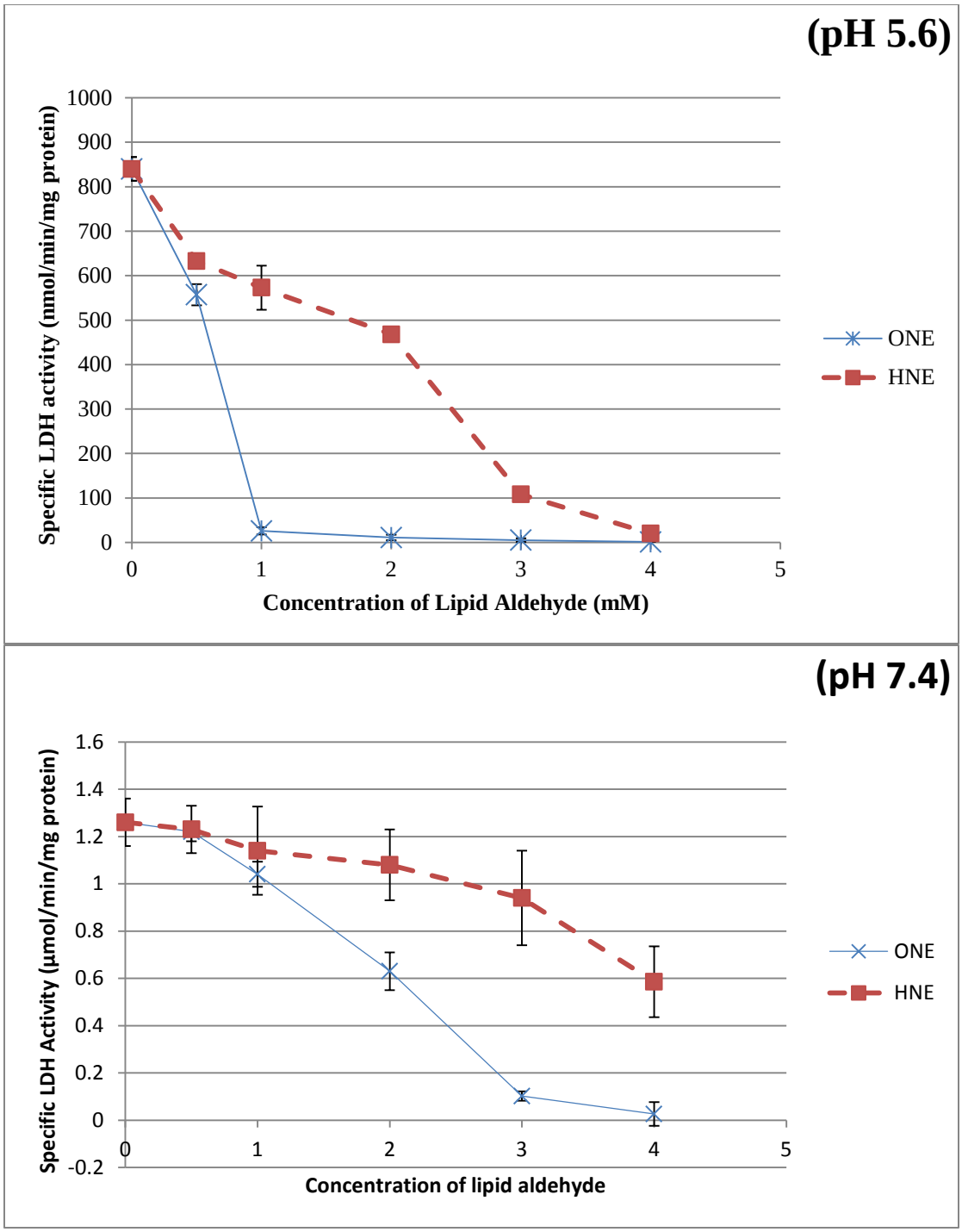

Figure 3.1. Effect of 4-ONE and 4-HNE incubation on specific LDH activity at pH 7.4 and 5.6 for the backwards (NADH forming) reaction. Enzyme was incubated for 72 hours at 37°C .

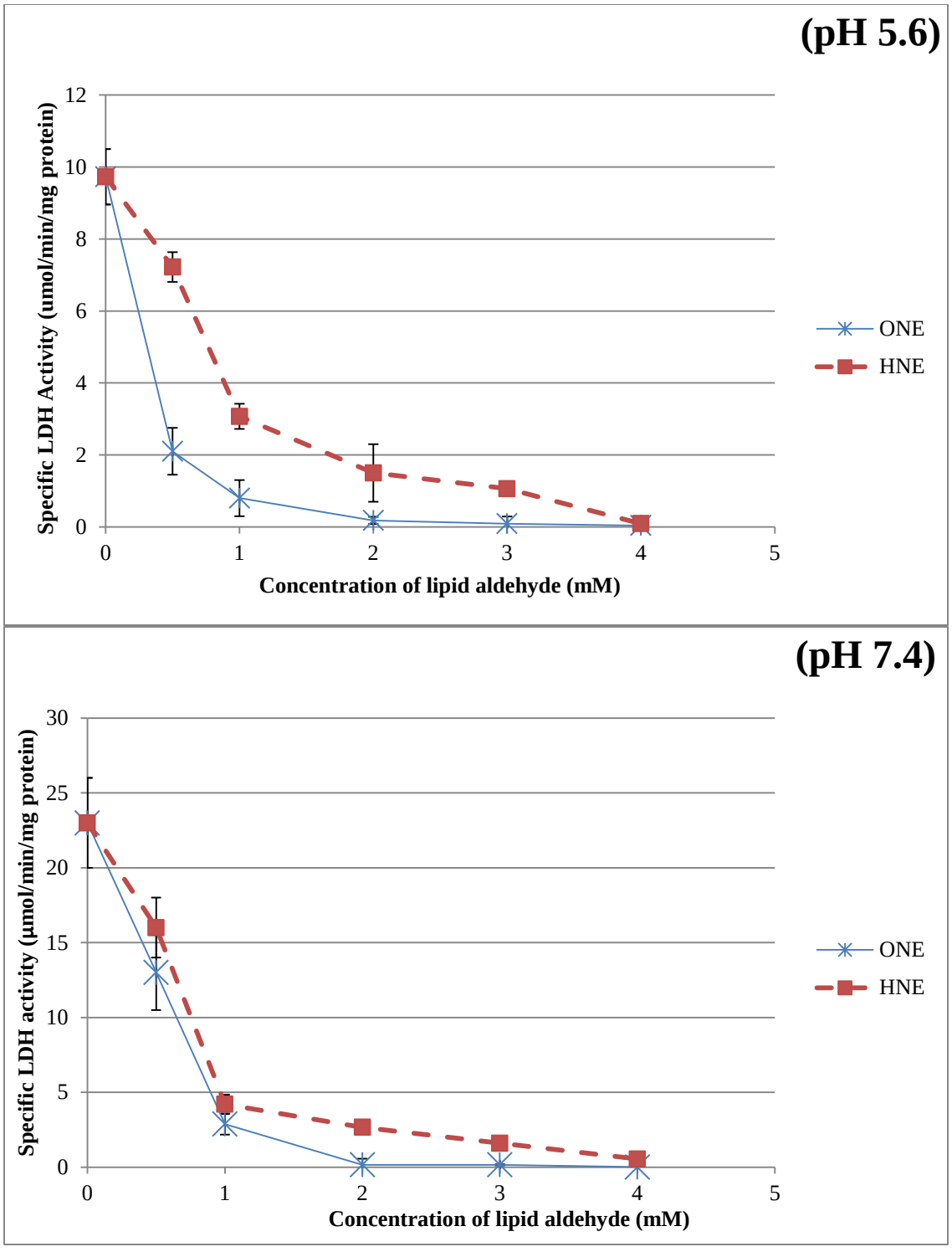

Figure 3.2. Effect of 4-ONE and 4-HNE incubation of specific LDH activity at pH 7.4 and 5.6 for the forwards (NADH consuming) reaction. Enzyme was incubated for 72 hours at 37°C.

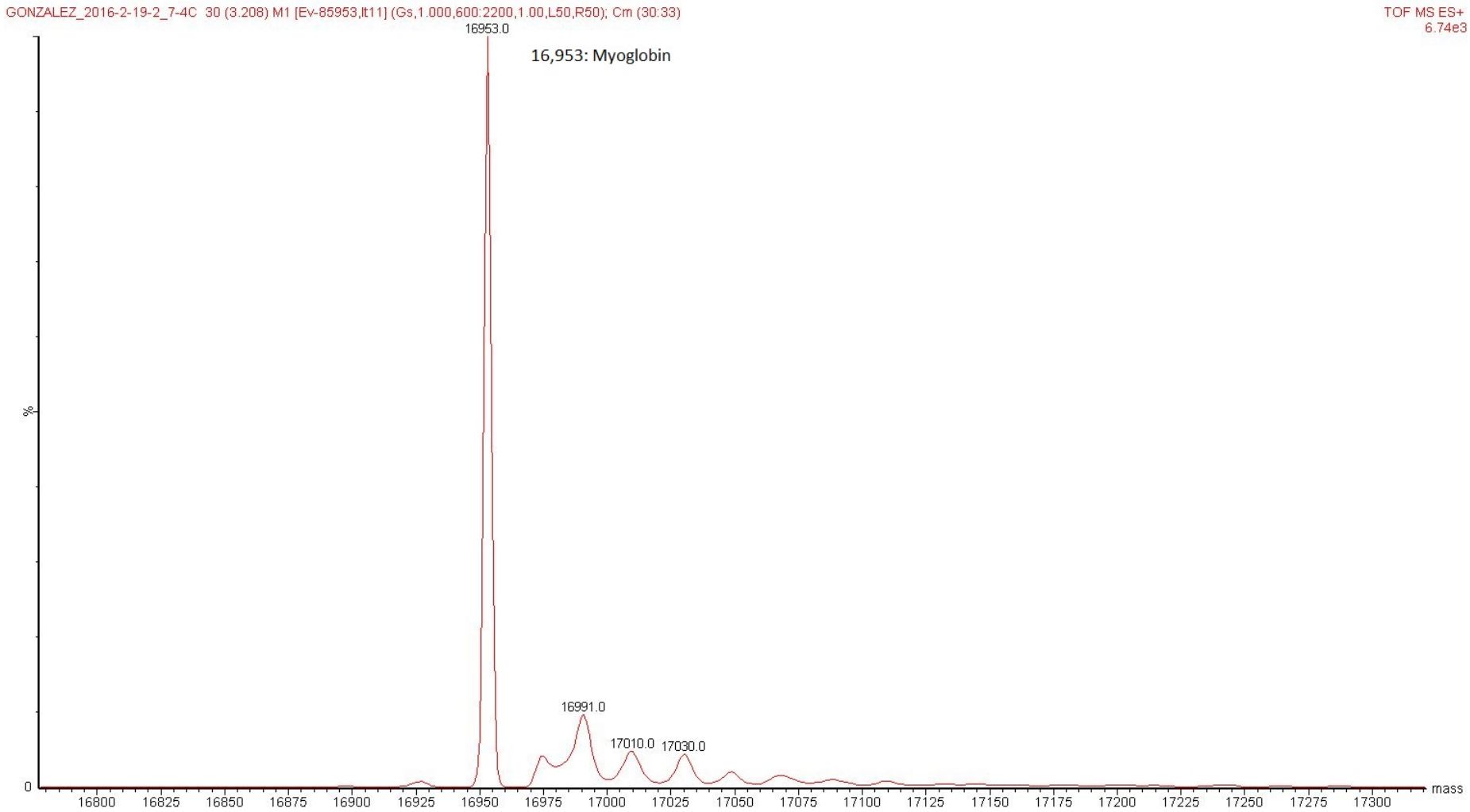

Figure 3.3 Mass spectrum of myoglobin incubated at pH 7.4.

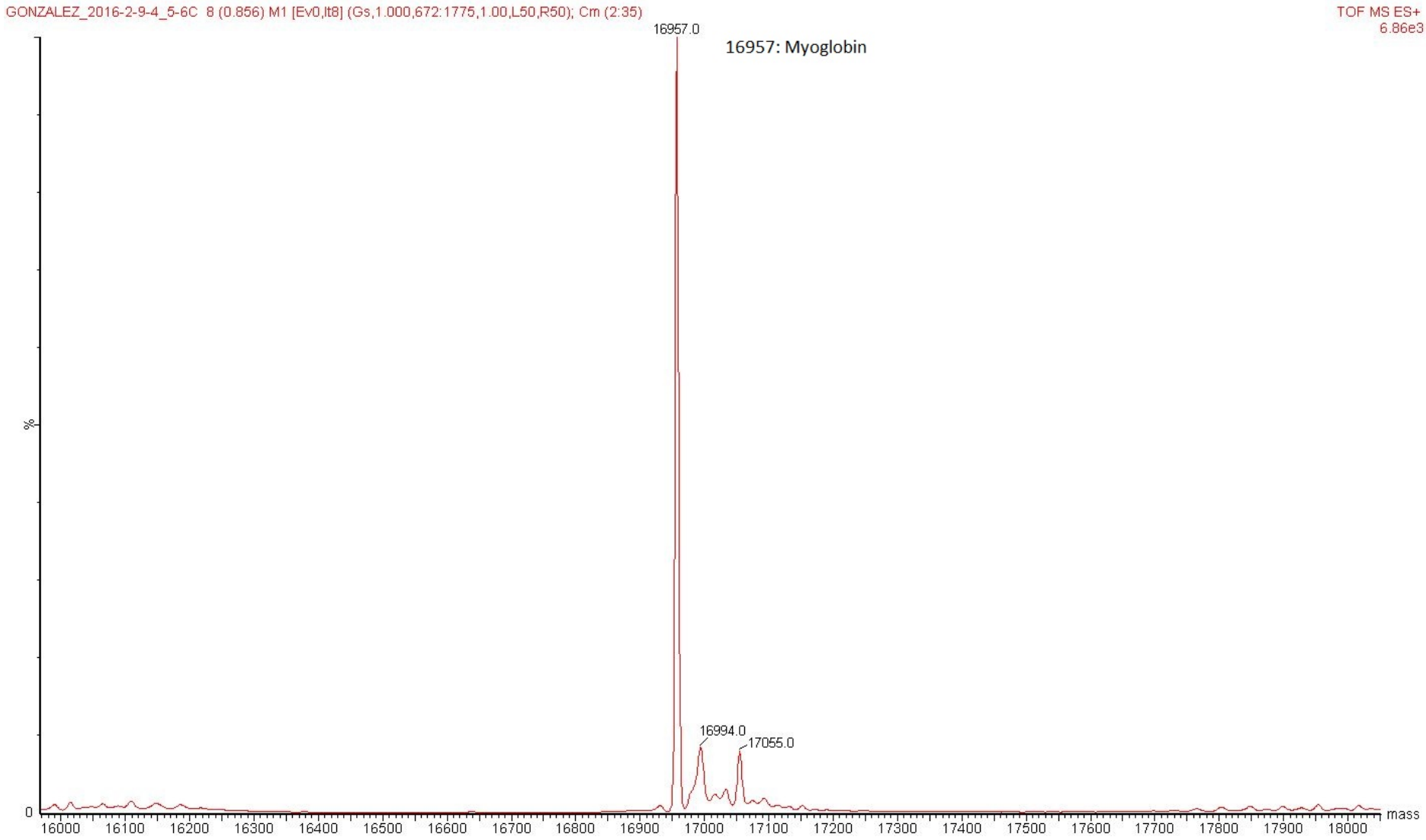

Figure 3.4: Mass spectrum of myoglobin incubated at pH 5.6

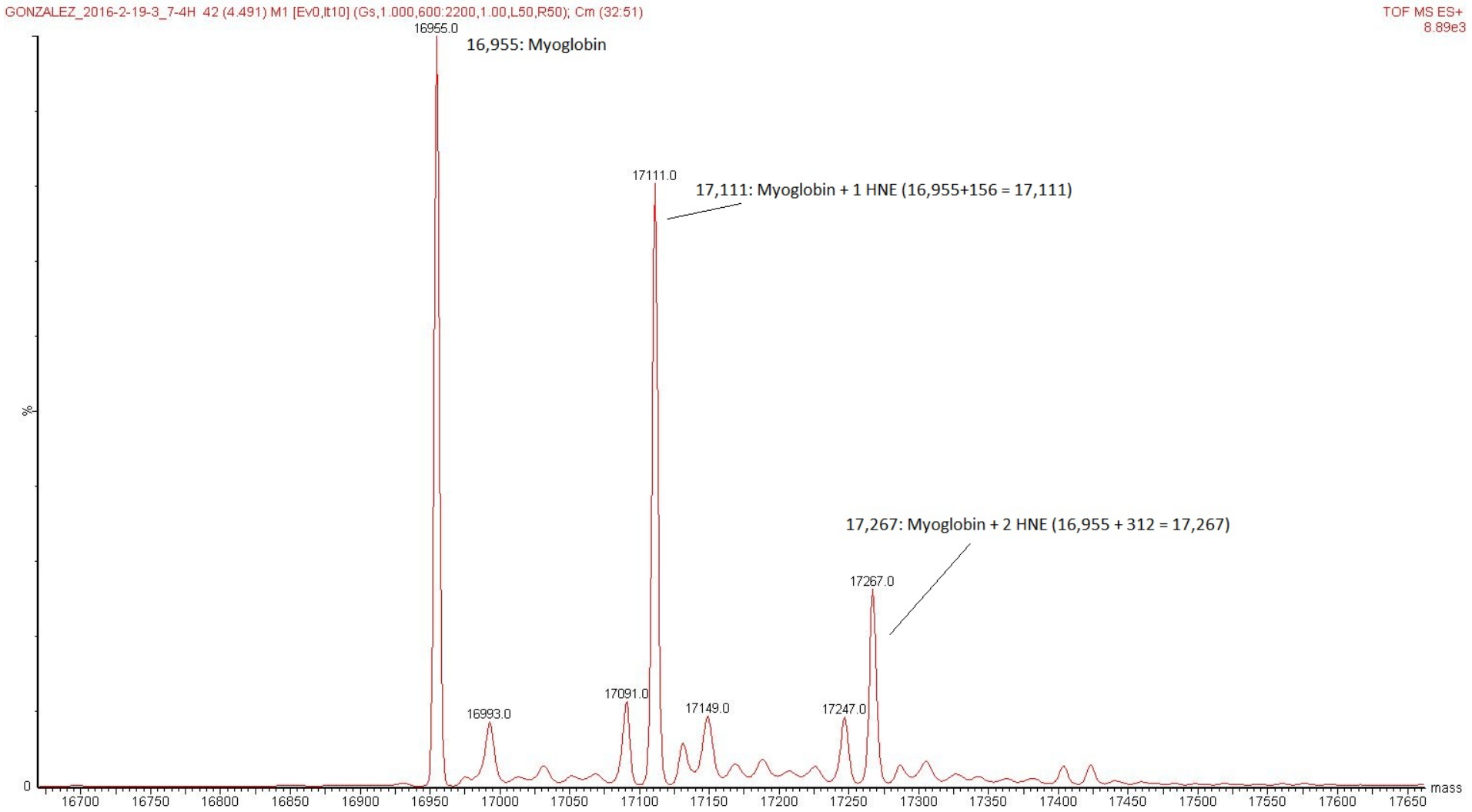

Figure 3.5: Mass spectrum of myoglobin incubated with 1 mM 4-HNE at pH 7.4.

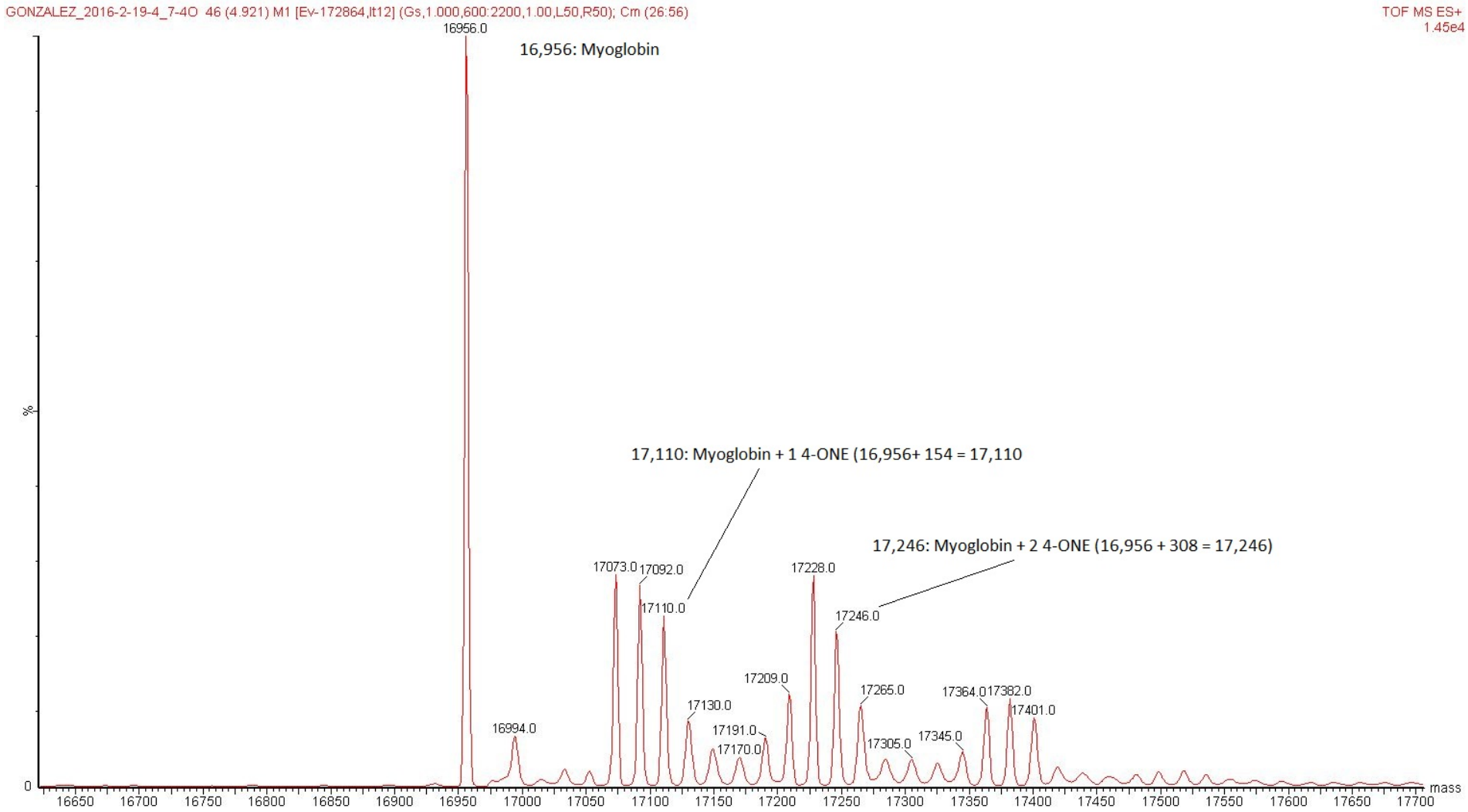

Figure 3.6. Mass spectrum of myoglobin incubated with 1 mM 4-ONE at pH 7.4.

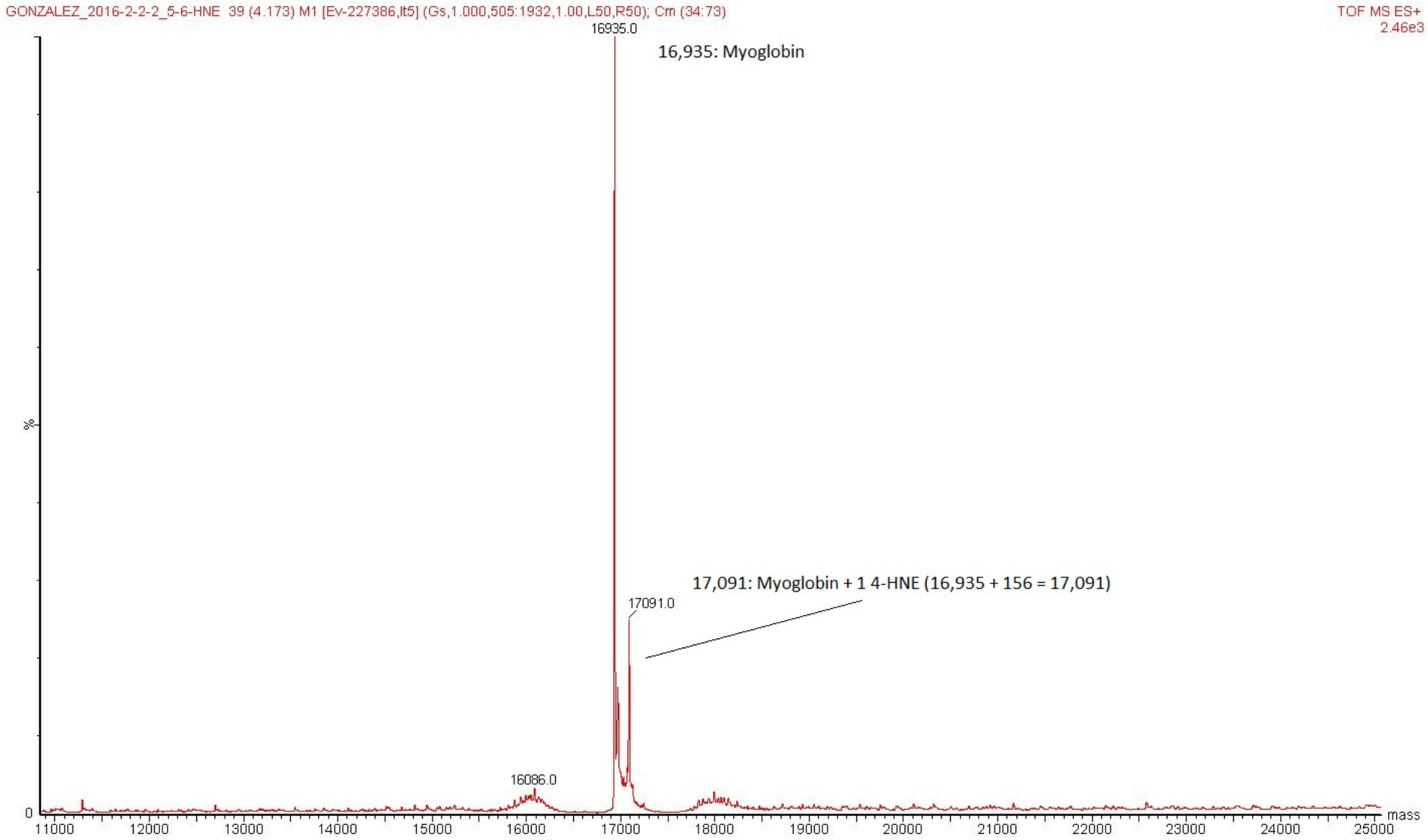

Figure 3.7. Mass spectrum of myoglobin incubated with 1 mM 4-HNE at pH 5.6.

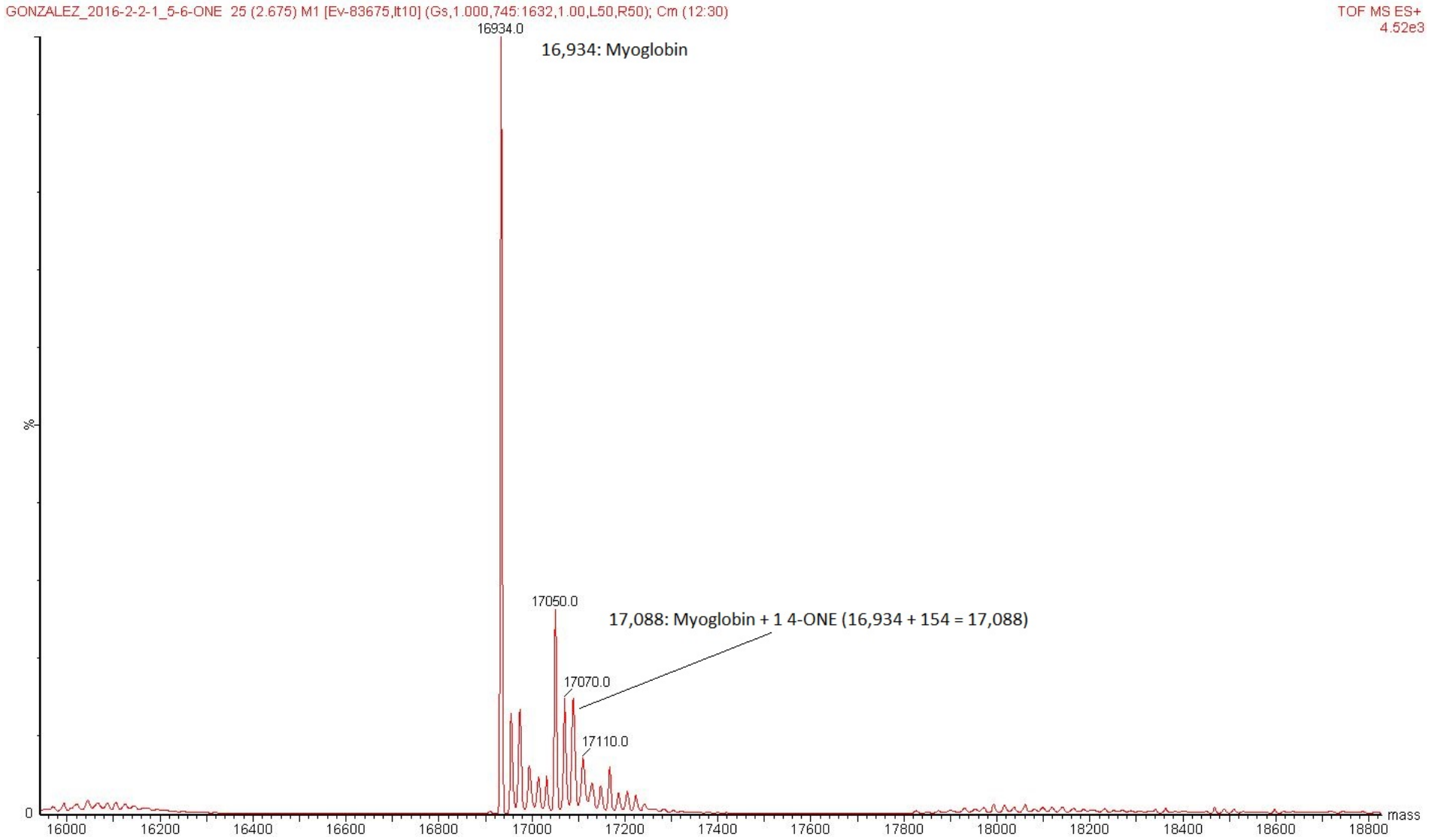

Figure 3.8. Mass spectrum of myoglobin incubated with 1 mM 4-ONE at pH 5.6.

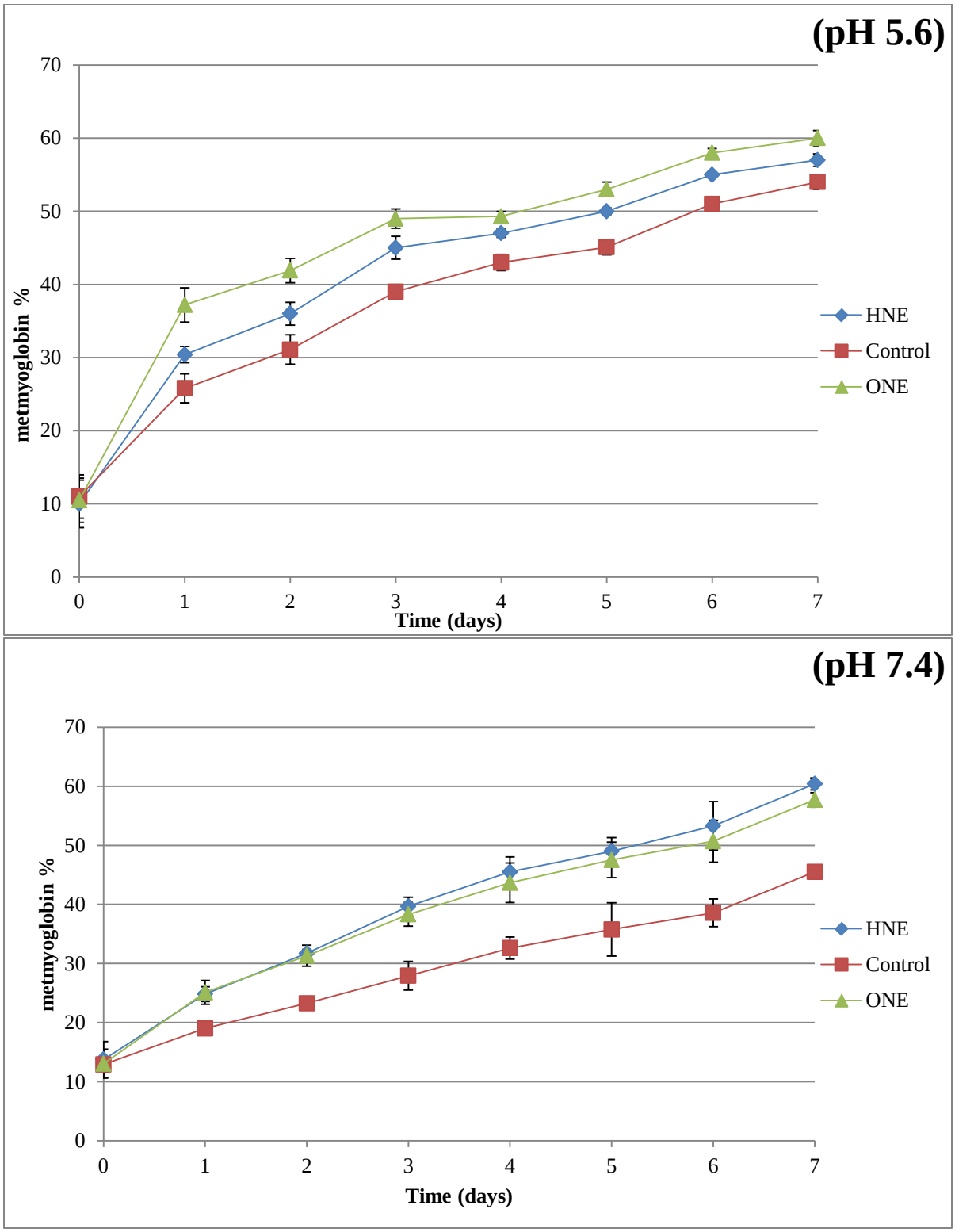

Figure 3.9. Redox instability induced by 4-ONE and 4-HNE in myoglobin at pH 7.4 and 5.6

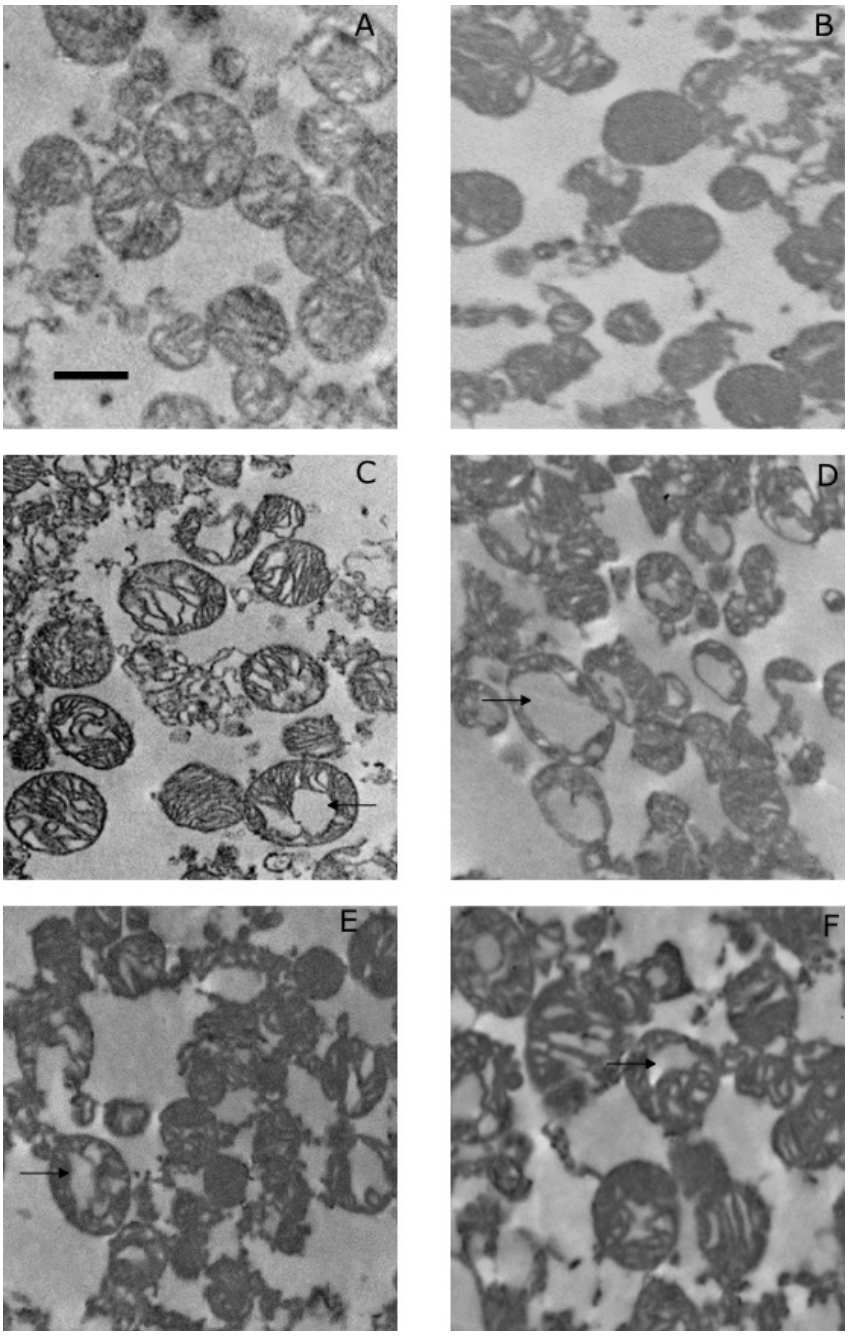

Figure 3.10: Electron micrographs of mitochondria incubated in a) pH 7.4 buffer, b)pH 5.6 buffer, c) pH 7.4 buffer with 0.4 mM 4-ONE, d) pH 5.6 buffer with 0.4 mM 4-ONE, e) pH 7.4 buffer with 0.4 mM 4-HNE, and f) pH 5.6 buffer with 4-HNE. The scale bar in panel A corresponds to 500 nm and is the same for panels A-F. Magnification = 10,000x. Arrows in panels C-F indicate mitochondrial vacuolization.

CHAPTER 4

CONCLUSIONS

4-ONE and 4-HNE both negatively affect LDH activity. 4-ONE had a more pronounced effect than 4-HNE on lactate dehydrogenase activity at both pHs (5.6 and 7.4) and in both reaction directions, with both aldehydes having significant effects compared to placebo at most concentrations. pH 5.6 facilitated LDH activity loss upon incubation with 4-HNE and 4-ONE, possibly due to protonation-mediated disruption of hydrogen bonding responsible for holding tetrameric LDH together.

4-ONE and 4-HNE both covalently bind to myoglobin, as observed by increases in molecular weight shown with mass spectroscopy. 4-ONE incubation produces a more complex pattern of signals, with adducts being formed with compounds other than intact 4-ONE.Physiological pH produces a greater amount of covalent modification by both 4-ONE and 4-HNE, possibly due to acidic conditions protonating nucleophilic residues that aldehydes bind to. Hydrogen bonding is a less important structural characteristic for a monomer like myoglobin compared to a tetramer like lactate dehydrogenase. 4-ONE and 4-HNE both induce significant redox instability in myoglobin at both pH 7.4 and 5.6.

4-ONE and 4-HNE both disrupt mitochondrial structure. 4-ONE induced more severe vacuolization as well as rupturing outer membranes entirely.