Accelerated aging affects shelf-stability, yield, sensory characteristics, cathepsin activity, and structure of collagen and troponin-T in lower quality beef cuts

by

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Abstract

Cooler aging beef cuts have been the standard method to increase tenderness and value of beef cuts. However, this technique is not time efficient as it may take days to weeks for the cuts to reach the desired level of tenderness. Accelerated aging (AA) is a new method that may significantly improve beef tenderness within just a few hours utilizing a vacuum packager and a warm water bath. However, there are little to no studies on impact of AA on meat quality parameters. Therefore, the aim of this study was to utilize AA at different temperature and time points to determine the impact of AA on shelf stability, yield, sensory characteristics, tenderness, and cathepsin proteolytic activity of two lower quality beef cuts. Triceps brachii (TB) and semimembranosus (SM) were collected and fabricated from 10 USDA Choice carcasses and assigned to one of six treatments: 3 d cooler aged (control), 21 d cooler aged, AA 49°C for 2 hours, AA 49°C for 3 hours, AA 54°C for 2 hours, and AA 54°C for 3 hours ($n=120$). Aerobic plate counts (APCs) were conducted on swabs from the steak surfaces and purge collected from the vacuum packaged bags before and after AA. Yield was determined before and after AA as well as before and after grilling. Instrumental color, Warner-Bratzler Shear Force (WBSF), trained sensory panels, proximate analysis, lipid oxidation, cathepsin zymography, troponin-T degradation, soluble/insoluble collagen content, total collagen in the purge, and perimysial peak transitional temperature and enthalpy were measured. The results showed that AA can decrease APC counts on meat surface and in purge ($P < 0.01$), decrease redness ($P < 0.01$), increase lightness ($P < 0.05$), and decrease yield ($P < 0.01$) of the steaks. Lower shear force measurements were also found in AA steaks compared to those from the control ($P < 0.01$), with the AA 54°C treatment being comparable to 21 d cooler aging. However, the trained sensory

panel determined AA steaks were less juicy ($P < 0.01$) and less flavorful ($P < 0.05$) than the control and 21 d cooler aged samples, and AA steaks were less tender ($P < 0.01$) even though there was lower connective tissue recorded ($P < 0.01$). There was no off-flavor in AA steaks ($P > 0.05$), although lipid oxidation was slightly higher in AA samples than in the control steaks ($P < 0.01$). The AA treatment stimulated cathepsin activity where AA 49°C for 3 hours displayed the highest activity ($P < 0.05$), which led to the additional degradation of troponin-T ($P < 0.01$) and solubilization of stromal proteins ($P < 0.01$). Although AA is an economical and time-efficient method to increase tenderness of lower-quality beef cuts, further research is needed to determine strategies to mitigate the loss of moisture and decrease in juiciness from AA treatments.

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List of Abbreviations

AA……Accelerated aging

APCs.....Aerobic plate counts

BCA.....Bicinchoninic acid

BHT.....Butylated hydroxytoluene

BF.....Biceps femoris

DMBA.....Dimethylaminobenzaldehyde

DTT.....Dithiothreitol

ECM.....Extracellular Matrix

EDTA.....Ethylenediaminetetraacetic acid

GT.....Gastrocnemius

HCl.....Hydrochloric acid

IgG.....Immunoglobulin G

LD..... Longissimus dorsi

LL.....Longissimus lumborum

LT.....Longissimus thoracis

MDA.....Malondialdehyde

MMPs.....Matrix metalloproteinases

PVDF.....Polyvinylidene difluoride

SDS.....Sodium dodecyl sulfate

SM.....Semimembranosus

ST.....Semitendinosus

TB……..…...Triceps brachii

TBARS.....2-thiobarbituric acid reactive substance

TBS.....Tris-buffered saline

WBSF.....Warner-Bratzler shear force

β.....Beta

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Dedication

I would like to dedicate this thesis to my grandma, Rose Kubiak. The person I am today is because of you and words cannot express how appreciative I am of your love and support.

Chapter 1 - Enhancing tenderness in beef by optimizing cathepsin activity

1. Introduction

Contrary to lamb and pork, tenderness is the main factor of consumer acceptability for beef (Warner et al., 2021), which consumers are willing to pay a higher price for steaks that are more tender (Boleman et al., 1997). Therefore, it has always been a priority for the industry to resolve the inconsistency of beef tenderness (Gonzalez & Phelps, 2018; Tarrant, 1998).

The muscle structure weakens during postmortem aging, which ultimately leads to a more tender product, and the process in which meat becomes tender is related to the effect of endogenous enzymes on muscle structure disruption (E. Huff Lonergan et al., 2010). For example, Huff-Lonergan et al. (1996) observed degradation in different myofibrillar proteins at different aging times from beef longissimus thoracis (LT), and Koulicoff et al. (2023) reported modifications in intramuscular connective tissue during extended aging. The enzymes that are believed to be involved in the meat tenderization process include calpain (Colle & Doumit, 2017), caspase (Kemp et al., 2006), 20S proteasome (Sentandreu et al., 2002), matrix metalloproteinases (MMPs) (Koulicoff et al., 2023), and cathepsin (Calkins & Seideman, 1988). Although calpains are suggested to play a major role in postmortem aging, there are discoveries of degradation products from myofibrillar and stromal proteins that are not explained with this enzyme alone, which led to the investigation of other enzymatic systems (Robert et al., 1999).

The enzymatic ability to degrade proteins depends on a multitude of factors (Saboury, 2009) which may include the presence of inhibitors (Wendt et al., 2004), temperature (Daniel et al., 2008), pH (Zhang et al., 2017), mineral content (Pasquet et al., 1996), and time postmortem (Veiseth et al., 2001). Due to the variability of tenderness and proteolytic activity among muscles

(Davis et al., 1979; Veiseth-Kent et al., 2018), understanding the mechanism to optimizing enzymatic activity can assist in producing more tender meat products. Therefore, this literature review will explore potential enzymes, their mechanism, and involvement in the postmortem aging process of meat.

2. Enzymes involved in tenderization of postmortem muscle

2.1 Calpain

One of the main enzymes involved in tenderization of postmortem muscle is calpain. Early studies have established this enzyme plays a key role in the degradation of myofibrillar proteins where the reduction of proteolytic capacity of calpain can result in a tougher meat products (Koohmaraie, 1992). There are two prominent isoforms of calpain in meat which are calpain 1 (also known as μ-calpain) or calpain 2 (also known as m-calpain). Calpains are calcium dependent enzymes where calpain-1 requires micromolar amounts of calcium to activate, and calpain-2 requires millimolar amounts of calcium to activate (Perrin & Huttenlocher, 2002). Studies have shown that calpain activity can be modified by cytosolic calcium concentration, which is regulated by mitochondria and sarcoplasmic reticulum (Dang et al., 2019). For example, Dang et al. (2020) inhibited mitochondria's ability to uptake calcium and increased cytosolic calcium concentration, which resulted in quicker calpain 1 activation. Additionally, Colle et al. (2018) injected calcium chloride in beef longissimus lumoburm (LL) and semimembranosus (SM) and found that calpain-2 was activated earlier postmortem with the increase in calcium concentration. The calpain-3 isoform has been identified, however, its role in meat tenderization has been determined to be minimal (Geesink et al., 2005).

One of the regulators of calpain is calpastatin, which limits calpain autolysis and its ability to degrade myofibrillar proteins (Geesink & Koohmaraie, 1999). Research has shown that

greater calpastatin level in meat can result in decreased consumer perception for tenderness and increase in Warner-Bratzler shear force (WBSF) (de Moura Souza et al., 2019; Whipple et al., 1990). On the other hand, pH of meat can further regulate calpains' activity. For example, activity of calpain-1 was greatest at a pH of 6.5 (Maddock et al., 2005), and calpain-2 displays the highest activity at a pH close to 7.0 (Kendall et al., 1993). Lomiwes et al. (2014) also reported higher calpain-1 activity in meat with higher (6.3-6.9) rather than lower (5.4-5.7) ultimate pH. Finally, Pomponio and Ertbjerg (2012) incubated pork LD muscles at 2, 15, 25, and 30°C, and Liu et al. (2014) incubated pork LD muscles at 0, 10, 20, 30, and 40°C. Both studies found high temperatures rapidly activated calpain activity. Moreover, Mohrhauser et al. (2014) isolated beef myofibrils and found higher calpain-1 activity at the higher temperature of 22°C than at 4°C. Overall, calpain activity decreases with an increase in calpastatin; however, calpain activity is heightened at higher temperatures, pH, and calcium concentrations.

2.2 Caspase

Though the calpain system is regarded as the predominant enzymatic method of postmortem proteolysis, there is a growing interest in the activities of other enzymes such as caspase as they may also be associated with the tenderization of meat during postmortem aging (Chen et al., 2015). The intracellular cysteine protease caspase has many isoforms including caspase-2 through caspase-10 and are involved in cellular apoptosis (Talanian et al., 1997). Caspase-3 is an effector caspase that is activated through cleavage of large and small subunits by an initiator caspase, which is activated through apoptosome association or assembly of a death-inducing signaling complex (Shi, 2004). Unlike the effector caspases, initiator caspases do not require cleavage to activate; instead, they are activated through dimerization (Green, 2022). The

isoforms that have been studied in postmortem storage of beef include caspase-3 and caspase-6. Huang et al. (2011) incubated beef muscles with caspase-3 and caspase-6 in a model system and resulted in minimal myofibrillar degradation. In addition, Underwood et al. (2008) found no detectable active caspase-3 in immunoblots nor any significant increases in its activity in beef longissimus thoracis and sternomandibularis during postmortem aging.

Zinc has been documented to inhibit caspase activity demonstrated by Perry et al. (1997) where inhibition of caspase-3 was observed *in vitro* through the addition of micromolar amounts of zinc. Chai et al. (1999) proposed a mechanism where zinc inhibits the activation of pro-caspase-3, meaning the enzyme is kept in its inactive zymogen precursor state. Huang et al. (2018) confirmed through the injection of zinc chloride that the activation of pro-caspase-3 was blocked in beef longissimus thoracis which were aged at 4°C for up to 7 days.

Parrish et al. (2013) described that caspases can also interact with other proteases as well as different types of caspases. Green (2022) showed that caspase-3 has the ability to cleave and activate pro-caspase-3, although the process is inefficient. Additionally, calpains can reduce caspase activity through cytochrome c by preventing the cleavage of pro-caspase-3 into its active subunit (Lankiewicz et al., 2000). In another *in vitro* study by Wolf et al. (1999), calpain reacted with pro-caspase-3 for 15 min resulting in inactive pro-caspase-3 fragments. Due to the limited amount of information gathered on the caspase system, there are still many contradictions and unknowns on the role of caspase in postmortem tenderization of meat system.

2.3 20S Proteasome

The 20S proteasome possesses catalytic β-subunits (Heinemeyer et al., 2004), and the protease is activated by releasing the propeptides that surround the catalytic subunits (Huber et

al., 2016). The role of the 20S proteasome in living tissue is to degrade proteins to regulate protein concentrations and maintain homeostasis of the cell (Latham et al., 2014). Calpains are unable to replicate the 20S proteasome's degradation pattern (Dutaud et al., 2006), which are extremely small fragments of myofibrillar proteins. However, the proteasome's ability to degrade proteins is directly related to calpain's disruption of the myofibril (Houbak et al., 2008). Goll et al. (2008) described proteins are unable to enter the catalytic portion of the proteasome unless they are already unfolded, displaying the necessity for calpain to cleave myofibrils into myofilaments before further degradation by the proteasome.

Although 20S proteosome's optimal pH is at pH of 8, the decrease in pH during rigor mortis did not seem to affect its stability in the postmortem environment which was observed by Lamare et al. (2002) in beef rectus abdominis muscle aged for 7 days. Thomas et al. (2004) aged ostrich meat for 12 days and similarly found high proteasome activity at the end of the aging period. Additionally, Otsuka et al. (1998) reported high activity in purified proteasome from rabbit muscle at the lower pH of 5, though activity was based on the substrates. The 20S proteasome lost most of its activity after pork LL has been frozen and then chilled as seen in the study by Zhang and Ertbjerg (2018). On the other hand, Lamarre et al. (2009) held wolf fish at 4°C for 1 hour and found high activity in the 20S proteasome. Additionally, Woods and Storey (2006) froze wood frogs at -2.5°C for 24 hours, and they found impaired proteasome functionality was impaired after the freezing. More research should be performed to better understand the 20S proteasome's activity in different handling conditions such as cookery method or storage method.

2.4 Matrix Metalloproteinase

Matrix metalloproteinases (MMPs) are enzymes which are associated with the extracellular matrix (ECM) with the ability to degrade collagen and elastin (Vincenti, 2001). Zinc ions are present in the catalytic as well as the activation sites in the structure of pro-MMPs, where the cysteine residue releases the zinc ion in the active site resulting in enzyme activity and a release of the pro-peptide (Fridman et al., 2003). Calcium is also essential in MMPs for substrate recognition by changing the molecular structure of MMP (Meraz-Cruz et al., 2006).

This is supported in the study conducted by Koulicoff et al. (2023) where changes were observed in collagen structure and aggrecan fragmentation due to MMPs activity during extended aging of beef longissimus lumborum (LL), gluteus medius (GM), and gastrocnemius (GT). Similarly, Kubota et al. (2003) found increased collagen solubilization in chilled storage of flounder due to continuous MMP-9 activity. Sylvestre et al. (2002) suggested MMP-2 likely degrades collagen in lamb longissimus and semimembranosus (SM) muscle due to its activity present through 21 days of postmortem aging. This is confirmed by Pambuka et al. (2007) who aged ostrich meat for 21 days and found an overall increase in MMP activity over the aging period. The impact MMPs have on meat quality have not been extensively studied (Sentandreu et al., 2002); however, its ability to degrade connective tissue postmortem provides the potential to reduce background toughness in meat (Purslow, 2018).

3. Cathepsin

3.1 Background

Cathepsins are a type of lysosomal protease which a slightly acidic environment is required for its optimal activity (Turk et al., 2012). The cathepsins are first synthesized as

proenzymes before activating through cleavage of the N-terminal propeptide (Sentandreu et al., 2002). Cysteine cathepsins are predominantly endopeptidases; however, they may also exhibit other activity such as carboxydipeptidases (cathepsin B) or aminodipeptidases (cathepsin C), which determines how substrates are cleaved (Turk et al., 2012). The most abundant cathepsins in muscles include aspartic cathepsin D as well as the cysteine cathepsins B, H, and L (Boland et al., 2018). In living tissue, cathepsins play key roles in protein degradation, energy metabolism, and immune responses (Yadati et al., 2020), both inside and outside of the cell (Mendoza-Palomares et al., 2008). Unlike calpain which degrades Z-line proteins, cathepsins attack collagen (Burleigh et al., 1974), myosin, actin, myosin heavy chains, and troponin (Jiang, 1998). Because of cathepsin's unique degradation of stromal and myofibrillar proteins, improving native cathepsin activity may assist in improving meat tenderness.

3.2 Regulation

Similar to other enzymes, cathepsin activity is affected by many factors in the environment. Zeece et al. (1986) incubated bovine myofibrils and increased pH from 5.5 to 7.5 and decreased the temperature from 37°C to 15°C, which they reported these modifications significantly reduced cathepsin activity in the myofibril. On the contrary, Beltrán et al. (1997) reported no differences in cathepsin activity in bovine LT at a higher ultimate pH (pH > 6.3). Lomiwes et al. (2014) aged beef LD muscles up to 28 days at -1°C and categorized them into high (pH ≥6.2), intermediate (pH 5.8-6.19), and low (pH ≤ 5.79) ultimate pH, which they reported the highest cathepsin activity was found in the lower pH samples. Cathepsins are found in the lysosome, where the environment is acidic (Casey et al., 2010). Aranishi et al. (1997) purified cathepsin from carp (*cyprinus carpio*) and measured the peptide hydrolyzing activity of

the cathepsin from pH 4 to 8 and temperature range from 20 to 70°C. They found the carp cathepsin's optimal pH was 6 and the optimal temperature was 45°C, which the same optimal temperature of cathepsin was also confirmed in sea cucumber by Sun et al. (2011). However, Sharma et al. (2007) purified cathepsin from goat brain and reported a higher optimal temperature of 55°C after measuring within the range of 20 to 70°C. Finally, Hu et al. (2023) utilized cathepsin purified from beef spleen to measure peptide hydrolysis activity and found a significant decrease in cathepsin activity from 60°C to 80°C due to denaturation of the enzyme's structure.

Toldrá and Etherington (1988) reported the activity of cathepsin B and L decreased by 40-79% after 20 days of storage but cathepsin D was an exception and did not change. However, Gil et al. (1998) described no differences in cathepsin activity over the 14 days storage period at 4°C in pork longissimus muscles. These results agree with the description by Kaur et al. (2020) who reported no change in cathepsin activity in beef brisket during storage for 14 days at 4 °C. After thawing mackerel surimi samples by water from frozen storage of -40°C, Jiang et al. (1997) found cathepsin activity decreased but only by about 20%, which suggested stability of the enzyme in cool environments.

Metal ions concentration can also affect cathepsin activity. Shen et al. (2021) found zinc ion can inhibit cathepsin activity in golden pompano followed by iron, copper, and calcium. Zhang et al. (2020) partially substituted the salt with different combinations of metal ions in dry-cured pork butts and found partial inhibition of cathepsin with the addition of zinc chloride. Jiang et al. (1994) also used dry-cured pork butts as a model and found zinc chloride partially inhibited activity, but magnesium chloride increased cathepsin's activity. Although there are many research published on how pH, temperature, storage, and presence of metal ions can affect

cathepsin activity in meat products, there are still many unanswered questions that needed further exploration on the topic.

3.3 Cathepsin Affects Meat Tenderness

Understanding the mechanism behind cathepsins' activity in meat may lead to improved tenderness for lower quality meat cuts. Cathepsin's role in postmortem proteolysis is contradictory in literature. Due to their lysosomal location, Hopkins and Taylor (2004) speculated cathepsins are not fully released during the cooler aging process thus do not contribute to tenderness development. This idea is challenged by Kemp et al. (2010) describing that the membrane of the lysosome can be disrupted by low pH levels of postmortem muscle. When cathepsin is added to beef in the form of freeze dried beef spleen extract, Cohen et al. (1982) found degradation in the sarcolemma, which led to myofibrillar protein degradation and leakage of sarcoplasmic proteins. Furthermore, Calkins and Seideman (1988) concluded that cathepsin accounted for 35 to 58% of the variation in tenderness of 14 days cooler aged beef muscles. In contrast, Uytterhaegen et al. (1992) described cathepsins did not play a role in tenderization of beef samples aged for 12 days.

Although it is unclear if the cooler aging environment can support notable cathepsin activity in meat or not, it is clear that cathepsin activity can be stimulated by minor heat treatments. Sous vide cooking methods are becoming popular as a way to increase meat tenderness (Modzelewska-Kapituła et al., 2019), which typically entails vacuum-packaging and heating food products in warm water bath (Bailey, 2018). Kurth (1986) and Buckow et al. (2010) utilized pressure-heat treatments at 60°C, which stimulated cathepsin activity due to heat. Sikes et al. (2010) demonstrated that there was improved tenderness in steaks subjected to these

treatments. Christensen et al. (2013) conducted sous vide cookery on beef semitendinosus up to 19 ½ hours at 53°C and 63°C and concluded a change in connective tissue properties due to cathepsin activity leading to no differences in WBSF between young bulls (10-12 months old) and cows (4-6 years old) at these temperatures and time points. Kaur et al. (2020) suggested the improvement in meat tenderness from sous vide cooking is due to improved cathepsin activity hydrolyzing collagen peptides that were already weakened by the moderate heat. Davey and Gilbert (1976) found shear force values decreased as sous vide temperature increased to 65°C; however, above this temperature cathepsin starts to denature and will not degraded proteins as efficiently (Davey & Niederer, 1977). Burleigh et al. (1974) purified cathepsin from human liver and found the enzyme degraded soluble and insoluble collagen extracted from rat liver, and Etherington (1976) similarly found cathepsin purified from beef spleen degraded collagen preferentially at 28°C. Because of the high level of activity cathepsins exhibit in heat up to 70°C (Spanier et al., 1990), sous vide at lower temperatures has the potential to improve tenderness (E. Tornberg, 2005).

Because of the effectiveness of cathepsin activity to improve meat tenderness, other methods are researched to further enhance its tenderizing effect. Chotigavin et al. (2023) combined sous vide at 60°C with pressure at 101, 200, or 300 kPa for up to 4 hours and reported higher tenderness in samples cooked under higher pressure. On the other hand, Chian et al. (2021) used a combination of shockwave processing at 11 kJ/pulse and sous vide cooking at 60°C for 12 hours on beef briskets and reported no differences in the structure of the connective tissue with the addition of shockwave processing but instead saw higher disruption of the myofibrils. Hyperbaric storage is a new storage method involving high pressure with the main goal to inhibit microbial growth (Moreira et al., 2015) and is proposed to improve safety and

quality of food products with the potential to replace refrigeration (Fernandes et al., 2015). Santos et al. (2021) reported higher cathepsin activity in ground beef that was in hyperbaric storage with conditions of 60 mPa at 10°C and 75 MPa at 25°C rather than refrigeration at 0.1 MPa at 4°C, which suggests a possible role of hyperbaric storage in increasing tenderness of whole muscle.

4. Conclusion

The traditional method to enhance enzymatic activity is through cooler aging, and calpain has been extensively researched as the main contributor for the development of meat tenderness during the cooler aging process. However, other enzymes are suggested to play a role in tenderness development such as caspase, the 20S proteasome, MMPs, and cathepsin. Enzymatic activity is influenced by external factors to where modifying the temperature or pH can significantly affect its ability to degrade substrates. Because cooler aging can take weeks to complete, there is an increase in interest in other methods that require minimal process to improve tenderness. Cathepsin is gaining popularity due to its strong activity in warm temperature treatments, and maximizing this activity can potentially increase tenderness of meat products in a short period of time (hours instead of days). Accelerated aging (AA) is a new technique proposed to increase meat tenderness by enhancing cathepsin activity using a warm water bath over a short period of time. Steaks which are AA can be frozen or kept at 4°C before they are grilled to the desired internal temperature. Therefore, this research aims to evaluate the effect of AA at different temperature and time points on shelf life, yield, sensory characteristics, collagen characteristics, myofibrillar degradation, and cathepsin activity.

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Chapter 2 - The effect of accelerated aging at different temperature and time points on shelf-stability, yield, sensory characteristics, cathepsin enzyme activity, and structure of collagen and troponin-T in lower quality beef cuts

1. Introduction

It has been well established that consumers place monetary value on tenderness of beef (Miller et al., 2001); however, Sullivan and Calkins (2011) showed that only around 17.5% of all beef muscles are considered tender. With the recent increase in beef prices (USDA, 2021), consumers are more interested in seeking out non-traditional beef cuts to offset the high cost of beef (Tonsor & Lusk, 2022). Unfortunately, many of these non-traditional beef cuts require further processing to achieve acceptable palatability (Brooks et al., 2000). Chun et al. (2020) described tenderness differences among muscles were driven by multiple factors including lipid content, connective tissue characteristics and myofiber structure integrity etc. In lower quality beef cuts, connective tissue may be the most important factor for tenderness determination. For example, higher collagen content, lower collagen solubility, and increased mature collagen crosslinks density have all been linked to increased toughness in beef (Li et al., 2022).

Cooler aging is an established method to improve tenderness by allowing the native proteolytic activity in beef to take its course during storage (Calkins & Seideman, 1988); however, it can take anywhere between 7 to 42 days of cooler storage time to reach the desired tenderness in tougher cuts (Bhat et al., 2018). Therefore, many alternative strategies have been proposed to quickly enhance the tenderness of beef (Pietrasik & Shand, 2005). And accelerated aging (AA) is a newer concept to enhance tenderness of beef in the culinary community

(Khoury, 2020). The proposed process is simple. Vacuum packaged beef cuts are incubated in warm water bath at ~50℃ for ~2 hours to enhance the enzyme activity and accelerate the aging process. After AA, the steaks can be immediately frozen or refrigerated until it is ready to be cooked. The meat is then cooked with the intended dry heat cookery method such as grilling or pan frying to the desired degree of doneness.

Based on past research in sous vide cooking, it has been hypothesized that the enzyme cathepsin is the main contributor to the increased tenderness seen in sous vide/AA steaks as cathepsin activity is optimized at these higher temperature (Dominguez-Hernandez & Ertbjerg, 2021). On the other hand, there is no study to the best of the author's knowledge on the effects of AA on meat quality and safety. Therefore, the objective of this study was to determine the effect of AA at different temperature and time points on shelf life, sensory properties, yield, degradation of myofibrillar proteins, and collagen structure.

2. Materials and Methods

2.1 Sample Collection

Ten USDA choice carcasses were selected, and the shoulder clod (NAMP #114) and top round (NAMP #169) was collected from both sides at 2 days postmortem from a Midwest beef processor, vacuum packaged, and transported to Kansas State University meat lab. The next day, purge was aseptically collected from the primals into a 50 mL conical tube, and all accessory muscles were removed from the shoulder clod and top round leaving only the triceps brachii (TB) and semimembranosus (SM), respectively. The TB and SM from both sides of each carcass were further fabricated from anterior to posterior into eighteen 2.54 cm steaks, and the epimysium was removed. The steaks were vacuum packaged and assigned to one of six treatments: 1) 3 days aged (control); 2) 21 days cooler aged at 2 ± 2°C; 3) AA 49°C for 2 hours;

4) AA 49°C for 3 hours; 5) AA 54°C for 2 hours; 6) AA 54°C for 3 hours (Figure 1). Prior to fabrication, all equipment and tabletop surfaces were cleaned with 70% ethanol 30 min prior to fabrication to minimize microbial contamination.

Steaks designated for cooler aging were vacuum packaged and aged at 2 ± 2°C for 21 days. For the AA treatments, ten separate sous vide systems (AN500-10, 1000 W, Anova Applied Electronics, San Francisco, CA) were utilized. The AA 49°C treatments were conducted first before adjusting the sous vide system to 54°C for the AA 54°C treatments. After incubation, steaks were submerged in an ice water bath for 10 min. The sous vide treatments were not applied to the control and cooler aged treatments.

Purge was aseptically collected from steaks designated for WBSF/microbial analysis into a 15 mL conical tube and were swabbed with the 3M Environmental Scrub Sampler Stick with neutralizing buffer (3M, Saint Paul, Minnesota) on the posterior side prior to AA and on the anterior side after AA. After collecting purge for microbial analysis, the samples were re-vacuum packaged and post-AA loss was determined by the following equation:

$$Post\ AA\ loss\ (\%) = (1 - \frac{weight\ after\ AA}{weight\ before\ AA}) \times 100$$

Samples designated for shear force and sensory analysis were immediately frozen at -20°C. The biochemical samples were cut into cubes, snap frozen in liquid nitrogen, and pulverized to a fine powder using commercial blenders (model 51BL32, Waring Commercial, Torring, CT) before storage in -80°C. Purge was also collected from steaks designated for biochemical analysis in 15 mL polypropylene conical tube and stored in -80°C for collagen content analysis.

2.2 Microbial Analysis

All swabs and purge were kept at 4°C overnight prior to plating. Prior to plating, the swabs were hand stomached to release the buffer. For both purge and swabs, samples were serially diluted 1:10 to 10^{-3} in 9 mL of sterile 1X PBS. One mL from each dilution (10^{-1}, 10^{-2} or 10^{-3}) were enumerated in duplicate using APC petrifilm (FB1171, 3M, Saint Paul, Minnesota) at 37°C for 48 hours. The 3M interpretation guide was followed for counting, and colonies were transformed to log CFU/mL.

2.3 Objective color measurements

Prior to pulverization, steaks designated for biochemical analysis were overwrapped with plastic wrap and allowed to bloom for 30 min. A Hunter Lab Miniscan spectrophotometer (D65, 2.54 cm aperture, 10° observer, Hunter Lab Associates Laboratory, Reston, VA) was utilized to collect L* (lightness), a* (redness), and b* (yellowness) values by following methods outlined by the AMSA Color Guidelines (King et al., 2022). Each sample was scanned 6 times at various locations of the steak, and the color values were averaged.

2.4 Trained panel

The training procedure followed closely to the protocol described by Farmer et al. (2022) as well as the AMSA Research Guidelines for Cookery and Evaluation (AMSA, 2016). Briefly, four training sessions were conducted over 1-week period, and panelists were selected from Kansas State University meat science faculty, graduate students, and staff. During the training sessions, panelists were trained to assess initial juiciness, sustained juiciness, myofibrillar tenderness, connective tissue amount, overall tenderness, beef flavor intensity, and off-flavor of four different beef muscles (psoas major, longissimus lumborum, TB, and SM) cooked at

different temperatures in the range of 55°C to 77°C representing rare to well-done in order to create variation and anchor panelists on the various scales.

A total of 20 trained panels were conducted with six samples served in each panel. Steaks were thawed at 2 ± 2°C for 24 hours. Steaks were grilled on Cuisine Art Griddle Deluxe Clamshells (Cuisine Art, Stamford, CT) to an internal temperature of 71°C, and the internal temperature was monitored by piercing at the geometric center with a thermometer probe (Thermapen MK4; Thermoworks, American Fork, UT). After 3 min of rest time, cooked steaks were cut into 1.3 cm x 1.3 cm x 2.54 cm cubes, and samples were served to eight panelists in a predetermined order. Each panelist was provided with a tablet, napkins, an expectorant cup, a water cup, apple slices, and unsalted crackers in their booth. Each panelist was given two cubes of a warm-up sample to standardize the pallet before receiving the six experimental samples. Steaks were rated on a continuous line scale on electric tablets (Lenovo TB-8505F, Morrisville, NC) with a digital survey (Qualtrics Software, Provo, UT, USA) for initial juiciness, sustained juiciness, myofibrillar tenderness, connective tissue amount, overall tenderness, beef flavor intensity, and off-flavor. The descriptive terms (0 = extremely dry/extremely tough/none present/extremely bland; 50 = neither dry nor juicy/neither tough nor tender/moderate amount/mild flavor; 100 = extremely juicy/extremely tender/ extremely abundant/extremely intense flavor) described the midpoint and both ends of the anchor.

2.5 Warner-Bratzler shear force (WBSF)

The WBSF procedure followed closely to the procedure described in AMSA Research Guidelines for Cookery and Evaluation (AMSA, 2016). Steaks were prepared the same as for the trained panels; however, steaks were covered with plastic wrap and cooled at 2 ± 2°C for 24 h

after cooking. Before and after cooking, steaks were weighed to calculate cook loss with the following equation:

$$Cook\ Loss\ (\%) = \frac{(raw\ weight - cooked\ weight)}{raw\ weight} \times 100$$

Six cores were taken parallel to the muscle fiber at a 1.27cm diameter. An Instron Universal Testing System (Model5569; Instron Corporation, Norwood, MA) with a V-shaped blade set to a speed of 250 mm/min with a load cell of 100 kg was used to shear cores perpendicular to the muscle fiber. The shear force (kgf) values for the six cores were averaged to calculate the final WBSF for each sample.

2.6 Proximate Analysis

For moisture analysis, aluminum pans were labeled and pre-dried in a forced-air oven (Isotemp, Thermo Fisher Scientific, Pittsburg, PA, USA) set to 100°C for 30 min and weighed. The exact weight of ~5 g of powdered sample was weighed into the aluminum pan and recorded. Wet pans were transferred to the same forced-air oven set to 100°C for 24 h and cooled for 30 min in a desiccator before the final weights were obtained. Percentage moisture was calculated through the difference of wet and dry sample weights over the wet weight of the sample.

Lipid content was obtained through the method described by Folch et al. (1957) with modifications. Glass tubes (12 x 75 mm) were labeled and dried for 30 min in a forced air oven (Isotemp, Thermo Fisher Scientific) set to 100°C before recording the weight. Exact measurements of ~0.5 g of powdered sample were measured into 15 mL polypropylene conical tubes, and 1.6 mL of ultrapure water, 4 mL chloroform, and 4 mL methanol was added. Samples were shaken in a Wrist Action Shaker (Model; 75 Burrel Corporation, Pittsburg, PA, USA) for 10 min before the addition of 2 mL of 0.74% KCl solution in ultrapure water. Samples were centrifuged at 1,000 x g for 5 min, and 1 mL of chloroform was extracted from the bottom layer

and transferred to a pre-dried 12 x 75 mm glass tube. A nitrogen evaporator (REACTI-VAP III #TS-18826, Thermo Fisher Scientific) was used to evaporate the chloroform, and the tubes containing the lipids were transferred into the same forced-air oven set to 100°C for 30 min to evaporate potential moisture picked up through the process. Lipid percent was calculated by dividing the calculated lipid weight over the sample weight x 100.

For protein content, exact weights of ~0.5 g of powdered sample was recorded in a ceramic crucible and loaded into the TruMac N (LECO Corporation, St. Joseph, MI) calibrated using EDTA standards. Percent protein was calculated by multiplying percent nitrogen by the nitrogen conversion factor of 6.25.

2.7 Lipid oxidation

Lipid oxidation was measured by 2-thiobarbituric acid reactive substance (TBARS) analysis. Exact weights of ~0.3 g of powdered samples in pre-filled bead homogenizer tubes were recorded, and 1.4 mL of thiobarbituric acid: trichloroacetic acid solution (TBA/TCA; 20 mM: 15% in ultrapure water) and 0.1 mL of 3% butylated hydroxytoluene (BHT) in ethanol was added. Samples were homogenized in a bead homogenizer (Bead Blaster 24; Benchmark Scientific) at 6.5 m/second for 45 seconds, and the content was transferred to a 1.5 mL microcentrifuge tube. Samples were centrifuged (Centrifuge 5810 R; Eppendorf, Hamburg, Germany) at 4,000 x g for 5 min, and 1 mL of sample was transferred into 12 x 75 mm glass tubes. The glass tubes were incubated in a hot water bath (model 2872, Precision, Thermo Fisher Scientific) set at 70°C for 30 min and cooled in a cold-water bath for 5 min. A 96 wells plate was prepared and 200 µL of sample was pipetted and read using a spectrophotometer (Eon; BioTek Instruments Inc., Winooski, Vermont) at 532 nm. The sample malondialdehyde (MDA) concentration was calculated by using a standard curve containing 0-25 µM of malondialdehyde

bis (156731000, Thermo Fisher Scientific), and the final sample was expressed as mg MDA per kg of muscle tissue.

2.8 Sarcoplasmic protein extraction

One and a half mL of extraction buffer (20 mM Tris-HCl, 125 mM NaCl, 1% Triton X) was added to 0.2 g of powdered sample in prefilled bead homogenizer tubes and homogenized using a bead homogenizer (Bead Blaster 24; Benchmark Scientific) at 6.5 m/second for 30 seconds. Samples were centrifuged (Centrifuge 5810R; Eppendorf) at 16,000 x g in 4°C for 20 min, and the supernatant was collected and designated as the sarcoplasmic protein stock. Protein concentrations were determined by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instruction. The concentrations of the sarcoplasmic protein stock was adjusted to 2,000 µg protein/mL with extraction buffer.

2.9 Cathepsin zymography

Adjusted sarcoplasmic protein samples were mixed with Laemmli non-reducing SDS sample buffer (J63615; Alfa Aesar, Haverhill, MA) at a 1:1 ratio. In all gelatin gels used for the zymography (Novex Zymogram 10% Plus Protein Gels, Invitrogen, Carlsbad, CA), the first well was loaded with 5 µL of protein ladder (#83-660, Prometheus ProSignal Full-Range Prestained Protein Ladder, Merck & Co., Rahway, NJ), the second well was loaded with 0.3 µg of cathepsin B reference, and the third well was loaded with 30 µg of a reference sample (a 3-day aged TB). Thirty µg of sarcoplasmic protein from each sample was loaded into each remaining well, and proteins were separated by gel electrophoresis in a Mini Gel Tank (Invitrogen) filled with tris-glycine SDS running buffer at a constant 125 volts for 180 min at 4°C. After separation, gels were incubated twice in a renaturing buffer (65 mM Tris base, 20% v/v glycerol, pH 7.4) at room temperature for 30 min. After decanting the renaturing buffer, gels were equilibrated with

activity buffer (50 mM sodium acetate, 100 mM sodium chloride, 5 mM EDTA, 2 mM DTT, at a pH of 5.5) for 30 min, followed by replacing with a fresh set of activity buffer and incubated at 37°C for 24 hrs. Following incubation, gels were washed in ultrapure water for 10 min before staining with Coomassie blue (0.2% Coomassie blue G250, 40% methanol, 10% glacial acetic acid, and 50% water) for 30 min. Gels were destained with destaining solution (30% methanol, 10% acetic acid, and 60% water) first three times for 5 min each, then two more times for 30 min each. Gels were rinsed with ultrapure water for 5 min before images were taken using the iBright Imaging System (FL1500, Thermo Fisher Scientific) and analyzed with the iBright Analysis Software (Thermo Fisher Scientific). Three distinct bands were present (Figure #2) where band 1 was at ~60 kDa, band 2 was at ~50 kDa, and band 3 was at ~30 kDa. Relative activity was calculated by dividing band intensity by the band intensity of the reference sample.

2.10 Myofibrillar protein extraction

Myofibrillar proteins were extracted using the method described by Hammond et al. (2022). Approximately 0.2 g of powdered sample was weighed into pre-filled bead homogenizer tubes, and 1.5 mL of ice cold, ultrapure water was added and homogenized at 6.5 m/second for 30 seconds. Samples were transferred into 1.5 mL microcentrifuge tubes and centrifuged (Centrifuge 5810R; Eppendorf) at 4,000 x g in 4°C for 5 min. The supernatant was discarded, and pellet was re-suspended in 1 mL ice cold, ultrapure water and re-centrifuged (Centrifuge 5810R; Eppendorf). The rinsing step was repeated three times to remove sarcoplasmic proteins. After centrifuging the last repetition, all visible liquid was removed and 1 mL of extraction buffer (0.1 M Tris-HCl, 1.25 mM EDTA, 2% SDS buffer) was added and vortexed. The samples were centrifuged (Centrifuge 5810R; Eppendorf) at 4,000 x g for 5 min, and the supernatant was transferred to a new tube and designated as the myofibrillar protein stock. Protein concentrations

were determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's ins, then concentrations were adjusted to 2,000 µg/mL with extraction buffer.

2.11 Troponin-T degradation

Adjusted myofibrillar protein samples were added to 2x Laemmli reducing SDS sample buffer (Alfa Aesar, Haverhill, MA) at a 1:1 ratio, and heated at 95°C for 5 min. Five µg of protein was loaded into a 10% tris-glycine gel (Thermo Fisher Scientific), and myofibrillar proteins were separated by gel electrophoresis in a Mini Gel Tank (Invitrogen) filled with tris-glycine SDS running buffer at a constant 180 volts for 50 min. The separated proteins on the gels were transferred to a polyvinylidene difluoride (PVDF) membrane using a transfer pack (iBlot 2 PVDF Regular Stacks, IB24001, Invitrogen) in an iBlot 2 Gel Transfer Device (Invitrogen) set to 20 V for one min, 23 V for four min, and 25 V for two min for the transfer. Following the transfer, membranes were blocked in OneBlock Western-FL Blocking Buffer (Genesee Scientific) overnight, and incubated in anti-troponin-T IgG1 primary antibody from mouse (JLT-12; Booster Bio, Pleasanton, CA) at a 1:2,000 dilution in blocking buffer (Genesee Scientific) for two hours. The membrane was washed in 1x TBS-Tween for five min, and this was repeated three times before washing once in TBS for five min. The Goat Anti-Mouse IgG Alexa Fluor Plus 488 (A32723, Invitrogen) secondary antibody was added to the membrane at a 1:2,000 dilution in blocking buffer (Genesee Scientific) for one hour. The membrane was washed in 1x TBS-Tween for 5 min, repeated three times before washing with TBS once for 5 min. Images were taken with a chemiluminescent mode using the iBright FL1500 imager (Thermo Fisher Scientific) and analyzed using the iBright Analysis Software (Thermo Fisher Scientific). The

percent of troponin-T degraded was determined by dividing the band intensities of the identified intact or degraded bands (traditional or further degraded) by all bands within the same lane.

2.12 Determination for purge, soluble and insoluble collagen content

Collagen was prepared as described by Avery et al. (2009) with modifications. Exact weights of ~0.1 g of powdered sample in 13 x 100 mm glass tubes were recorded, and 1.2 mL of ultrapure water was added. Samples were incubated at 80°C while shaking in a water bath (model 2872, Thermo Fisher Scientific) for 90 min and centrifuged at 3,000 x g for 12 min at room temperature. The supernatant was transferred to a new 13 x 100 mm glass tube and was identified as the soluble collagen portion, and the pellet was identified as the insoluble fraction. Both the soluble and insoluble portion was further dried at room temperature using a Vacuum Evaporation System (RapidVap; Labconco Corporation, Kansas City, MO, USA) set to 53% speed, 0 mbar vacuum, for 1-2 hours. The soluble portion was hydrolyzed in 1 mL, and the insoluble portion was hydrolyzed in 2 mL of 6N HCl in a forced air oven (Isotemp, Thermo Fisher Scientific) set to 115°C for 24 hours. After hydrolysis, the HCl was evaporated out at room temperature using the Vacuum Evaporation System (RapidVap; Labconco Corporation) set to 53% speed, 0 mbar vacuum, and run until the HCl was completely evaporated (~ 5-6 hours). To calculate total collagen content in the purge, 1 mL of purge was evaporated and hydrolyzed following the same procedure as described for soluble collagen. The dried insoluble collagen, soluble collagen, and purge were rehydrated in 500 µL of ultrapure water, then stored at -80°C until hydroxyproline analysis.

Collagen concentration was determined by hydroxyproline as described by Hammond et al. (2022) with modifications. Ten µL of the rehydrated insoluble sample was diluted 1:200 in ultrapure water in 16 x 100 mm glass tubes. For the soluble and purge samples, 50 µL of the

rehydrated sample was diluted 1:40 in ultrapure water in 16 x 100 mm glass tubes. Two mL of diluted purge/soluble/insoluble collagen samples were incubated in 1 mL of the Chloramine-T oxidant reagent (6 mM of chloramine-T hydrate in buffer solution; 140 mM citric acid monohydrate, 37.5 mM sodium hydroxide, 660 mM sodium acetate trihydrate, and 29% 1-propanol, pH 6.0) for 20 min at room temperature, followed by adding 1 mL of dimethylaminobenzaldehyde (DMBA) color reagent (60 mM DMBA, dissolved in 21% perchloric acid, 65% two-propanol, and 14% ultrapure water) and incubated at 60°C in a water bath (model 2872, Precision, Thermo Fisher Scientific) for 30 min. After removal from the water bath, the tubes were cooled in a cold water bath for 3 min, and 200 µL of the content was pipetted into a 96 well plate in duplicate. Plates were read using a spectrophotometer equipped with a spectrophotometer (Eon; BioTek Instruments Inc.) set to 558 nm, and hydroxyproline was calculated by using a standard curve containing 0-3 µg/mL of trans-4-Hydroxy-L-proline (121780100, Thermo Fisher Scientific). The collagen was calculated using a conversion factor of 7.14 from the calculated hydroxyproline content, and the final sample was expressed as mg collagen per g of muscle tissue.

2.13 Perimysial Peak Transitional Temperature and Enthalpy Measurement

Perimysial fraction extraction was determined through the method described by Light and Champion (1984) with modifications. Five hundred mg of powdered sample was weighed in pre-filled bead tubes and one mL of 0.05 M $CaCl_2$ was added before homogenization using a bead homogenizer (Bead Blaster 24, Benchmark Scientific) at 6.5 m/second for 30 seconds. The perimysial fraction was collected after samples were filtered through a 1 mm sieve (VWR International, Radnor, PA) and transferred into a microcentrifuge tube containing 1X PBS. Samples were blotted with filter paper to remove unbound water and five to ten mg was weighed

into a TZero Pan (TA Instruments, New Castle, DE) and sealed with a TZero hermetic lid (TA Instruments). Both sample and reference pans underwent a temperature program which heat the sample at 5°C/min increments starting with room temperature and ending at 80°C using a differential scanning calorimeter (DSC Q1000; TA Instruments). Data was analyzed using the TA Universal Analysis Software (TA Instruments) to determine enthalpy, onset temperature, and peak transitional temperature (Figure 4).

2.14 Statistical Analysis

All data was analyzed using the PROC GLIMMIX procedure in SAS as a split-plot. The model included the muscles as the whole-plot factor, the treatments as the sub-plot factor, and the muscle × treatment interaction. The experimental unit was each individual carcass, and the degrees of freedom were estimated using the satterhwaite approximation. The least significant difference was used to detect differences among means at 5% level.

3. Results and Discussion

3.1 Microbiological analysis

There were no interactions between muscles and treatment in swab APC ($P > 0.05$). However, there was a main effect found for treatment which showed a reduction in APC on the steak surface after AA regardless of treatments ($P < 0.01$; Table 2). However, samples from AA 54 °C for 3 hours exhibited an increase in swab APC compared to those from AA 54 °C for 2 hours and AA 49 °C for 3 hours ($P < 0.05$) but was not different from the swab APC from AA 49 °C for 2 hours ($P > 0.05$). Furthermore, there were interactions between muscles and treatments for purge APC ($P < 0.01$; Table 1). Prior to AA, purge from TB had greater purge APC than those found in SM purge ($P < 0.01$); however, a reduction in purge APC was found after AA regardless of treatment or muscle types ($P < 0.01$).

The biggest issue identified by industry professionals in AA have been the meat submerged within the "Food Temperature Danger Zone (5 – 60 °C)" (Hajmeer & Cliver, 2002) for an extended time period of time, which could lead to safety and shelf-stability concerns. Kilibarda et al. (2018) described sous vide cooking should not be performed under 54.4°C and should be maintained for 6 hours at minimum to properly reduce the growth of pathogenic bacteria. However, Stringer and Metris (2018) emphasized the lack of information available on bacterial behavior during mild sous vide cooking. Therefore, one of the main objectives of this study was to understand the effects of the AA treatments on general microbial growth.

The results from this study indicated that even at 49°C for only 2 hours, aerobic bacterial counts significantly decreased. This is further supported by Vaudagna et al. (2002) who sous vided beef semitendinosus (ST) muscles at 50 – 65 °C for 1.5 - 6.5 hours and found that even sous vided at 50 °C for 1.5 hours can decrease microbial counts. Furthermore, Salaseviciene et al. (2014) also demonstrated a reduction in bacterial counts when they sous vided pork longissimus dorsi muscles at 53°C for 5 hours. These results indicated that AA at 49/54°C for 2/3 hours will not boost microbial growth, with evidence to perhaps even decrease microbial growth. Finally, AA treated steaks are meant to be subjected to additional heat treatments such as grilling or pan frying, so AA should not be considered as a process which may increase food safety risk.

3.2 Objective color

There were interactions between muscle and treatments for both L* ($P < 0.05$) and a* values ($P < 0.01$; Table 1). The L* values for TB were highest for AA 49°C for 2/3 hours and AA 54°C for 2 hours. This is followed by AA 54°C for 3 hours with the lowest L* in the control and cooler aged samples ($P < 0.01$). The L* values for SM were highest for AA 49°C for 2

hours, followed by AA 49°C for 3 hours and AA 54°C for 2/3 hours. The cooler aged and control had the lowest L* values in the SM ($P < 0.05$). Between the muscles, L* was highest in the AA 49°C for 3 hours and AA 54°C for 2 hours samples. Furthermore, a* values for TB were highest for the control and AA 49°C for 2/3 hours samples, followed by the cooler aged samples, with the lowest a* values in the AA 54°C 2/3 hours samples ($P < 0.01$). The a* values for SM were highest for the control and AA 49°C for 2 hours, followed by AA 49°C for 3 hours and AA 54°C for 2 hours samples, and the cooler aged and AA 54°C for 3 hours samples were the lowest ($P < 0.05$). Between the muscles, a* was highest in the SM for the control samples; however a* was highest in the TB for the 21 d cooler aged samples. There were no interactions between muscle and treatment for b* values ($P > 0.05$). However, there was a treatment effect ($P < 0.01$; Table 2) and a muscle effect ($P < 0.01$; Table 3). The highest b* values were found in AA 49°C for 2/3 hours and AA 54°C for 2 hours samples, followed by AA 54°C for 3 hours, then the control, with the lowest b* values in cooler aged samples ($P < 0.05$). Between the muscles, SM had higher b* values than the TB ($P < 0.01$).

Similar to our findings, García-Segovia et al. (2007) reported higher L* values in beef pectoral muscles sous vided at 70°C for 15 - 60 min as compared to the raw samples. Furthermore, Roldán et al. (2013) also found that lamb loins sous vided at higher temperatures (in this case 80°C) were darker than those sous vided at lower temperature (60°C). However, both Gámbaro et al. (2023) and Becker et al. (2016) found no differences in L* values among beef hind shanks sous vided at 55-75°C for 2-24 hours or pork loins sous vided at 53-58°C for 20 hours, respectively. Bertram et al. (2006) described that myosin heads denature extensively between the temperature range of 40 - 50 °C, which can affect the interaction between myofibrillar proteins and the water holding capacity of meat, thus resulted in more free water

present on the meat surface. Rees et al. (2003) further explained that when there is water present on the meat surface, it could reflect more light, thus increase in L* values readings. Finally, Purslow et al. (2020) and Swatland (2004) further confirmed sarcoplasmic and myofibrillar proteins denaturation are likely to contribute to scattering of light, which can also result in increased lightness in cooked meat products.

Overall, our a* results are in agreement with the a* values reported by many sous vide studies. Roldán et al. (2013) showed that lamb loins decreased in a* values when sous vided in higher temperatures (70-80°C) compared to the lower temperature (60°C). On the other hand, Christensen et al. (2012) reported decreased redness in sous vide cooked ST from beef as temperature increased from 53 to 58 °C. Gámbaro et al. (2023) further stated that the decreased a* values are correlated with higher percentage of denatured myoglobin as sous vide temperature and time increased. It was interesting to note that the AA 49°C for 2 hours treatment did not affect a* as compared to the control in this present study. Sen et al. (2014) showed that only 25.7% of myoglobin was denatured at 51 °C, and Moriyama and Takeda (2010) described that the helical structures of myoglobin are perfectly reformed upon cooling to 25 °C from heat treatment below 75 °C. This indicated that AA at lower temperature may not affect redness of meat, which is indicator of wholesomeness for the consumers (Font-i-Furnols & Guerrero, 2014).

Again, Roldán et al. (2013) found that b* values increased when lamb loins were subjected to higher temperatures and longer heating times, and García-Segovia et al. (2007) also reported increased b* in beef pectoral muscles sous vided for any amount of time (15-60 min) at any temperature (60-80°C) when compared to those for the raw samples. Both Farmer et al. (2022) and Lybarger (2022) found meat color as one of the most important factors in consumer

purchasing decisions for both ground beef and fabricated short loins. Although it is clear that AA at higher temperatures and longer time had shown to decrease the redness of beef, it is important to point out that AA treatments are meant to be conducted at consumer households or food services as a post-purchase treatment. Therefore, more research is needed to better determine how AA treatments may affect consumer purchasing decisions.

3.3 Post-AA and cooking loss %

There was an interaction between muscle and treatment for post-AA loss ($P < 0.01$; Table 1). The post-AA loss is defined as % sample loss as purge after the AA treatments. For all AA treatments, the TB samples had less post-AA loss than those for the SM samples ($P < 0.01$). Furthermore, AA 49°C for 2/3 hours samples regardless of muscles had less post-AA treatment loss than those from 54°C for 2/3 hours samples, and AA 54°C for 2 hours samples had less post-AA treatment loss than those for the AA 54°C for 3 hours samples in SM samples only ($P < 0.01$). There were no interactions between muscles and treatments in cooking loss ($P > 0.05$); however, there were main effects for both treatments ($P < 0.01$; Table 2) as well as muscles ($P < 0.01$; Table 3). Cook loss is defined as the % loss of each treated muscle after proper cooking (grilled to an internal temperature of 71 °C in the present study). Interestingly, the least cook loss was found in AA 54°C for 3 hours samples ($P < 0.01$), followed by AA 54°C for 2 hours and AA 49°C for 2/3 hours samples ($P < 0.05$), with the control and cooler aged samples displaying the most cook loss ($P < 0.05$). The TB samples had lower cook loss than those from the SM samples ($P < 0.01$).

Cook loss of meat products is a key factor in production efficiency and sustainability and has huge impact on the economic viability of the production system (Cheng & Sun, 2008). Furthermore, research has shown that meat cook loss is correlated with juiciness of the product,

which impacts consumer satisfaction (Toscas et al., 1999). In the present study, there was greater loss from samples subjected to AA treatments with higher temperature and at longer time periods. García-Segovia et al. (2007) sous vided beef pectoral muscles at 60 to 80°C for 15 to 60 min and also found greater loss in samples heated at higher temperatures for longer periods of time. In addition, Palka and Daun (1999) reported that there was not only an increase in cook loss with increase in cooking temperature of beef ST, but they also noted a decrease in muscle fiber diameter. Similarly, Bendall and Restall (1983) observed a decrease in muscle fiber diameter compared to the raw samples when beef psoas major were heated to 60°C, and the water within the cells were expelled and accumulated between the muscle fiber bundles.

The lower yield at higher temperatures/longer times during AA is likely due to the heat denaturation of myofibrillar proteins with lower heat tolerance, which can lead to the observed lower water holding capacity (Murphy & Marks, 2000). Bertram et al. (2004) reported that myosin denatures as low as 43°C in pork, and this noted myosin denaturation can significantly affect the water-holding capacity within the muscle fiber. On the other hand, both Zhang et al. (2006) and Lawson (2004) reported that increase in fluid loss in pork can also be caused by the degradation of integrin, which binds to connective tissue components. The degradation of integrin can result in the detachment of the cell membrane from the cytoskeletal proteins thus leading to the formation of drip channels (Lawson, 2004).

On the other hand, this present study showed that the samples that have been through higher AA temperature and longer time had less cooking loss after grilling. Hodgson et al. (1991) found that the pork loins with higher moisture content (75%) displayed higher cook loss than those with lower moisture content (70%). Our samples that had gone through AA have lost some moisture (4-12%) from the treatments; therefore, it is likely that these post AA samples had

lower moisture % to begin with thus resulted in less cook loss. Additionally, Vaudagna et al. (2002) has shown that there are two phases of water loss during the cooking of meat, which one phase occurs during 45 - 60 °C, and the other phase of shrinkage occurs from 60 - 90 °C. The AA samples displayed less loss likely due to which the AA samples have already undergone the first water loss phase during the AA treatment, however; the control and cooler aged samples had to go through both phases during the cooking process. Finally, Delgado-Suárez et al. (2016) sourced SM and TB from the US and Mexico and found no differences in cook loss between the two muscles, which differed from our findings.

3.4 Objective Tenderness

There were no interactions between treatments and muscles for WBSF ($P > 0.05$). However, there was a treatment effect (Table 2), where the control had the highest shear force, followed by AA 49°C for 2/3 hours, with the lowest shear force found in the cooler aged and AA 54°C for 2/3 hours samples ($P < 0.01$). There was no muscle effect for WBSF ($P > 0.05$).

Part of the reason for sous vide technology to gain in popularity is due to its ability to increase tenderness of meat products (Uttaro et al., 2019). In our study, all steaks that were AA treated had lower WBSF compared to the control. Similar to our findings, both Jwa et al. (2020) and Franke (2022) reported lower WBSF values in beef striploins that were sous vided at 55 - 57 °C for 1 - 5 hours, then grilled to 71°C compared to the samples that were only grilled without the sous vide treatment. In addition, Naqvi et al. (2021) reported sous vide cooking at higher temperatures (75°C) and longer times (18 hours) resulted in lower WBSF values than those sous vided in lower temperatures (55°C) and shorter times (1 hour), which agreed with our finding as we saw an improvement in tenderness from the 49°C for 2 hours to the AA 54°C for 2/3 hours samples.

Martens et al. (1982) described myofibrillar protein denaturation occurs around 50°C, and meat tenderness usually begins to increase due to collagen denaturation at 50-60°C. The lowered shear force values at AA 54°C than AA 49°C is likely due to temperature induced collagen denaturation. However, our study showed that even the AA 49°C improved sample tenderness compared to the control, and this temperature is not high enough for collagen solubilization to occur. Kathuria et al. (2022) described sous vide as a technology that may be used to improve enzymatic activity in meat products, and cathepsins are believed to be the enzyme responsible for the noted decrease in WBSF for sous vide products due to their known thermal resistance property (Christensen et al., 2011). Therefore, it is likely there were also some enzymatic activity in our AA samples that were modifying the muscle structure in addition to the heat.

3.5 Sensory Analysis

For all sensory parameters, there were no interactions between muscle and treatment present ($P > 0.05$). There was a treatment effect for initial and sustained juiciness (Table 2) where the cooler aged samples were the juiciest, followed by the control, with all AA treatments receiving the lowest juiciness rating ($P < 0.01$). There were no differences among the AA treatments samples for initial or sustained juiciness ($P > 0.05$). There was also a muscle effect (Table 3) where TB samples had higher initial ($P < 0.01$) and sustained ($P < 0.01$) juiciness than those from the SM.

O'Quinn et al. (2018) described consumer acceptance is based upon tenderness, juiciness, and flavor with none more important than the other. Therefore, determining how AA affects these parameters is crucial in the creation of a favorable product for the consumer. It was clear that the AA samples were not as juicy as the control or cooler aged samples, which is in

agreement with Scott (2023) who also found a decrease in juiciness in beef serratus ventralis steaks that were sous vided at 51.6°C for 12 hours as compared to their control or cooler aged at 2°C for 25 days samples. It is important to point out that all steaks used in that study (both sous vide and cooler aged steaks) were further cooked to 71°C prior to the panels similarly to our study. Harris (1976) described juiciness of meat to be influenced by moisture released as well as amount of saliva produced during chewing. During AA, the water holding capacity of the meat is impacted due to denaturation of myosin (Bertram et al., 2006). Perhaps, the additional moisture loss as noted from the AA treatment process negatively impacted the juiciness scores of AA samples.

There was a treatment effect for overall and myofibrillar tenderness ($P < 0.05$; Table 2). The cooler aged samples received the highest tenderness rating followed by AA 54°C for 3 hours, where the control, AA 49°C for 2/3 hours, and AA 54°C for 2 hours samples all received similar tenderness ratings ($P < 0.05$). There was also a muscle effect (Table 3) for muscles where the TB was rated to be more tender than the SM ($P < 0.01$). There was a treatment effect for connective tissue amount ($P < 0.01$; Table 2). The control and AA 49°C for 2/3 hours had the highest connective tissue scores, followed by the AA 54°C for 2 hours, with the cooler aged and AA 54°C for 3 hours samples received the lowest connective tissue scores ($P < 0.01$). There was also a muscle effect (Table 3) where the SM was rated with more connective tissue than the TB ($P < 0.01$).

Gámbaro et al. (2023) also reported similar results where increased tenderness was observed in beef shanks with increased cooking times (2-24 hours) when sous vided at 55-75°C. Furthermore, Park et al. (2020) also observed increased tenderness and decreased perceived connective tissue amount in sous vided chicken breast at 60-70°C for 1-3 hours compared to the

control chicken breast cooked in an oven to 71°C. Although our WBSF result demonstrated tenderness improvement for all AA samples compared to the control, the trained panel showed only a slight improvement in tenderness in the AA 54°C for 3 hours samples but no differences in the other AA treatments in the present study. Bouton et al. (1975) described traditional WBSF measurements to mostly account for the strength of the myofibrils; however, the connective tissue strength may not be accurately represented, thus not accurately reflecting the taste panel tenderness ratings. Shackelford et al. (1995) further demonstrated that shear force measurements were unable to accurately determine the sensory differences among 10 different beef muscles.

The average shear force that consumers are able to distinguish differences in have been recorded by ASTM (2011) to be around 0.5 kg, so the differences in force may not be apparent enough for panelists to detect. The variation in juiciness may have further skewed the differences in tenderness scores between WBSF and the trained panelists. Mathoniere et al. (2000) showed that a panelist's ability to assess tenderness of meat is impeded without ample of liquid present. In a study to create soft meat products for people with swallowing difficulties, Tokifuji et al. (2013) described moisture within the muscle structures contribute to the overall tenderness. Therefore, there is likely a correlation between the loss of moisture and the perceived increase in toughness compared to the shear force values for the AA samples. Finally, it is important to point out Foster et al. (2011) showed that aroma and flavor may influence salivation, which may influence a panelist's perception of tenderness of a meat product. Overall, measuring sensory tenderness is more complex than measuring objective tenderness as there are many factors that can affect a person's perception of tenderness.

Both Panea et al. (2018) and Wu et al. (2021) emphasized the importance of collagen solubility and collagen crosslinking in determining meat texture rather than amount of collagen.

Mortensen et al. (2012) described the highest tenderness would be achieved if steaks are kept at a temperature high enough for collagen solubilization, which EVA Tornberg (2005) describes 53-63°C as the temperature collagen denatures. In the present study, lower connective tissue amount was detected in AA 54°C for 3 hours compared the other AA treatments, which is likely due to an Increase in collagen solubility from the heat (Kong et al., 2008) or potential structural modification from the proposed enzymatic activity (Berge et al., 2001).

In regard to beef flavor intensity, there was a treatment effect ($P < 0.05$; Table 2) where the control and AA 49°C for 2 hours had the most intense flavor, and the AA 54°C for 2/3 hours were the most bland ($P < 0.05$), where the cooler aged and AA 49°C for 3 hours samples were not differ from any of the treatment groups ($P > 0.05$). There was a muscle effect (Table 3) where the TB had a more intense beef flavor than SM ($P < 0.01$). There were no differences in off-flavor intensity ($P > 0.05$).

In the present study, beef flavor intensity was preserved in AA 49°C samples as they have similar beef flavor intensity as the control and cooler aged samples, however, beef flavor intensity decreased in AA 54°C samples. Antoniewski and Barringer (2010) showed that reducing purge loss can better maintain the flavor of meat products; additionally, Yu et al. (2023) explained complex protein degradation products that contribute to meat flavor can be expelled within purge. Therefore, the increased purge loss from the AA 54°C samples may lead to this noted decreased beef flavor intensity. However, Ruiz-Carrascal et al. (2019) reported higher lamb flavor in sous vided lamb chops that have been through a oven roasting step compared to samples that only went through sous vide cooking, implying a potential way to increase the flavor intensity of sous vide steaks. Finally, there was no incidence of off-flavors reported in this

study likely due to the inhibition of lipid oxidation from the vacuum packaging (Reineccius, 1979).

3.6 Proximate analysis

There was an interaction between treatment and muscle for moisture ($P < 0.01$; Table 1). In TB, the control and cooler aged samples had higher relative moisture percentages compared to all AA treatments ($P < 0.01$). In SM, the control and cooler aged samples had the highest moisture percentages, followed by AA 49°C for 2/3 hours and AA 54°C for 2 hours, with AA 54°C for 3 hours displaying the lowest moisture percentages among all ($P < 0.01$). It was interesting to note that TB had higher moisture percentage than those from SM only when they were subjected to AA 54°C for 3 hours ($P < 0.01$).

There were no interactions between treatment and muscle in fat percentages ($P > 0.05$). There was a treatment effect (Table 2), where the AA 54°C for 3 hours samples had higher fat percentages than those from the control, cooler aged, AA 49°C for 2/3 hours samples, and AA 54°C for 2 hours samples ($P < 0.01$). There was also a muscle effect (Table 3) where the TB had higher fat percentage than those from the SM ($P < 0.05$). There was no interaction between treatment and muscle for protein percentages ($P > 0.05$). However, there was a treatment effect (Table 2) where AA 54°C for 2/3 hours had the highest protein percentages, followed by AA 49°C for 2/3 hours, with the control and cooler aged samples had the lowest protein percentages among all ($P < 0.01$). There was a muscle effect (Table 3) where the SM had higher protein percentages than those from the TB ($P < 0.01$).

Naqvi et al. (2021) also found moisture decreased with increased temperature as well as increased cook length of beef biceps femoris (BF) and ST, and Garcia-Linares et al. (2004) reported lower moisture content in salmon sous vided at 90°C for 10 min than the raw sample,

leading to an increase in relative fat and protein content. As mentioned earlier, moisture can be lost due to increased pressure within the muscle cell from the heat induced perimysium and cytoskeletal proteins shrinkage (Tornberg, 2013). This expelled free water can then be collected into the extracellular space (Honikel, 2004), and this free water can be easily lost as purge (Huff-Lonergan & Lonergan, 2005). Aaslyng et al. (2003) described initial juiciness is dependent on moisture content, while sustained juiciness is determined by moisture as well as intramuscular fat. In our study, all AA samples had the lower juiciness scores compared to the control and cooler aged samples, and this finding was supported by our moisture content results. However, it is apparent that the slight increase in fat content in the AA 54°C for 3 hours was not able to overcome the negative impact on palatability from the moisture loss.

3.7 Lipid oxidation

There was no interaction between treatments and muscles for lipid oxidation ($P > 0.05$), but there was a treatment effect (Table 2) where the AA 54°C for 3 hours samples had the highest MDA content, followed by the AA 49°C for 2/3 hours and AA 54°C for 2 hours samples, with the lowest in those from the control and cooler aged samples ($P < 0.01$). There was no muscle effect for lipid oxidation ($P > 0.05$).

Cropotova et al. (2019) sous vided Atlantic mackerel at 70-80°C for 10-20 min and stored the fillets at 4°C for 1-9 days and found that sous vided mackerel had higher MDA content compared to the control fillets which were not sous vided. In addition, Vaudagna et al. (2002) sous vided beef ST muscles at 50-65°C for 1.5-6.5 hours before storage at 1°C up to 55 days and found that samples processed at higher temperatures possessed higher lipid oxidation values than those processed at lower temperatures after 13 days of storage. These findings are similar to the findings from the current study, which Lalas (2008) and Ruiz-Carrascal et al.

(2019) both explained that the heating process can result in the formation of lipid oxidation compounds due to the release of pro-oxidants during heating. However, Yuan et al. (2023) discovered that raw Russian sturgeon steaks had lower MDA than those that have been sous vided at 40°C for 20 min, but the sous vided samples maintained the similar MDA level throughout the display period and ended with lower MDA values compared to the raw samples by day 7.

Furthermore, our sensory data showed no detection of off-flavors in any of the AA samples. This is likely because the highest MDA values detected in this study was only at 0.7 mg MDA/kg of meat, and Resconi et al. (2012) reported consumers can only detect off-flavor in samples containing MDA level greater than 2.2 mg MDA/kg of meat. Unfortunately, no retail display was conducted for this study, and future studies should include either retail display or refrigeration storage of AA steaks to document the shelf-life of AA treated steaks.

3.8 Cathepsin activity

The gelatin zymography resulted in 3 distinct bands: band 1 (60 kDa), band 2 (50 kDa), and band 3 (30 kDa) as shown in Figure 2. There was no interaction between treatments and muscles for any of the bands ($P > 0.05$). For band 1, there was a treatment effect (Table 2), where the highest activity was seen in AA 49°C 2/3 hours samples compared to the those from rest of the treatments ($P < 0.01$). There were no differences between the control, cooler aged, and AA 54°C for 2/3 hours samples ($P > 0.05$). There were no muscle differences for band 1 ($P > 0.05$). There was also a treatment effect for band 2 (Table 2), where highest activity was seen in AA 49°C for 3 hours and AA 49°C for 2 hours, followed by the control, 21 d cooler aged samples, and AA 54°C for 2 hours, with the lowest activity seen in AA 54°C for 3 hours samples ($P < 0.05$). Similarly, there was no muscle effect for band 2 ($P > 0.05$). Finally, there were

treatment differences for band 3 ($P < 0.01$; Table 2) where the highest activity was seen in AA 49°C for 3 hours samples ($P < 0.05$), followed by the 21 d cooler aged, AA 54°C for 2/3 hours, and AA 49°C for 2 hours samples, and the lowest activity was seen in the control samples ($P < 0.05$). There was also a muscle effect ($P < 0.05$; Table 3) with TB having the higher activity than SM.

Ertbjerg et al. (2012) measured calpain and cathepsin activity in pork longissimus dorsi sous vided at 25-70°C from 2 min to 24 hours. While calpain-1 and calpain-2 significantly reduced in activity at 40 °C and completely ceased in activity at 55°C within a few min of incubation, cathepsin B and L remained active for 24 hours at 55°C (Ertbjerg et al., 2012). Though not mentioned within the materials and methods, we also did not detect any calpain activity through casein zymography in AA 49°C treatment samples during our preliminary test runs ($n=3$). This likely means the measurable decrease in shear force in the AA samples was not due to calpain activity. In a thorough review on the contribution of proteolytic enzymes to meat tenderness, Lana and Zolla (2016) pointed out that the role of cathepsins' role in postmortem meat tenderization is still debatable, mainly due to their confinement in the lysosomes. For example, Ouali et al. (1987) showed that cathepsin D did not exhibit the ability to degrade actin on rabbit myofibrils through SDS-PAGE. However, Hughes et al. (2000) later found that cathepsin D can hydrolyze actin into peptides by using reverse-phase high performance liquid chromatography.

There is a renewed interest in cathepsin'' role for sous vide cooking due to its known resistance to heat. Hayes et al. (2001) reported different isoforms of cathepsin may have different heat stabilities, which may explain the differences in activity among the 3 zymography bands found in this study. L. Kaur et al. (2020) sous vided beef brisket at 50-70°C for 1-24 hours and

found cathepsin H had no measurable activity at 55°C for 1 hour and lose its activity quickly at 50°C after 5 hours, while cathepsin B and L displayed the highest activity when heated for 1 hour at 50 and 55°C, respectively. On the other hand, Wang et al. (2013) cooked duck breast in a water bath to various internal temperatures from 30-90°C and reported cathepsin B and L activities to decrease above 50°C, while cathepsin D activity can sustain higher temperature which its activity decreased above 70°C. The most abundant cathepsins isoforms in muscles are cathepsin B, D, H, and L (Boland et al., 2018), and the main cathepsins researched in regards to meat tenderization are B, L, and oftentimes D (Hopkins & Geesink, 2009).

On the other hand, the bands may also represent cathepsin light chain, cathepsin heavy chain, preprocathepsin, or procathepsin instead of multiple cathepsin isoforms. Cathepsins begin their journey as "preprocathepsin" when they are first synthesized in the endoplasmic reticulum (Erickson et al., 1981). They will later be cleaved into the inactive procathepsin form, transported into the lysosome and mature into the active two-chain form of light and heavy chain cathepsins (Kobayashi et al., 1992; Yasuda et al., 2005). Studying brain tissue extracts through western blot, Sivaparvathi et al. (1996) utilized an anti-rabbit cathepsin D antibody and reported bands from pro-cathepsin D along with the heavy and light chain forms of cathepsin D. Similarly, Hou et al. (2002) utilized an anti-human cathepsin K antibody to study diseases in joint connective tissue and found bands both from pro-cathepsin K along with active form of cathepsin K.

Our study utilized purified cathepsin B as a reference, but the molecular weight and band patterns did not match the reference's expected characteristics. Hawkes et al. (2010) warns of potential differences in molecular weights in standards bought from commercial sources as well as how the running conditions can affect the MW of the proteins on gel (Pungercar et al., 2009).

In this present study, the AA treatment increased the activity for all three bands present in the gels (60 kDa, 50 kDa, and 30 kDa bands). Unfortunately, we did not have the proper resources to determine whether the bands were from a synergistic effect from 2 or more cathepsin isoforms or just simply procathepsin and/or heavy and light chains from the same cathepsin isoform.

3.9 Proteolysis analysis

Interestingly, the degradation pattern of the AA samples were slightly different from those from the traditional cooler aging. In addition to the traditional intact band (37 kDa) and the degraded band (28 kDa), there was a set of further degraded bands (21 kDa) present on the troponin-T western blot as seen in Figure 3. There were no interactions, nor were there any muscle effects for the traditional, degraded or the further degraded bands ($P > 0.05$). However, there was a treatment effect for the intact band (Table 2) with the highest percent in the control, followed by AA 49°C for 2/3 hours and AA 54°C for 2/3 hours samples, and the cooler aged samples had the lowest relative percentage ($P < 0.01$). There was a treatment effect for the traditional degraded bands ($P < 0.01$; Table 2) with the highest relative percentage in the cooler aged samples, and the relative percentages of the traditional degraded bands for the rest of the treatments were not different from each other ($P > 0.05$). There was also a treatment effect for the further degraded bands (Table 2) with all AA samples showing higher relative percentages in the further degraded bands than those from the control and cooler aged samples ($P < 0.05$).

Troponin is located on thin filaments (actin) in the muscle fiber (Ohtsuki, 2007), and troponin T represents the connection between tropomyosin to the entire troponin complex (Ohtsuki & Morimoto, 2008). It is well established that troponin-T degradation is positively correlated with tenderness of meat (Hopkins & Thompson, 2002). Penny and Dransfield (1979) described decreased toughness as troponin T is degraded in conditioned beef, and Elisabeth Huff

Lonergan et al. (2010) recognized the correlation of the appearance of degraded troponin T to increased tenderness. Although calpains are traditionally associated with the degradation of troponin-T (Di Lisa et al., 1995), calpains' contribution to AA is likely minimal in this study due to its sensitivity to temperature above 40 °C. However, cathepsins have also been reported to degrade myofibrillar proteins. Mikami et al. (1987) incubated beef myofibrillar proteins with cathepsin L and reported degradation of the myosin heavy chain, myosin light chain, troponin T, troponin I, α-tropomyosin, titin, nebulin, C-protein, and α-actinin. Zeece et al. (1986) also recorded degradation of troponin T from beef myofibrils incubated in cathepsin D, *in vitro*. Finally, Noda et al. (1981) incubated myofibrils with and without cathepsin B and visualize myofibril degradation from the enzymatic activity of cathepsin B utilizing a phase-contrast microscopy.

One of the most unexpected findings from this study was the distinct difference in troponin-T degradation patterns between the AA treatments and the cooler aged samples. The additional degradation bands past the traditional 28 kDa degraded band shown in our AA images are likely due to further segmentation of troponin-T from enzymatic activity differ from calpains. For example, Bhat et al. (2020) reported troponin-T degraded into smaller fragments than the traditional degradation pattern from aging in an in-vitro gastrointestinal digestion study. The additional degradation bands from troponin-T have also been reported in Li et al. (2020), which they sous vided hairtail fish (Trichiurus lepturus) at 40-68°C for 10-40 min. In addition, Matsumoto et al. (1983) incubated rabbit muscle with cathepsin D and also noticed a troponin-T degradation pattern very similar to our finding where the intact bands were located at 37 kDa, and the degraded bands were located at 24 kDa, 20 kDa, and 11 kDa. In addition to cathepsin D, cathepsin B had also been reported to degrade troponin-T into fragments of 21 kDa, 12 kDa, and

10 kDa in rabbit skeletal muscle (Matsuishi et al., 1992). However, Schwartz and Bird (1977) pointed out that cathepsin D can degrade additional myofibrillar proteins that are known to resist calpain activity such as myosin and actin into additional fragments, but cathepsin B was unable to accomplish that. Additional research is needed to investigate the effect of AA treatments on the degradation pattern of other myofibrillar proteins.

3.10 Collagen content

There was an interaction between treatment and muscle in soluble collagen content ($P < 0.01$; Table 1). Within the TB, AA 54°C for 2 hours samples had the highest soluble collagen content, followed by AA 54°C for 3 hours, with the rest of the treatments having the lowest soluble collagen content ($P < 0.01$). However, no treatment differences were found within SM ($P > 0.05$). In addition, the TB AA 54°C for 2/3 hours samples had higher soluble collagen content than those in SM ($P < 0.01$). There were no interactions between treatment and muscle for insoluble collagen content ($P > 0.05$). However, there was a treatment effect (Table 2), which all AA samples had greater insoluble collagen content than those from the control and cooler aged samples ($P < 0.01$). There were no differences in insoluble collagen content between the control and cooler aged samples, nor were there differences among the samples from AA treatments ($P > 0.05$). There was no muscle effect for insoluble collagen content ($P > 0.05$). There were no interactions between treatment and muscle for total collagen in the purge ($P > 0.05$). There was a treatment effect (Table 2), which the AA 54°C for 3 hours had the highest purge collagen content, followed by AA 54°C for 2 hours, AA 49°C for 2/3 hours, with the lowest purge collagen content in the control and cooler aged samples ($P < 0.05$). There was also a muscle effect (Table 3) where TB had higher collagen content in the purge than those in SM ($P < 0.01$). Finally, there was an interaction between treatment and muscle in total collagen content

($P < 0.05$; Table 1). Within the TB, the highest collagen content was in the AA 54°C for 2/3 hours treatments, followed by AA 49°C for 2 hours, with the lowest total collagen content found in those from the control, cooler aged, and AA 49°C for 3 hours samples ($P < 0.05$). Interestingly, there were no differences among treatments for total collagen content within the SM ($P > 0.05$). Between the muscles, the TB had higher total collagen content in the AA 54°C for 2/3 hours samples compared to those from SM ($P < 0.01$).

Similar to our findings, both Naqvi et al. (2021) and Karki et al. (2022) reported higher soluble collagen in various beef muscles sous vided at higher temperature and longer time compared to samples sous vided at lower temperature and shorter time. However, many sous vide studies have also shown an increase in total collagen and insoluble collagen content from beef samples sous vided at higher temperature and longer time compared to samples sous vided at lower temperature and shorter time (Chang et al., 2011; Wang et al., 2022). This increase in soluble, insoluble and total collagen content found in AA samples from this study can easily be explained by the additional moisture loss from AA 54°C, which the collagen content was more concentrated within the AA 54°C samples.

Perhaps, what is more interesting about the collagen results is the modification of collagen solubility in AA samples, which all AA samples had an increase in purge collagen concentration. Savage et al. (1990) described that although purge is mainly composed of sarcoplasmic proteins, the degradation of myofibrillar and stromal proteins can also lead to their presence within purge. Scott and Pearson (1978) incubated isolated collagen from beef skin with cathepsin D and reported cleavage of the collagenous peptides from cathepsin D. However, different isoforms of cathepsin have also been reported to degrade collagen differently. Lecaille et al. (2007) who reported differences between cathepsin K and L's substrate specificity which

led to a different degradation pattern of cathepsin L's degradation of collagen I and II than when cathepsin K attacked collagen I and II.

Purslow (2018) described during fast heating, collagen denaturation occurs in the range of 62-67°C and in slower heating methods this can occur in 55-60°C. Furthermore, Chang et al. (2011) showed that heat-induced collagen structure modification in beef was recorded at temperatures of 60°C and above in water bath heating. This likely means that the increase in collagen solubilization in our AA samples, especially at 49°C, is likely due to enzymatic activity rather than the heat. The data retrieved on total collagen in the purge provided a link for a more comprehensive understanding of how collagen in beef was solubilized and released during the AA process.

3.11 Perimysial peak transitional temperatures

There were no interactions between treatment and muscle present in any parameter measured by the differential scanning calorimeter ($P > 0.05$). There was a treatment effect for perimysial peak denaturation temperature ($P < 0.01$; Table 2) where the AA 54°C for 3 hours had the highest temperature, followed by the control and AA 54°C for 2 hours, then the AA 49°C for 2/3 hours, with the 21 d cooler aged samples had the lowest temperature among all ($P < 0.05$). Additionally, there was a muscle effect ($P < 0.05$; Table 3) where the SM had a higher perimysial denaturation temperature than TB. There was a treatment effect for the onset temperature ($P < 0.05$; Table 2), with the highest in AA 54°C samples ($P < 0.05$) compared to those from the rest of the treatments ($P < 0.05$). There were no differences between AA 54°C for 2/3 hours and AA 49°C for 3 hours ($P > 0.05$), nor were there any differences between the control, cooler aged, and AA 49°C for 2/3 hours samples ($P > 0.05$). There was also no muscle effect ($P > 0.05$).

The differential scanning calorimeter can provide information on the modifications of thermal properties and protein stability due to collagen structure change during AA (Gill et al., 2010; Weber & Salemme, 2003). In our study, peak perimysial denaturation temperature and onset temperature of the samples increased with increasing temperature in AA treatments. Similarly, Latorre et al. (2019) found that beef ST muscles sous vided at 60°C for 1 hour displayed higher thermal stability in the remaining undenatured collagen compared to those from the raw samples. Bächinger and Davis (1991) reported that the amino acid sequence is the main determinant of thermal stability of collagen, which Burjanadze (1982) further elaborated that the position of the hydroxyproline plays the most important role in collagen stability. Past research has shown that cathepsins can cleave collagen at different sites (Aguda et al., 2014; Brömme et al., 1996), but there is no information on how the cleavage of collagen molecule by cathepsins may affect collagen thermal stability.

On the other hand, Friess and Lee (1996) showed higher denaturation temperatures in collagen which has been air-dried as compared to wet collagen molecules. This is similarly noted by Capella-Monsonís et al. (2018) who reported less hydrated collagen molecules record higher onset and peak denaturation temperatures due to the increased stability of compacted collagen molecules. Perhaps, the lower moisture content in AA treated samples can result in increased thermal stability of the collagen fibers due to decreased in moisture content.

4. Conclusion

This study showed that AA may improve microbial safety, proteolytic enzyme activity from cathepsin, and tenderness of lower quality beef cuts; however, sensory aspects of the meat such as juiciness, beef flavor intensity, and shelf stability may decrease from the AA process. The increased cathepsin activity in AA treatments is likely responsible for the increased

solubilization of collagen and the additional degree of degradation of troponin-T. Further studies are needed to better understand how myofibrillar proteins and connective tissue structure may be altered due to cathepsin activity. The AA method is a promising technique to increase the eating quality of lower quality beef cuts; however, additional strategies to minimize moisture loss such as AA with marinate are needed to make AA feasible for the meat industry.

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Table 1. Lightness (L*), redness (a*), moisture, soluble collagen, and total collagen of two beef muscles assigned to one of six treatments (*n=120*). Microbial purge aerobic plate counts from two beef muscles assigned to one of five treatments (*n=100*). Yield after AA from two beef muscles assigned to one of four treatments (*n=80*).

		Treatment							
Measurements	**Muscle**	**Cooler Aged 3 d**	**Cooler Aged 21 d**	**AA 49°C 2 hrs**	**AA 49°C 3 hrs**	**AA 54°C 2 hrs**	**AA 54°C 3 hrs**	**SEM[1]**	**P-value**
L*									
	TB	38.12^{Ac}	39.75^{Ac}	47.82^{Aa}	48.07^{Aa}	46.13^{Aa}	42.45^{Ab}	0.90	< 0.05
	SM	38.12^{Ad}	41.49^{Acd}	46.89^{Aa}	44.42^{Bb}	43.41^{Bbc}	41.59^{Ac}		
a*									
	TB	16.82^{Bab}	15.36^{Ab}	17.57^{Aa}	15.76^{Aab}	12.82^{Ac}	11.50^{Ac}	0.71	< 0.01
	SM	19.18^{Aa}	11.43^{Bc}	17.48^{Aa}	15.43^{Ab}	14.61^{Ab}	12.39^{Ac}		
Microbial Purge APC (log CFU/mL)									
	TB	3.16^{Aa}	N/A	1.03^{Ab}	0.70^{Ab}	0.96^{Ab}	1.04^{Ab}	0.14	< 0.01
	SM	2.82^{Ba}	N/A	0.95^{Ab}	1.07^{Ab}	0.80^{Ab}	0.80^{Ab}		
Moisture (%)									
	TB	72.97^{Aa}	72.22^{Aa}	70.16^{Abc}	69.86^{Abc}	69.19^{Ac}	69.49^{Ac}	0.46	< 0.05
	SM	71.97^{Aa}	72.44^{Aa}	69.61^{Abc}	70.63^{Ab}	69.61^{Abc}	67.82^{Bd}		
Post-AA Loss (%)									
	TB	N/A	N/A	4.80^{Aa}	5.21^{Aa}	8.53^{Ab}	9.27^{Ab}	0.38	< 0.01
	SM	N/A	N/A	9.41^{Ba}	8.60^{Ba}	10.93^{Bb}	12.86^{Bc}		
Soluble Collagen									
	TB	1.57^{Ac}	1.20^{Ac}	1.29^{Ac}	1.11^{Ac}	3.78^{Aa}	2.61^{Ab}	0.38	< 0.01
	SM	0.67^{Aa}	0.68^{Aa}	0.88^{Aa}	0.77^{Aa}	0.74^{Ba}	1.01^{Ba}		
Total Collagen									
	TB	7.29^{Ac}	6.87^{Ac}	9.34^{Abc}	8.19^{Ac}	12.36^{Aa}	11.52^{Aab}	0.97	< 0.05
	SM	7.39^{Aa}	7.29^{Aa}	8.54^{Aa}	9.00^{Aa}	8.39^{Ba}	8.12^{Ba}		

$^{A-B}$Least sqauare means without a common superscript differ within the column

$^{a-d}$Least sqaure means without a common superscript differ across the row

[1]Standard error of the least square means

Table 2. Main effect of treatment for Warner-Bratzler shear force (WBSF), yellowness (b*), cook yield, fat content, protein content, lipid oxidation, insoluble collagen, total collagen in the purge, onset and peak denaturation temperature, sensory scores evaluated by trained panelists, troponin-T degradation, and cathepsin zymography of two beef muscles subjected to one of six treatments (*n=120*).

Measurements	Treatment						SEM	P-value
	Cooler Aged 3 d	Cooler Aged 21 d	AA 49°C 2 hrs	AA 49°C 3 hrs	AA 54°C 2 hrs	AA 54°C 3 hrs		
Microbial Swab (log CFU/mL)	1.25^{a}	N/A	0.45^{bc}	0.17^{c}	0.17^{c}	0.73^{b}	0.16	< 0.01
WBSF (kg)	4.46^{a}	3.85^{cd}	4.18^{b}	4.04^{bc}	3.87^{cd}	3.73^{d}	0.12	< 0.01
b*	14.99^{c}	13.82^{d}	19.22^{a}	18.24^{a}	18.3^{a}	17.04^{b}	0.40	< 0.01
Cook Loss (%)	23.53^{c}	22.48^{c}	20.81^{b}	20.36^{b}	19.64^{ab}	18.24^{a}	0.50	< 0.01
Fat (%)	4.99^{bc}	4.64^{c}	5.46^{bc}	4.98^{bc}	5.80^{ab}	6.42^{a}	0.35	< 0.01
Protein (%)	22.28^{d}	22.63^{d}	24.10^{c}	24.51^{bc}	25.16^{a}	25.00^{ab}	0.24	< 0.01
Lipid Oxidation (mg MDA/kg)	0.25^{c}	0.28^{c}	0.5^{b}	0.44^{b}	0.51^{b}	0.67^{a}	0.07	< 0.01
Insoluble Collagen (mg/g)	6.22^{b}	6.11^{b}	7.88^{a}	7.80^{a}	7.87^{a}	7.90^{a}	0.50	< 0.05
Total Collagen in the Purge (mg/mL)	0.29^{d}	0.32^{d}	0.41^{c}	0.44^{c}	0.54^{b}	0.69^{a}	0.03	< 0.01
Peak Denaturation Temperature (°C)	64.77^{ab}	63.98^{d}	64.22^{cd}	64.49^{bcd}	64.77^{ab}	65.29^{a}	0.25	< 0.01
Onset Temperature (°C)	60.00^{bc}	59.78^{c}	60.30^{bc}	60.56^{abc}	60.77^{ab}	61.35^{a}	0.32	< 0.05
Sensory Panel[2]								
Initial Juiciness	48.13^{b}	56.82^{a}	40.49^{c}	42.75^{c}	40.66^{c}	39.89^{c}	1.91	< 0.01
Sustained Juiciness	41.47^{b}	49.73^{a}	34.28^{c}	35.71^{c}	34.21^{c}	32.59^{c}	1.83	< 0.01
Myofibrillar Tenderness	51.69^{c}	63.64^{a}	50.70^{c}	50.69^{c}	53.52^{c}	57.95^{b}	2.07	< 0.01
Connective Tissue	26.60^{ab}	16.68^{c}	27.13^{a}	23.58^{ab}	22.88^{b}	17.06^{c}	2.12	< 0.01
Overall Tenderness	43.99^{c}	58.06^{a}	41.38^{c}	42.85^{c}	46.49^{c}	51.84^{b}	2.41	< 0.01
Beef Flavor	32.55^{a}	31.68^{ab}	32.69^{a}	31.09^{ab}	29.91^{b}	29.59^{b}	1.04	< 0.05
Troponin-T Degradation (%)								
Intact bands	31.19^{a}	10.03^{c}	27.21^{ab}	26.53^{ab}	28.94^{ab}	22.55^{b}	3.28	< 0.01
Traditional degraded bands	66.12^{b}	84.10^{a}	62.09^{b}	61.62^{b}	59.09^{b}	64.92^{b}	4.30	< 0.01
Further degraded bands	3.16^{b}	7.05^{b}	11.60^{a}	12.78^{a}	13.50^{a}	14.01^{a}	1.87	< 0.01
Cathepsin Zymography (fold change)								
Band 1 (60 kDa)	1.33^{b}	1.28^{b}	2.00^{a}	1.95^{a}	1.45^{b}	1.27^{b}	0.30	< 0.01
Band 2 (50 kDa)	1.09^{bc}	1.07^{bc}	1.23^{ab}	1.46^{a}	1.15^{bc}	0.91^{c}	0.25	< 0.05
Band 3 (30 kDa)	1.41^{c}	2.58^{ab}	2.26^{b}	3.19^{a}	2.77^{ab}	2.63^{ab}	0.46	< 0.01

[a-d]Least sqaure means without a common superscript differ across the row

[1]Standard error of the least square means

[2]Sensory scores: 0=extremely dry/tough/bland or none; 50=neither dry nor juicy/neither tough nor tender/neither bland nor intense/moderate amount; 100=extremely juicy/tender/abundant/intense

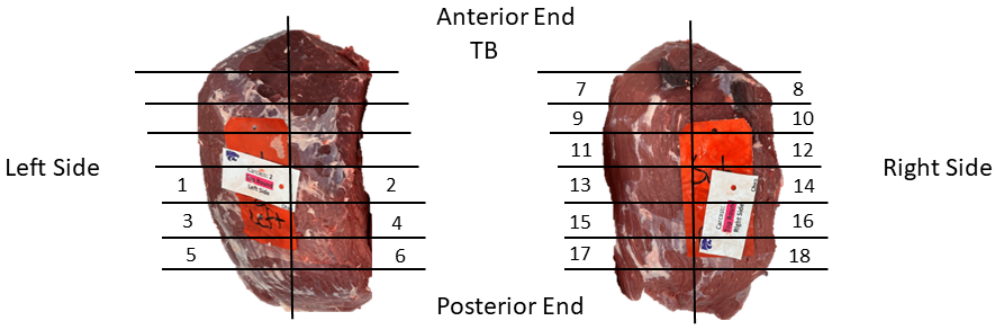
Table 3. Main effect of muscle for yellowness (b*), cook yield, sensory scores evaluated by trained panelists (initial juiciness, sustained juiciness, myofibrillar tenderness, connective tissue amount, overall tenderness, and beef flavor), proximate analysis (fat and protein content), total collagen in the purge, peak denaturation temperature, and band 3 of cathepsin zymography between two beef muscles subjected to one of six treatments (*n=120*).

Measurements	Muscle		SEM[1]	P-value
	TB	SM		
b*	15.97[b]	17.90[a]	0.24	P < 0.01
Cook Loss (%)	19.92[a]	21.77[b]	0.29	P < 0.01
Sensory Panel[2]				
Initial Juiciness	51.36[a]	38.21[b]	1.27	P < 0.05
Sustained Juiciness	44.58[a]	31.41[b]	1.23	P < 0.01
Myofibrillar Tenderness	60.56[a]	48.84[b]	1.66	P < 0.01
Connective Tissue Amount	18.13[b]	26.51[a]	1.74	P < 0.01
Overall Tenderness	54.63[a]	40.25[b]	1.90	P < 0.01
Beef Flavor	32.16[a]	30.35[b]	0.79	P < 0.01
Proximate Analysis				
Fat Content (%)	5.64[a]	5.13[b]	0.24	P < 0.05
Protein Content (%)	23.26[b]	24.63[a]	0.19	P < 0.01
Total Collagen in the Purge (mg/g)	0.50[a]	0.40[b]	0.02	P < 0.01
Peak Denaturation Temperature (°C)	64.37[b]	64.75[a]	0.19	P < 0.05
Cathepsin Zymography Band 3 (30 kDa)	2.72[a]	2.23[b]	0.39	P < 0.05

[a-b]Least sqaure means without a common superscript differ across the row

[1]Standard error of the least square means

[2]Sensory scores: 0=extremely dry/tough/bland or none; 50=neither dry nor juicy/neither tough nor tender/neither bland nor intense/moderate amount; 100=extremely juicy/tender/abundant/intense



1 – AA 54°C for 2 hours for trained panel analysis
2 – AA 54 °C for 3 hours for trained panel analysis
3 – AA 49 °C for 2 hours for trained panel analysis
4 – AA 49 °C for 3 hours for trained panel analysis
5 – Cooler aged 3 days (control) for trained panel analysis
6 – Cooler aged 21 days for trained panel analysis
7 – Cooler aged 21 days for biochemical analysis
8 – Cooler aged 3 days (control) for biochemical analysis
9 – AA 49 °C for 2 hours for biochemical analysis
10 – AA 49 °C for 3 hours for biochemical analysis
11 – AA 54 °C for 2 hours for biochemical analysis
12 – AA 54 °C for 3 hours for biochemical analysis
13 – AA 54 °C for 2 hours for WBSF/microbial analysis
14 – AA 54 °C for 3 hours for WBSF/microbial analysis
15 – AA 49 °C for 2 hours for WBSF/microbial analysis
16 – AA 49 °C for 3 hours for WBSF/microbial analysis
17 – Cooler aged 3 days (control) for WBSF/microbial analysis
18 – Cooler aged 21 days for WBSF/microbial analysis

Figure 1. Representative image of the fabrication map for triceps brachii (TB) from both sides of the beef carcass.

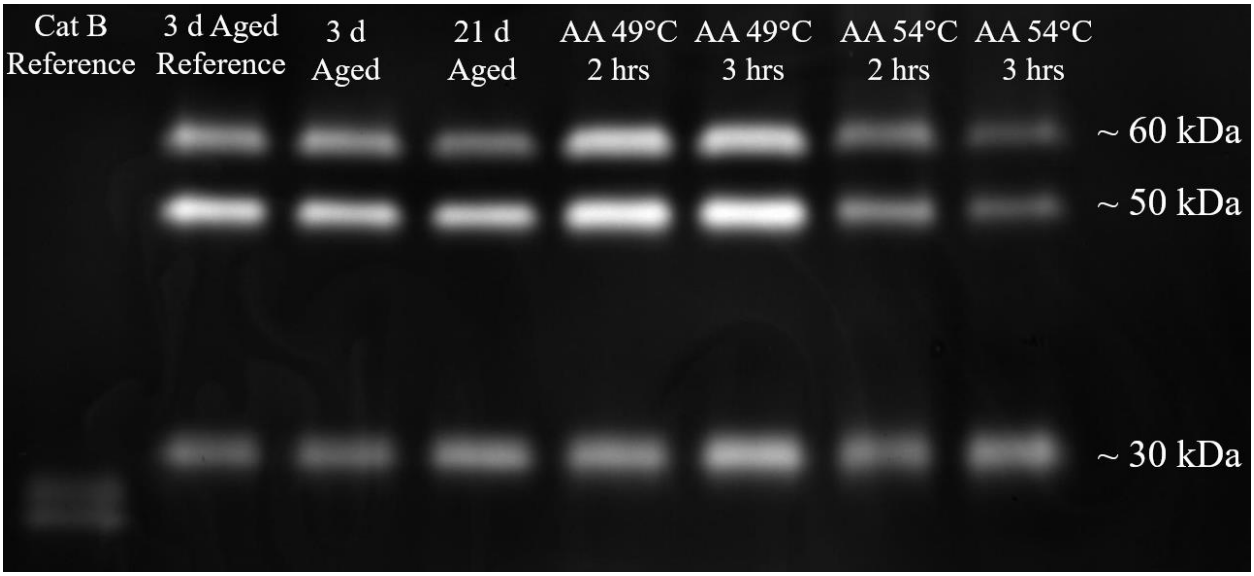


Figure 2. Representative image of cathepsin zymography.

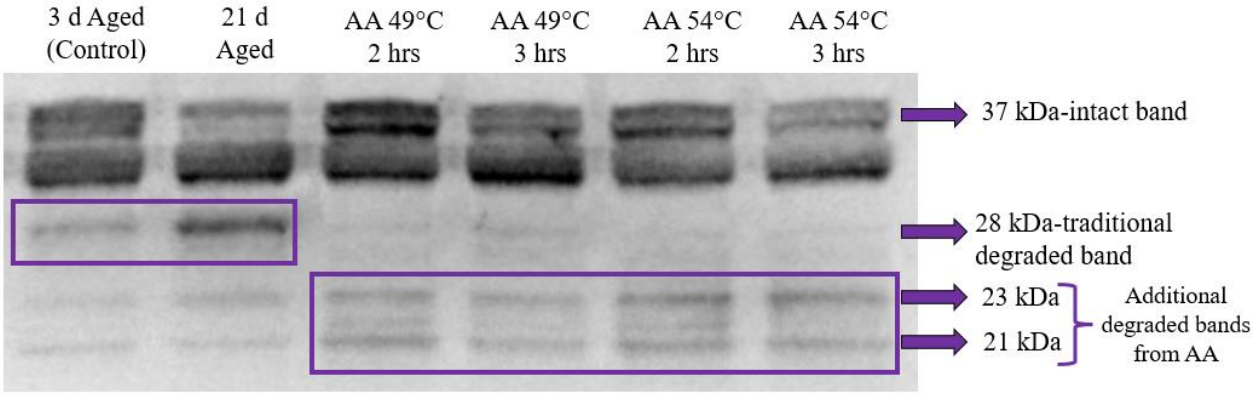


Figure 3. Representative image of troponin-T degradation.

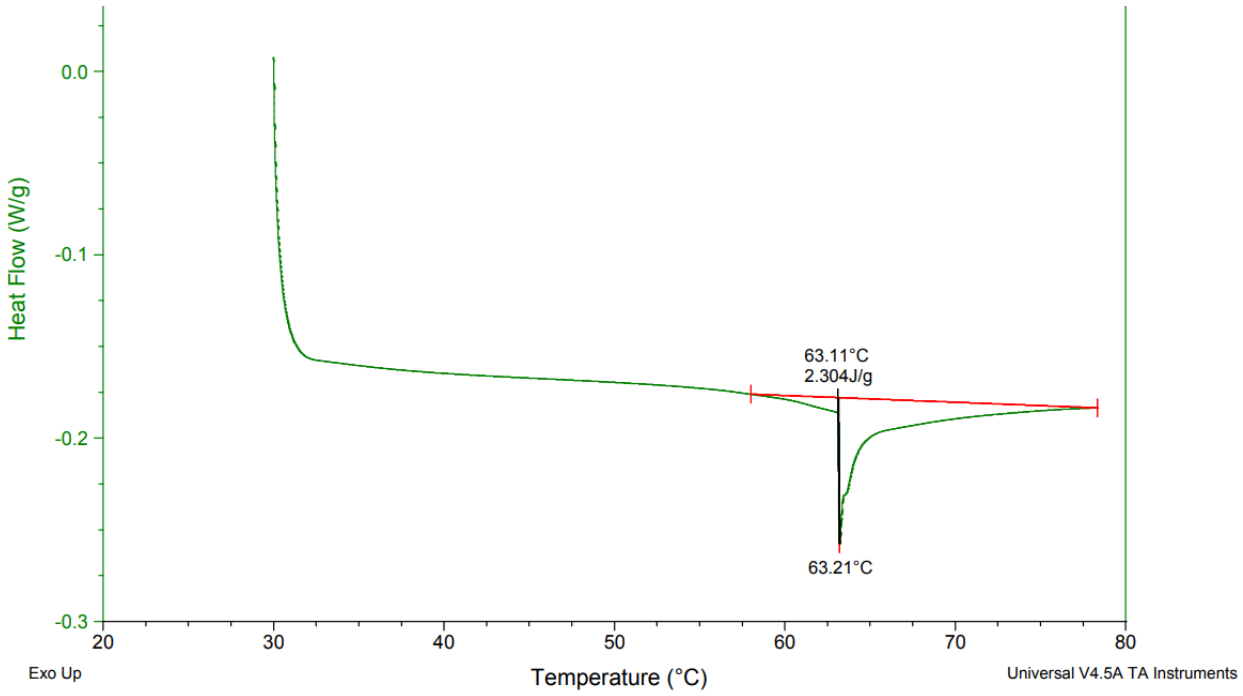


Figure 4. Representative image of perimysial enthalpy from the differential scanning calorimeter.