Mechanism and Control of Beef Toughening during Retail Display in High Oxygen Modified Atmosphere Packages

Lasika S. Senaratne
University of Nebraska-Lincoln, lasika_ss@yahoo.com

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MECHANISM AND CONTROL OF BEEF TOUGHENING DURING RETAIL DISPLAY IN HIGH OXYGEN MODIFIED ATMOSPHERE PACKAGES

by

Lasika Shyamalie Senaratne-Lenagala

A DISSERTATION

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MECHANISM AND CONTROL OF BEEF TOUGHENING DURING RETAIL DISPLAY IN HIGH OXYGEN MODIFIED ATMOSPHERE PACKAGES

Lasika Shyamalie Senaratne-Lenagala, Ph.D.

University of Nebraska, 2012

Advisor: Chris R. Calkins

This research was conducted to elucidate the mechanism and factors (diet, aging time, antioxidant level, and retail display time) affecting beef toughening in retail display under high oxygen atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂). In the first study, effects of HiOx-MAP on color and lipid stability of beef m. longissimus lumborum steaks from yearlings fed corn or 30% wet distillers grains plus soluble (WDGS) diets supplemented with (150 mg/kg) or without (0 mg/kg) AGRADO-PLUS (AG) antioxidants were studied. Steak color-shelf life was extended ($P < 0.0001$) by HiOx-MAP compared to oxygen permeable film packages (PVC-OW). Steaks from corn+AG-supplemented cattle had ($P \leq 0.05$) less discoloration and lower lipid oxidation levels at the end of retail display than steaks from all other diets. However, effects of AG on reducing steak discoloration and lipid oxidation when feeding WDGS could not be seen ($P > 0.05$). In the second study, mechanism and factors affecting beef toughening due to HiOx-MAP during retail display were investigated. High oxygen atmosphere packages oxidized proteins ($P < 0.10$) and formed protein aggregates ($P = 0.01$) thereby making steaks tougher ($P <.0001$). Also, HiOx-MAP reduced ($P = 0.001$) troponin-T degradation in beef. Protein aggregates were formed mainly by polymerization of myosin proteins mostly due to disulfide bonds. Retail display of steaks aged long-term increased protein
oxidation ($P < .0001$) and polymerization ($P < .0007$), thereby making beef tougher ($P < 0.06$). Feeding WDGS also increased protein oxidation ($P = 0.002$) and polymerization ($P < 0.07$); however, tenderness was improved ($P = 0.06$). Dietary supplementation of AG toughened beef due to protein oxidation ($P < 0.04$) and decreased ($P = 0.001$) proteolysis by decreasing ($P = 0.007$) sarcoplasmic free-calcium levels. In the third study, tenderness and protein oxidation gradient in the beef $m.\ triceps\ brachii$ roasts were studied. Protein oxidation, aggregation, and toughening occurred at a descending gradient from the outside to the inside of beef roast packaged in HiOx-MAP systems ($P < .0001$).
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INTRODUCTION

According to the 2010 National Meat Case Study, case-ready packages represent about 66% of total fresh meats in the U. S. (NMCS, 2010). Among different case-ready packages, high oxygen modified atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂) is widely used in the fresh meat retail markets as it holds the desirable red color of meats for a longer time (Jakobson and Bertelsen, 1999; Cornforth and Hunt, 2008).

However, it is reported that HiOx-MAP packages negatively affect tenderness (Lund et al., 2007b; Zakrys et al., 2009; Huff-Lonergan et al., 2010; Kim et al., 2010; Lagerstedt et al., 2011), juiciness (Lund et al., 2007b; Zakrys et al. 2009; Resconi et al., 2012), flavor (Sørheim et al., 2004; Clausen, 2004; Madsen and Clausen, 2006; Grobbel et al., 2008; Bingol and Ergum 2011), and nutritional value (Lund et al., 2011) of muscle foods during retail display. These properties are considered as the most important organoleptic properties of meat which influence consumer satisfaction.

The literature available (Seyfert et al., 2005; Lund et al., 2007b; Bornez et al., 2010; Lagerstedt et al., 2011) hints that the decrease in tenderness of meat packaged in HiOx-MAP is due to oxidation of proteins. Oxidation due to HiOx-MAP could mainly occur in myofibrillar proteins and enzymes responsible for postmortem tenderization process in meats during retail display. As proteins are oxidized they could change their molecular structure and form intra/inter cross-links (di-sulfide or dityrosine) to produce protein aggregates (Lund et al., 2011). Also, enzymes involved in the postmortem tenderization process could get oxidized and impede their proteolytic activities (Huff-Lonergan et al., 2010). Both circumstances mentioned above could reduce the residual post-mortem aging
or tenderization process and form aggregates in beef during retail display, eventually making beef less tender.

Radicals formed during lipid oxidation as well as intact and denatured ferric hemochromogens produced during myoglobin oxidation could initiate protein oxidation in postmortem muscles. However, in HiOx-MAP systems myoglobin is available as oxymyoglobin in which hemochromogen is present as the ferrous form (non-catalytic form). Therefore, myoglobin oxidation of meats packaged in HiOx-MAP systems would have less impact on protein oxidation than lipid oxidation. Previous literature (Viljanen et al., 2004; Vuorela et al., 200; Estevez et al., 2008; Park et al., 2006) also showed that lipid-derived radicals and hydroperoxides, produced from lipid oxidation, more rapidly promoted protein oxidation in muscle foods than products formed from pigment oxidation. Any factor which could increase levels of lipid oxidation in meats would likely increase protein oxidation. Such factors could include feeding wet distiller grains plus solubles (WDGS; Senaratne. 2009; de Mello Jr., 2010), longer aging and retail display time (Senaratne, 2009), and low antioxidant status in meats (Estevez and Cava, 2004; Sante-Lhoutellier et al., 2008; Senaratne, 2009).

There is very little information available on the mechanism of protein oxidation related to beef toughening in HiOx-MAP systems. Therefore, there is more to discover about protein oxidation especially its mechanism, factors affecting oxidation (diets, aging time, retail display time, antioxidant status, muscle fiber orientation, and tissue depth etc.) and control measures of beef toughening due to HiOx-MAP systems. Thus, the objectives of this project are;
❖ To study the effects of HiOx-MAP systems on color and lipid stability of short- and long-term aged beef from cattle fed WDGS diets containing synthetic antioxidant supplementation.

❖ To elucidate the mechanism and factors affecting protein oxidation related beef toughening due to HiOx-MAP systems during retail display and its control measures by dietary antioxidant supplementation.

❖ To investigate the gradient and levels of protein oxidation within beef roasts due to HiOx-MAP systems during retail display.

❖ To investigate the effects of lipid oxidation levels on postmortem tenderization of beef.
LITERATURE REVIEW

In 2010, the National Meat Case Study showed that case-ready packages represent 66% of total retail fresh meats in the United States (NMCS, 2010). This rapid increase in case-ready fresh meats is due to numerous benefits offered by case-ready packaging for both retailers and consumers over the conventional system. Fresh meats in the United States retail markets are normally packaged in oxygen permeable polyvinyl chloride film packages (PVC-OW) or modified atmosphere packages (MAP).

Vacuumed-packaged meats have been marketed widely in other countries. Due to the dark purplish color (deoxymyoglobin) of the meat, vacuum-packaged meats are not very popular among the U.S. customers. Since PVC films are permeable to oxygen, the meat surface, in contact with oxygen, converts myoglobin muscle pigments into attractive bright cherry red color pigments, oxymyoglobin (Landrock and Wallace, 1955). However, PVC-OW packages are not only more prone to punctures during handling, but also discolor rapidly within 5 to 7 days due to pigment oxidation and formation of brown color (metmyoglobin). When surface discoloration or metmyoglobin exceeds 40% in beef and 50-75% in pork, retail meats are normally unattractive to customers or discarded (Greene et al., 1971; Asensio et al., 1987). Rapid discoloration of meat in PVC-OW is mainly due to the occurrence of low oxygen concentration in the packages by limiting oxygen diffusion though PVC film (George and Stratmann, 1952; Sorheim et al., 1995). According to Mancini and Hunt (2005), oxygen levels at 0.15 – 2.0% cause rapid discoloration; therefore, the oxygen levels in meat packages must be less than 0.15% to prevent rapid discoloration. Also, PVC-OW packages favor rapid spoilage from bacterial
growth (Pierson et al., 1970; Gill, 1983; Sorheim et al., 1995; Seideman and Durland, 1983).

High Oxygen Modified Atmosphere Packaging

Packages containing 70-80% oxygen and 20-30% carbon dioxide and sealed with a polyamide-polyethylene film are used for case-ready meats (Eilert, 2005). The nylon and polyethylene film layers provide strength, sealability, and gas- and water vapor-barrier properties (John et al., 2004). Compared to PVC-OW packages over high oxygen modified atmosphere packages (HiOx-MAP), meats in HiOx-MAP hold the desirable red color for 10-14 d (Cornforth and Hunt, 2008). Carbon dioxide gas at > 20% levels in MAP systems provides bacteriostatic and fungistatic properties (Moir et al., 1993; Nissen et al., 1996; Luno et al., 2000; Alam and Goyal, 2006). Antimicrobial properties are provided by dissolving carbon dioxide in water and fats (Blickstad et al., 1981) at low temperature (1°C). However, Daniels et al. (1985), Dixon and Kell (1989), and Farber (1991) revealed that bacteriostatic properties of CO₂ are due to direct impacts on microbial cell membrane functions and inhibition of microbial cellular enzymes by altering intercellular pH.

Effects of High-Oxygen Modified Atmosphere Packages on Muscle Food Quality

Color Stability. Fresh meat color, cherry red color, is one of the important factors considered by consumers at retail stores to determine its freshness at the point of purchasing (Liu et al., 1996; Faustman et al., 1998). The level of oxygen present in packages is important for color stability in meats. Elevated oxygen levels used in HiOx-
MAP stabilize oxymyoglobin pigments in meats and reduce metmyoglobin formation (Cornforth and Hunt, 2008). According to Cornforth and Hunt (2008) at 50-80% levels of oxygen, fresh longissimus dorsi beef steaks maintain good color stability for 10 days at 3ºC, compared to 20% oxygen level (Jakobson and Bertelsen, 1999). Pork chops packaged in 80% oxygen-enriched atmosphere with 20% carbon dioxide had significantly lower metmyoglobin formation than pork chops packaged in vacuum pouches and in 80% air and 20% carbon dioxide packages (Asensio et al., 1988).

Retail display temperature also influences color stability of meat. Sorheim et al. (1999) showed that beef loin steaks, ground beef, and pork chops packaged in 70% O₂ and 30% CO₂ at 4ºC had lower discoloration rates and higher a* values (redness) compared to similarly packaged beef at 8ºC. Jakobson and Bertelsen (1999) also reported that beef steaks stored in 50-80% oxygen with carbon dioxide maintained higher color stability for 10 days at 3ºC. However, they showed that raising temperature to 5ºC decreased color stability to 6½ days.

The color stability of meats in HiOx-MAP also depends on the physical state of meat. Belcher (2006) reported ground beef in HiOx-MAP had shorter color stability (for 10 – 12 d) compared to whole muscle steaks in HiOx-MAP (12 – 16 days). Type of species which meat comes from also affects the color stability. Pork chops stored in 80% O₂ and 20% CO₂ had metmyoglobin concentration below 30% even after 15 day at 1ºC (Ordonez and Ledward, 1977). At different temperatures, pigment denaturation levels of cooked beef are also affected by the packaging systems used during retail display. At 49, 57, 66, 71, 79 ºC cooking temperatures, patties from raw beef stored in 80% oxygen and 20% carbon dioxide had higher myoglobin denaturation percentage and lower a* values.
than patties from carbon monoxide and vacuum packaged beef (John et al., 2004). This premature browning of patties from beef stored in high oxygen packaging systems may be an issue regarding food safety. Seyfert et al. (2004) also reported that HiOx-MAP increased premature browning in ground beef patties. Different muscles packaged in HiOx-MAP act differently regarding color stability. Beef strip loin (m. longissimus dorsi), eye of round (m. semitendinosus) and clod heart (m. triceps brachii) steaks packaged in HiOx-MAP discolored faster compared to those packaged in vacuum or ultra-low oxygen and carbon monoxide systems (Grobbel et al., 2008).

Another factor influencing color stability of meats packaged in HiOx-MAP is postmortem aging time. O’Keefe and Hood (1981) and Senaratne (2009) reported that beef aged for 1 week was more color stable than meat aged for 3 - 4 weeks. This was explained as a reduction of metmyoglobin reducing activity in beef which was aged for longer periods (O’Keefe and Hood, 1981). Faustman (1990) further explained this due to the decrease in activity of metmyoglobin reductase, needed to enzymatically reduce cytochrome b₅, which is an important factor in enzymatically reducing metmyoglobin during the process of metmyoglobin reducing activity (MRA) of meats. The loss of mitochondrial function during aging also affects subsequent color stability of meats as it directly influences the oxygen consumption rate of meat. O’Keefe and Hood (1982) reported beef aged for only 3 days had more unstable color than meat aged for a week. They theorized this was due to the higher oxygen consumption rate of 3-d old meat, relative to 7-day old meat, which prevents formation of a deep layer of oxymyoglobin and subsequently causes rapid discoloration.
**Lipid stability and off-flavor.** According to Greene et al. (1969) and Faustman (1990), lipid and pigment oxidation are closely related as an increase in one causes a similar increase for the other. However, this relationship is not valid for meat packaged in high oxygen levels (Resconi et al. 2012). In an oxygen saturated atmosphere, color and lipid stability of meat behave differently.

Ladikos and Lougovois (1990) and Calkins and Hodgen (2007) extensively discussed that lipid oxidation capacity in meats depends on phospholipid composition, level of polyunsaturated fatty acid level (PUFA), and amount of metal ions, oxygen, salt and other prooxidants. Lipid peroxidation creates non-radical products known as volatile compounds, such as aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones which eventually develop rancid off-flavors in meat (Frankel, 1984; Erickson, 2002; Campo et al., 2006). Cayuela et al. (2004) reported that the use of 70% oxygen in packaging systems serves as a pro-oxidant for fatty acids and cholesterol oxidation in pork compared to vacuum packaging. Knock et al. (2006) also demonstrated that increased level of salt in an injection-enhancement solution increased rancid flavors in beef.

Zakrys et al. (2007) stated that lipid oxidation in beef loin steaks (measured by malonaldehyde production; thiobarbituric acid reactive substances, TBARS) increased with the increased levels of oxygen in the packaging system. Zhang and Sundar (2005) reported that TBARS values were relatively low when oxygen level in the packaging system was below 25%. However, the TBARS values significantly increased with increasing levels of oxygen from 35 to 55% in the packaging system. There were some contradictory results reported by Ordonez and Ledward (1977) in early studies done on
packaging systems. They reported that TBARS values of meats occurred at the same rate in air and in air-mixtures containing 89, 90, and 100% oxygen levels. Bingol and Ergum (2011) reported that ostrich meat packaged with air or higher CO\textsubscript{2} levels had lower lipid oxidation rate during the 10-day storage period compared to meat in HiOx-MAP. Asensio et al. (1988) also showed that pork loin chops packaged in high oxygen atmosphere systems had higher lipid oxidation levels compared to pork chops stored in air mix packages. However, some studies (Zakrys et al., 2009; Resconi, et al., 2012) reported that TBARS values of strip steaks were higher for 50 or 60% O\textsubscript{2} containing packages than those in 80 or 70% O\textsubscript{2} level packages after 8 d retail display.

Jakobson and Bertelsen (1999) reported that lipid oxidation levels (rancidity) in meat samples stored at 35, 65, 80% oxygen levels were higher compared to rancidity levels at 20% oxygen. In addition, they stated that lipid oxidation levels increased with increasing retail display temperature and time. Lagerstedt et al. (2007) also reported that ground meat in HiOx-MAP rapidly increased TBARS values during 8 days at 4 ºC under retail display conditions. Similar results were reported by Formanek et al. (2001) in a study where beef patties were packaged in high oxygen atmosphere and stored under refrigerated conditions.

Sensory panelists found that steaks packaged in carbon monoxide (CO) MAP and vacuum packages had more desirable beefy flavor and had lower off-flavor levels compared to steaks in HiOx-MAP systems (Grobbel et al., 2008). Others (Tørngren, 2003; Sørheim et al., 2004; Clausen, 2004; Madsen and Clausen, 2006) also reported that beef steaks packaged in HiOx-MAP developed more off-flavors (warmed-over flavors) than steaks in vacuum packages during retail display. Jackson et al. (1992) also
demonstrated that steaks in HiOx-MAP systems developed stronger off-odors and contained high levels of methyl thiirane, ethylacetate, bezene, and 1-heptene in the packages after 1 – 2 weeks of storage than steaks in 100% CO₂ or vacuumed packages. At 49, 57, 66, 71, 79 °C cooking temperatures, patties from raw beef stored in 80% oxygen and 20% carbon dioxide had higher lipid oxidation levels than beef in carbon monoxide or vacuum packaging systems (John et al., 2004). Sorheim et al. (1999) reported that ground beef, beef loin steaks, and pork chops rapidly developed off-odors compared to those in low CO/ high CO₂/high N₂ atmosphere packages. Touraille and Girard (1985) reported that beef packed in HiOx-MAP for 5 days produced soapy and rancid flavors. Insausti et al. (2002) found 53 volatile compounds in beef packaged under 60% O₂/30% CO₂, and 10%N₂ atmosphere during 15 d of cooler storage. Out of those 53 compounds, 2,3,3-trimethylpentane, 2,3,5-trimethylhexane, 3-octane, 3-methyl-2-heptene, 3-octene, 2-octene, and 2-propanone increased with increased display period and dimethyl sulfide decreased. Kerler and Grosch (1996) related a decrease in dimethyl sulfide to the increase in warmed-over flavors. Resconi et al. (2012) identified several volatile ketones (2,3-butanedione, 2-octanone, 2,3-pentanedione, 2-heptanone, and 4-methyl-2-pentanone) and aldehydes (pentanal, 2-methyl-butanal, 2-furfurylthiol, 1-octen-3-ol, and 2-methylpropyl-acetate) in strip loins stored in HiOx-MAP.

Juiciness. Ultimate juiciness of beef depends on water-holding capacity of beef (Offer and Trinick, 1982). According to Offer and Trinick (1982), water holding capacity of beef is important for two reasons; first, economic and the secondly, for consumer satisfaction. Excess drip during retail display makes unattractive appearance and also drip loss, together with losses during cooking, reduces the servable size of meat.
Bertram et al. (2001) and Huff-Lonergan and Lonergan (2005) explained that most of the water in fresh meat is free or physically entrapped in inter- and intra-myofilamental spaces via capillary forces and a small percentage is tightly bound to proteins by hydrogen bonds in the cellular microstructure; therefore, any alteration in the intracellular structure influences the water holding capacity of muscles. Rate and extent of pH decline, ionic strength, phosphate level, proteolysis, and protein oxidation mainly affect the ability of meat to retain moisture (water holding capacity) during handling, storage, and cooking (Bendall et al., 1988; Night et al., 1988; Huff-Lonergan and Lonergan, 2005; Sheard et al., 2005).

Recent studies have shown that protein oxidation occurred in postmortem muscles during processing, storage, and packaging influence water-holding capacity of raw meat. Chemical modifications in myofibrillar proteins due to oxidants cause a reduction in proteolysis and an increase in protein aggregation (Rowe et al., 2004; Huff-Lonergan and Lonergan, 2005). These chemical alterations in muscle microstructure due to protein oxidation by HiOx-MAP systems increase purge or drip loss of meat during storage and cooking loss during cooking, compared to skin-packaged meats (Lund et al., 2007). Liu et al. (2009) found a reduction in myofibrillar hydration capacity after exposing to hydroxyl radicals was due to myosin oxidation. They further explained the main cause for reduction in hydration capacity of myofibrils was due to a decrease in transverse expansion due to myosin cross linking by disulfide bonds. In 2010, Liu et al. reported that oxidation of myofibrillar proteins with $\text{H}_2\text{O}_2$ significantly enhanced hydration; however, it drastically reduced water-holding capacity and increased cooking loss. Therefore, treatment with $\text{H}_2\text{O}_2$ eventually caused a reduction in product yield. They further
explained the main reason for the increase in hydration capacity was due to enlargement of intracellular spacing in oxidized muscles tissues which worked as canals for water diffusion.

Waliwander et al. (2012) showed that drip loss was higher for beef strip loin steaks in HiOx-MAP compared to vacuum-packaged steaks during 14 d of cooler storage. This may influence ultimate juiciness of steaks after cooking. This finding was revealed by Zakry et al. (2009) and Resconi et al. (2012). In those studies, consumer sensory panel rated beef strip loin steaks, which were packaged in 60, 70, or 80% oxygen contained packages, lower for juiciness compared to steaks in vacuum packaged or in 40-50% oxygen contained packages. Sorheim et al. (1996) reported that inclusion of 10 – 25% oxygen into the packaging system did not significantly affect the purge loss in pork loin sections but they found that packages containing 100% CO2 drastically increased drip loss compared to vacuum-packages. Wicklund et al. (2006) reported, phosphate-enhanced pork chops packaged in HiOx-MAP had more purge loss and lower juiciness ratings than pork chops stored in carbon monoxide packages. Linares et al. (2008) reported, drip losses were lower in lamb longissimus dosi packed with carbon monoxide and higher in samples in 70% O2/30%CO2 packages.

However, D’Agata et al. (2010) reported, packaging systems (PVC-OW, vacuum or 60 % O2 containing packages) did not significantly affect the water holding capacity of beef. Similar results were reported by Doherty et al. (1996) that lamb shoulder primals packaged in MAP (80% O2/20%CO2, 50%CO2/50% N2 or 100%CO2) had less drip loss than primals in vacuum packages during cooler storage. One plausible reason for having less drip loss would be due to being packaged them as primals so that the surface area
exposed to oxygen was low and eventually protein oxidation was low. Pietrasik et al. (2006) also reported that vacuum-packaged and injected (sodium chloride and sodium tripolyphosphate) beef had higher moisture loss as purge during 2 week of dark cooler storage than beef packaged in 70% O₂ and 30% CO₂ atmosphere packages.

Some studies reported that patties prepared from those meats packaged in HiOx-MAP systems were less juicy than patties made from vacuum-packaged ground beef. Jayasingh et al. (2002) reported that patties produced from ground beef stored for 6 or 10 days in HiOx-MAP had less juiciness than patties made from vacuum-packed ground beef. Torngren (2008) also reported that patties made from beef packed in 70%O₂ and 20%CO₂ reduced juiciness and increased cooking loss, compared to patties made from beef overwrapped with oxygen permeable packages.

**Nutritional quality.** Muscle foods are considered as a major source of protein with high biological values in the human diet. The oxidation of myofibrillar proteins in muscle foods may considerably reduce the nutritional value. Nutritional value deterioration in muscle foods due to protein oxidation occurs due to modification in essential amino acid side chains, cleavage of peptide bonds, formation of covalent protein-protein cross linked derivatives, and reduction in digestibility (Stadtman and Berlett, 1997; Xiong, 2000; Lund et al., 2011).

Oxidation of amino acid side chains in muscle proteins forms mainly carbonyl groups and other derivatives. According to Stadtman and Berlett (1997), arginine, cysteine, histidine, leucine, methionine, phenylalanine, tyrosine, tryptophan, threonine, arginine, proline, glutamic acid and lysine are more prone to oxidize and form carbonyls or other derivatives that other amino acids. Among them, histidine, leucine, methionine,
phenylalanine, tryptophan, threonine, and lysine are known as essential amino acids in the human diet and their modification due to oxidation may lead to a significant depletion of their availability in food. Oxidation of some amino acids, especially cysteine and tyrosine, forms disulfide and dityrosine bonds which eventually lead to protein aggregates (Stadtman and Berlett, 1997). Recent studies extensively discussed formation of protein aggregates due to oxidation and its effect on proteolytic degradation in meat (Xiong, 2000). Oxidization of amino acids can alter the secondary and tertiary structure of proteins and lead to change in physiological properties of amino acids, specially solubility and hydrophobicity (Davies, 2001).

Lack of proteolytic degradation of protein aggregates may affect protein digestibility (Morzel et al., 2006; Sante-Lhoutellier et al., 2007; Sante-Lhoutellier et al., 2008a; Sante-Lhoutellier et al., 2008b). Morzel et al. (2006) oxidized myofibrillar proteins isolated from porcine *m. longissimus* muscles by adding different concentrations of Fenton’s reagent (iron catalyst and hydrogen peroxide) and then tried to digest oxidized proteins with papain enzyme. They found that rate of proteolysis of oxidized proteins by papain declined significantly at higher degrees of protein oxidation in myofibrillar proteins. Kristensen et al. (1997) supported this by reporting that oxidative modification of myosin produced high molecular weight aggregates and subsequently reduced its susceptibility to proteolytic degradation by cathepsin B enzyme. Sante-Lhoutellier et al. (2007) reported that oxidized myosin protein were resistant to gastric and pancreatic proteases, such as pepsin, trypsin, and α-chymotrypsin. Sante-Lhoutellier et al. (2008) showed that thermal processing also increased oxidation in myofibrillar proteins. Myofibrillar proteins thermally oxidized at 100°C for 5-45 min had lower pepsin degradation rate compared to...
non-thermally oxidized myofibrillar proteins. However, meat so high in protein and such a good source of balanced amino acid and essential amino acid and that mild oxidation might not have much of a nutritional impact on the overall diet.

**Tenderness.** Tenderness is considered as one of the most important consumer palatability traits and directly or indirectly determines the final meat price (Miller et al., 2001). According to MacCormick (2009), the development of tenderness depends on the integrity of the skeletal muscle cell structure and the activity of endogenous proteases. Therefore, the ultimate tenderness of fresh meat depends on degree of muscle protein degradation during aging, especially the myofibrillar proteins titin, desmin, nebulin, vinculin, troponin-T, and some of other major and minor myofibrillar proteins (Goll et al., 1992; Huff-Lonergan et al., 1995; Taylor et al., 1995; Hopkins and Thompson, 2002; Lametsch et al., 2003; Koohmaraie and Geensink, 2006). Any practice or process which hinders postmortem aging or myofibrillar protein degradation could negatively impact final tenderness of meat.

In the last few years, many studies have been carried out to elucidate the effects of postmortem oxidation in muscle proteins, including functional proteins (calpain system) and myofibrillar proteins (myosin and actin) on meat quality, including tenderness and juiciness, flavor stability, and color stability, during aging. Several researches reported that oxidation processes in postmortem muscle converts some amino acid residues (especially histidine) to carbonyl derivatives, free thiol groups in cysteine residues to disulfide bonds, tyrosine groups to dityrosine bonds, and fatty acids to hydroperoxides (Levine et al., 1994; Martinaud et al., 1997; Xiong, 2000). These oxidative changes in proteins cause protein fragmentation, cross-linking, and aggregation. Those changes can
alter activities of proteolytic enzymes and myofibrillar proteins in the muscle microstructure and eventually reduce postmortem muscle tenderization.

Lund et al. (2011) reported that high oxygen-containing packaging systems increase protein oxidation in muscle foods and negatively affect their instrumental and sensory tenderness attributes. The severity of reduction in tenderness due to high oxygen-containing packages would largely depend upon the levels of oxygen in the packaging system. Zakrys et al. (2009) showed that >40% oxygen inclusion in MAP significantly increased Warner-Bratzler shear force (WBSF) values and sensory toughness in beef steaks. The most common level of oxygen used in modified atmosphere packages in the meat industry is around 70-80% as this level helps to hold cherry red color of meat for a longer time. Seyfert et al. (2005) reported that high oxygen concentrations (80%) in packages reduced sensory tenderness and WBSF values in beef to a greater extent than steaks in 80% \(N_2/CO_2\) packages. Lagerstedt et al. (2011) have also reported that beef strip steaks stored in HiOx-MAP have higher shear force and lower sensory tenderness values compared to steaks packaged in vacuum packages.

A decrease in tenderness due to high oxygen modified packaging systems was reported not only for beef, but also for meats from other species. Lund et al. (2007) showed that porcine *longissimus dorsi* steaks packaged in 70% \(O_2/30\% CO_2\) resulted in reduced tenderness compared to steaks in vacuum, skin packages. Bornez et al. (2010) reported that lamb *longissimus* portions stored in packages containing 70% \(O_2\) and 30% \(CO_2\) had higher shear force values than lamb portions in carbon monoxide-containing packages (69.3% \(N_2/30\% CO_2/0.7\% CO\)). Similar results were reported by Linares et al. (2008) in a study where suckling lamb portions packed in 70% \(O_2/30\% CO_2\)
compared to those stored in CO and CO\textsubscript{2} packages. Some sea-foods, such as red swamp crayfish, stored in 80% CO\textsubscript{2} / 10% O\textsubscript{2} / 10% N\textsubscript{2} containing MAP also had higher shear force values than PVC-OW packaged crayfish (Chen et al., 2008). In addition, there are some studies showing that foods prepared from meat stored in HiOx-MAP also had negative textural attributes compared to those made from meat in PVC-OW packages (Torngren, 2008).

**Mechanism of Muscle Protein Oxidation**

Reactive oxygen species or free radicals (hydroxyl radicals, peroxyl radicals, superoxide anions, hydrogen peroxides and nitric oxides) produced in postmortem muscles can extensively oxidized meat components (mainly lipids and proteins) and eventually change the chemical and physical structure of meat (Burton and Traber, 1990; Butterfield et al., 1998). Those reactive oxygen species and other oxidants can be formed within muscles due to intrinsic (metabolic functions) or extrinsic (processing) factors. In the living muscle cells, some mechanisms are available to prevent formation of free radicals. However, after slaughter, free radical accumulation increases within cells due to the collapse of intrinsic free radical preventive or scavenging mechanisms.

The process of protein oxidation is similar to the oxidation of lipids (Stadtman and Berlett, 1997; Lund et al., 2011). In the process of protein oxidation, a hydrogen atom in a protein molecule is abstracted by a reactive oxygen species and produces a protein carbon-centered radical (P\textsuperscript{*}). The protein carbon-centered radical is consecutively converted into a peroxyl radical (POO\textsuperscript{*}) in the presence of oxygen, and then to an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another protein molecule.
Further reacting with HOO\(^*\), alkyl peroxide generates alcoxy radical (PO\(^*\)) or its POH or hydroxyl derivative (Lund et al., 2011).

It is important to know what factors promote protein oxidation in muscle foods. Radicals formed during lipid peroxidation and intact and denatured ferric hemochromogens (metmyoglobin) produced during myoglobin oxidation can initiate protein oxidation in postmortem muscles (Lund et al. 2011). Faustman (1990) reported that rate of lipid oxidation is coupled with the rate of myoglobin oxidation in meat. However, under HiOx-MAP systems, myoglobin is available as oxymyoglobin in which heamochromogen is present in the ferrous form (the non-catalytic form) of pigment; therefore, it has less effect on lipid oxidation and eventually less effect on protein oxidation as well. Allen (2009) showed that myoglobin oxidation is less in 80% oxygen containing environment. Other than heme-iron, about 13-28% of total iron content in muscle is represented by non-heme iron (Lombadi-Boccia et al., 2002), which is known to be a good prooxidant for lipid and protein in beef muscles. On the other hand, numerous studies showed that lipid-derived radicals and hydroperoxides more rapidly promote protein oxidation than active heamocromogens formed from myoglobin oxidation (Viljanen et al., 2004; Vuorela et al., 2005; Estevez et al., 2008). Park et al. (2006) also reported that protein oxidation is more related to lipid hydroperoxidation system than metal catalyzed metmyoglobin oxidizing system. Therefore, we can conclude that protein oxidation in muscle foods due to HiOx-MAP is more dependent on the rate of lipid oxidation than the rate of myoglobin oxidation.

The significant and quantifiable changes created by protein oxidation in muscle foods are the formation of protein carbonyls, loss of sulfhydryl or free-thiol groups, and
formation of protein cross-links (Stadtman and Berlett, 1997; Lund et al., 2011). Those changes remarkably decrease eating quality of meat by decreasing protein solubility, meat tenderness, myofibrillar swelling and water-holding capacity or juiciness (Huff-Lonergan et al., 2010; Xiong, 2000).

**Formation of carbonyls.** Formation of carbonyl derivatives from oxidation of amino acid side chains during protein oxidation is well known. Oxidation of side chains of lysine, proline, arginine, and threonine residues has been shown to produce carbonyl (aldehydes and ketones) derivatives (Amici et al., 1989; Stadtman and Berlett, 1997). In addition to the direct oxidation of side chains of amino acids, 4-hydroxy-2-nonenal (HNE) produced from lipid peroxidation can react with the ε-amino group of lysine, the imidazole moiety of histidine, or the sulfhydryl group of cysteine residues in other proteins to introduce carbonyl groups to them (Uchida and Stadtman, 1993; Friguet et al., 1994; Nadkarni and Sayre, 1994).

These carbonyls formed are chemically stable; therefore, they can be stored and detected easily (Shacter, 2000). Carbonyls can be derivatized with dinitrophenyl hydrazone (DNPH) and detected by spectrophotometrically at 370 nm, by enzyme-linked immunosorbent assay, by high-performance liquid chromatography, or by Western blotting.

Most *in vitro* myofibrillar protein oxidation studies in model systems showed that carbonyl levels increased significantly when oxidation was induced by the Fenton reagent, containing iron catalyst and hydrogen peroxide (Sante-lhoutellier et al., 2007; Morzel et al., 2006; Martinaud et al., 1997). Formation of carbonyls in meat stored in HiOx-MAP is very well documented. Most studies support that HiOx-MAP systems
increase production of carbonyls in meat by increasing myofibrillar protein oxidation compared to vacuum, PVC-OW, or 100% N₂ packages (Lund et al., 2007; Leygonie et al., 2011; Zakry-Waliwander et al., 2012). However, some studies reported that packaging system with or without oxygen did not affect myofibrillar carbonyl levels in meat (Jongberg et al., 2011; Lagerstedt et al., 2011). Sante-Lhoutellier et al. (2008) reported that cooking also significantly increased carbonyl content in meat by releasing catalytic form of iron from myoglobin denaturation.

**Loss of sulphydryl or free-thiol groups.** Sulphydryl or free-thiol groups in cysteine amino acids are highly susceptible to oxidation in the presence of H₂O₂ or molecular oxygen (Stadtman and Berlett, 1997; Bloksma et al., 1963; Davies, 2003). Oxidation of thiol groups forms various oxidized products, sulfenic acid (RSOH), sulfinic acid (RSOOH), and disulfide cross-links or RSSR as follows (Winterbourn et al., 1999);

\[
\begin{align*}
RSH + H_2O_2 & \rightarrow RSOH + H_2O \\
RSOH + RSH & \rightarrow RSSR + H_2O \\
2RSH + O_2 & \rightarrow RSSR + H_2O
\end{align*}
\]

Davies (2003) reported that thiyl radicals (RS•) formed during oxidation can also produce RSSR disulfide cross-links in the presence of molecular oxygen as follows;

\[
RS^• + RS^- \rightarrow (RSSR)^• \xrightarrow{O_2} RSSR
\]

Determination of loss of thiol or sulphydryl groups in muscle foods is an indication of protein oxidation. The amount of sulphydryls in myofibrillar proteins can be spectrophotometrically measured at 420 nm after derivatized with Ellman’s or 5,5’-dithio-bis(2-nitrobenzoic acid) or DTNB reagent.
Packaging systems containing high levels of oxygen extensively decrease sulfhydryl contents in muscle foods compared to vacuum, PVC-OW, or other packages containing N₂ or CO₂ (Jongberg et al., 2011; Zakrys-waliwander et al. 2012). In metal-catalyzed protein oxidation systems, it has been shown that free thiols in myofibrillar proteins decreased with the increase concentration of oxidizing agents and the time duration of incubation (Morzel et al., 2006; Liu et al., 2009).

**Protein cross-linking.** Formation of intra- and inter-molecular cross-links of muscle proteins leads to aggregation or polymerization of proteins (Stadtman and Berlett, 1997). This is mainly achieved by formation of disulfide cross-links or dityrosine cross-links. Formation of disulfide cross –links has been extensively discussed under the previous topic. Dityrosine cross-links are formed by oxidation of tyrosine amino acid residue. Oxidation of tyrosine forms tyrosyl radicals which eventually react with another tyrosyl radical and form dityrosyl bond (Stadtman and Berlett, 1997; Lund et al., 2011). Formation of cross-links between protein molecules in muscle foods due to protein oxidation is responsible for major negative alterations occurring in meat quality (Xiong, 2000). Xiong et al. (2009) showed that disulfide cross-linking was more responsible for the cross-linking in porcine myofibrillar protein exposed to different oxidative environments (iron-catalyzed, linoleic acid-, and metmyoglobin-oxidizing systems) than dityrosine cross-links. Also, they reported that the systems which generated hydroxyl radicals and ferryl oxygen species were stronger in the cross-linking and aggregation of myofibrillar proteins than the system producing peroxide.

The most common method used to detect cross-linked proteins in a myofibrillar protein sample is the sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-
PAGE) without reducing agents. By using this technique, Lund et al. (2007, 2008a, b) and Kim et al. (2010b) have reported that heavy molecular weight protein bands were detected on gels when myofibrillar proteins, isolated from meats packaged in high oxygen conditions, were electrophoresed. They identified those heavy molecular weight protein bands as cross-linked myofibrillar proteins.

**Oxidation of myofibrillar proteins**

Many studies show that HiO$_2$-MAP systems negatively affect organoleptic and physical properties of retail meat, including tenderness, juiciness, flavor, protein solubility and digestibility (Lund et al., 2007; Grobbel et al., 2008; Kim et al., 2010b; de Mello Jr., 2010; Huff-Lonergan et al., 2010) which are the main factors of consumer satisfaction. Oxidation of myofibrillar proteins is one of the main issues which alter eating qualities of meat packaged under high oxygen conditions. It is reported that side chains of cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine, and methionine amino acid residues are more susceptible to reactive oxygen species (Amici et al., 1989; Stadtman and Berlett, 1997). Therefore, the nature of product formed (carbonyls or cross-linked products) due to protein oxidation depends upon the amino acid residues of the proteins involved in the process. It is reported that myofibrillar proteins are more susceptible to oxidation and among them myosin is the most susceptible, causing intra- or intermolecular cross-linking or aggregation of proteins (Bhoite-Solomon et al., 1992; Decker et al., 1993; Hanan and Shklai, 1995; Martinaud et al., 1997; Liu and Xiong, 2000; Stagsted et al., 2004; Kjaersgard et al., 2006; Lund et al., 2011). Plausible reasons for higher oxidation susceptibility of myosin molecules could be
due to being the most abundant protein type in a muscle cell (about 43% of total myofibrillar proteins) and also consisting of more oxidation susceptible amino acids (Swartz et al., 2009). Oxidation of amino acid residues in myosin proteins form disulfide and dityrosine bonds, within or between myosin filaments and causes protein aggregation (Rowe et al. 2004a, b; Lund et al., 2008a, b; Xiong, 2009; Zakrys-Waliwander et al., 2012). Xiong et al. (2009) showed that these cross-links were formed especially on tail portion of myosin heavy chains. However, it was reported that cross-linking occurs not only between myosin heavy chains but also between myosin heavy chains and other proteins, like titin (Kim et al., 2010a, b). Martinaud et al. (1997) showed that oxidation of myosin in meat occurred during aging as well. Formation of protein aggregates is considered as the main reason for decreasing protein solubility, tenderness, water-holding capacity or juiciness of meat (Huff-Lonergan et al., 2010).

**Oxidation of proteases**

**Calpains.** The key proteolytic enzyme system which is responsible for postmortem tenderization of muscles is the calpain system. Calpain system consists of isoforms of calcium-dependent cysteine proteases, (μ- and m-calpains) and their competitive inhibitor, calpastatin (Kooehmaraie, 1992; Goll et al., 2003). In general, μ-calpain needs between 3-50 μM of Ca²⁺ for half-maximal activity while m-calpain requires 200-1000 μM of Ca²⁺ for half-maximal activity (Cong et al., 1989). These two enzymes digest the same myofibrillar proteins, including troponin T, desmin, and titin in the process of postmortem meat tenderization without breaking down actin and myosin proteins (Dayton et al., 1976; Kendall et al., 1993; Huff-Lonergan et al., 1996). Suzuki (1990)
showed that µ- and m-calpain are composed of 80 and 28 kDa subunits. The 28 kDa and 80 kDa subunits are identical in both calpains. However, the 80 kDa subunits of µ- and m- calpains are encoded by different genes (Suzuki, 1990). The 80 kDa subunit has four domains: Domain I, the N-terminal domain, has no sequence homology to any known polypeptide. Domain II is the catalytic domain which contains a cysteine residue as similar to other cysteine proteinases. Domain III is not homologous to any other known protein. Domain IV is a calmodulin-like domain (Huff-Lonergan et al., 2010). Calcium is necessary for µ- and m-calpain activity but they get autolyzed when incubating with calcium. Autolysis reduces the 80 kDa subunit of µ-calpain to 76 kDa and m-calpain to 78 kDa. The 28 kDa subunit of both enzymes is reduced to 18 kDa (Huff-Lonergan et al., 2010). Extent of autolysis (brief or extended) of both enzymes leads to either to reduce their Ca^{2+} concentration requirement for activation or for their inactivation (Edmunds et al., 1991). Calpain autolysis occurs in both living and postmortem muscle cells (Goll et al., 1992). Autolyzed and unautolyzed calpain forms have proteolytic activity. However, the autolyzed form of µ-calpain shows a higher hydrophobicity and binds strongly to subcellular organelles, such as myofibrils (Boehm et al., 1998). Many studies have shown that both µ- and m-calpains have slower rates of activity against myofibrillar proteins at postmortem muscle pH and ionic strengths (Geesink et al., 1992; Kendall et al., 1993; Huff-Lonergan and Lonergan, 1999). Huff-Lonergan and Lonergan (1999) reported that changes in pH/ionic strengths might cause conformational changes in calpains which favor their hydrophobicity and aggregation on myosin substrates. However, those alterations could cause conformational changes in myofibrillar proteins that impede their degradation by calpains (Huff-Lonergan and Lonergan, 1999). Melody et al. (2004) and
Carlin et al. (2006) showed that rapid postmortem pH decline of muscle favors autolysis and activation of μ-calpains and proteolysis of myofibrillar proteins which speeds up the tenderization process. However, too rapid pH decline negatively affects the calpain activity and degradation of myofibrillar protein substrates as it denatures calpains and enzyme substrates (Bee et al., 2007; Barbut et al., 2008;). Goll et al. (2003) hypothesized that calpains degrade cytoskeletal proteins (titin and nebulin) and intermediate filament proteins (desmin) to release major myofibrillar proteins (actin and myosin) for further degradation by proteasome and lysosomes. Many studies support the contention that calpain enzymes are the major enzymes responsible for pre- and post-rigor proteolysis in muscles (Koohmaraie, 1992; Huff-Lonergan et al., 1996; Huff-Lonergan and Lonergan, 1999).

**Oxidation of Calpains.** One of speculated reasons for decreasing tenderness in meat packaged in HiOx-MAP could be due to the inactivation of calpains. As μ-calpain and m-calpain enzymes contain histidine and free-thiol containing residues (cysteine) at their active sites, they are prone to be oxidized and inactivated easily (Lametsch et al., 2008). Oxidation retards the proteolytic activity (i.e. degradation of titin, nebulin, troponin-T, desmin, and filamin; Harris et al., 2001; Rowe et al., 2004a, b) of μ-calpains and lowers the calpastatin activity on inhibition of μ- and m-calpains (Guttmann et al., 1997; Guttmann and Johnson, 1998; Carlin et al., 2006). This decrease in calpain activity due to oxidized conditions is more prominent at lower pH (pH < 6) and at higher ionic strength (295 mM NaCl; Carlin et al., 2006) which suggests normal postmortem muscles (pH of 5.6 and ionic strength of 295 mM) are susceptible to calpain oxidization when exposed to oxidation conditions. This was confirmed by Rowe et al. (2004a, b) in studies where meat
aged for different time periods was exposed to irradiation. They found that oxidation due to irradiation caused reduction in troponin-T and desmin degradation through inactivation of calpains.

**Controlling Strategies against Protein Oxidation**

Prevention of protein oxidation in meat is important as oxidation reduces or deteriorates some important meat palatability traits. One of the strategies previously used to prevent protein oxidation is application of antioxidants at ante- (feeding) or post-mortem (spray or in-processing formula) stages of meat production. However, the literature available on antioxidant protection against muscle protein oxidation is quite ambiguous. A handful of studies (Young et al., 2002; Estevez et al. 2006; Baron et al., 2009) have reported that some antioxidants considerably reduced protein oxidation and others have reported that there is no protection from antioxidants against protein oxidation.

Strategies used to control lipid oxidation in meat could be used to prevent protein oxidation, as protein oxidation due to HiOx-MAP is directly associated with lipid oxidation. These strategies include incorporation of antioxidants into muscles or muscle foods. Antioxidants can be incorporated into animal tissues thought dietary means or through direct incorporation into the muscle foods.

**Dietary strategies.** Oxidative stability of muscle foods directly depends on the composition (especially fatty acid composition) and antioxidant status (tocopherol, carotenoids, and polyphones) in the feed given to animals. Any feeding regime which increases the PUFA level in meat decreases oxidative stability of meat. The most recent
example for a feeding regime which increases oxidation potential by increasing PUFA levels in beef is corn wet distiller grains plus solubles (WDGS), a byproduct of ethanol production (Senaratne, 2009; de Mello Jr., 2010). It has been shown that only the wet distillers grain part, not the soluble part, of the corn distillers byproduct is responsible for the increase in PUFA which deteriorate color, lipid and flavor stability in beef (Senaratne, 2009; Haack, 2010; Varnold et al., 2012).

Several studies reported that inclusion of antioxidant supplements in the animal diet increases antioxidant level in meat and thereby increasing oxidation stability. Sante-Lhoutellier et al. (2008) reported that oxidation stability of meat depends on the balance between PUFA and antioxidant levels. They observed that lambs fed a concentrate diet had higher carbonyl content than animals fed pasture diet. They further showed that meat from pasture diets had higher levels of vitamin E than meat from concentration diets. Estevez and Cava (2004) also showed that liver pates from Iberian pigs fed grass and acorns had lower lipid and protein oxidation compared to those from white pigs intensively reared and fed a concentrated diet. They also showed that liver pate from white pigs had higher levels of PUFA and lower levels of vitamin E than pate from Iberian pigs. They also reported that the non-heme level in pate from white pigs increased rapidly during cooler storage compared to pate from Iberian pigs. Therefore, they concluded that lipid, protein and non-heme iron levels during refrigerated storage are closely related. Ventanas et al. (2006) reported that feeding pigs with diets enriched with oleic sunflower oil and vitamin E successfully inhibited lipid and protein oxidation. Also, they confirmed that lipid and protein oxidations are interrelated. Mercier et al. (1998) reported that turkey meat from birds fed soy oil significantly induced oxidation of protein
and vitamin E supplementation resulted in a significant decrease in protein oxidation (both carbonyls and loss of thiols). Rowe et al. (2004) also reported that vitamin E supplementation significantly decreased the number and extent of oxidized sarcoplasmic proteins in irradiated beef steaks. Rowe et al. (2004a, b) reported that antioxidants (α-tocopherol) protect μ-calpains from oxidation in early postmortem (2 days of postmortem). Therefore, increasing levels of proteolysis (degraded products of troponin T) could be seen in strip loins from steers fed α-tocopherol supplementation.

Other than vitamin E supplementation, Young et al. (2002) reported that feeding apple and broccoli to chicken reduced oxidation of myofibrillar proteins and lipid in cooked chicken liver. Baron et al. (2009) showed that feeding canthaxanthin from fish oil or vegetable oil sources effectively protected both protein and lipid oxidation in fish. However, several studies reported that there were no effects of dietary antioxidant treatments or lipid sources on protein oxidation (Haak et al., 2006; Smet et al., 2008).

**Postmortem application of antioxidants.** Addition of substances (α-tocopherol, plant phenols, and other synthetic antioxidants) to the meat during processing in order to mitigate oxidation is commonly practiced. Vuorela et al. (2005) reported that rapeseed and pine bark extracts inhibited protein oxidation by 42 and 64%, respectively, compared to controls. Sirinivasan et al. (1996) reported that protein carbonyl production in beef heart surimi-like materials treated with tripolyphosphate, propyl gallate and α-tocopherol reduced significantly. Sweetie et al. (1998) reported that minced chicken treated with butylated hydroxytoluene (BHT), sodium tripolyphosphate, sodium nitrite, tocopherol, ascorbic acid, and citric acid had significantly decreased carbonyl content. Estevez et al. (2008) showed that gallic acid, genistein, and cyanidin-3-glucoside were the most
efficient inhibitors of lipid and protein oxidation. They also stated the chemical structure of the phenolic compounds as well as the nature and conformation of the protein were significantly influential on the overall effect against protein oxidative reactions. Moreover, they explained that phenolic compounds protect myofibrillar and soluble protein against protein oxidation by scavenging lipid-derived radicals and by acting directly on proteins; therefore, the overall effect depends on the nature and consequences of the protein-phenolic interaction. In 2010, Estevez and Heinonen (2010) reported that in the presence of copper, α-tocopherol plus phenolics (gallic acid, chlorogenic acid, genistein, catechin, cyaniding-3-glucoside, and rutin) significantly decreased formation of α-aminoacidic and γ-glutamic semialdehydes carboxyls. In the same study they showed that antioxidant activity of α-tocopherol was affected by the addition of different oxidation initiators (Cu²⁺, Fe³⁺, Myoglobin), but addition of Fe³⁺/myoglobin diminished the antioxidant effect of α-tocopherol.

Rababah et al. (2004) showed that tertiary-butyl hydroquinone (TBHQ, followed by grape seed extract and green tea extract, had effective antioxidant properties against formation of carboxyls in chicken breast. Jongberg et al. (2011) reported that phenolic compounds in white grape extracts inhibited lipid oxidation, carboxyl formation, and myosin cross-linking in beef patties packaged in HiOx-MAP, except loss of thiols.

Baron et al. (2005) reported that hydrophilic Trolox (soluble α-tocopherol) was very effective in preventing lipid and protein oxidation. They further explained that lipophilic antioxidants were inefficient in preventing oxidation of proteins in aqueous solution but showed moderate antioxidative activity on proteins and lipids in a linolenic acid methyl ester system (lipid system).
Grobbel et al. (2008) reported that reduction in tenderness due to HiOx-MAP can be resolved by injection enhancing of meat with beef broth, potassium lactate, sodium phosphate, salt, and natural flavoring prior to packaging them under high oxygen conditions. Kim et al. (2010c) also reported that injection enhancement of beef longissimus and semimembranosus muscles with lactates and phosphates before HiOx-MAP improved instrumental tenderness and reduce protein polymerization. Seyfert et al. (2005) also showed reported that injection enhancement with water, salt, phosphates and rosemary significantly increased overall and myofibrillar tenderness and decrease oxidation. This would be due to increase in ionic strength and pH of enhanced muscles. Wu and Smith (1987) showed that an increase in ionic strength in muscles favors weakening of structural integrity of myofibrils by solubilizing many myofibrillar proteins. Brenner et al. (1984) reported that increased ionic strengths in meat had negative influences on interactions between thick and thin filaments and reduced cross-bridges between them. The other reason for the improvement in tenderness in pH-elevated meat would be due to an increase in water holding capacity. When water holding capacity of meat is high there are larger inter- and intra-myofibrillar spaces. Therefore, number of myofibrils per bite or sample to be cut through in the sensory and instrumental tenderness tests would be low. However, the exact mechanism for increasing tenderness in meat due to phosphate enhancement is not yet fully elucidated.

Most other studies relating antioxidants and HiOx-MAP effects on meat quality deterioration did not evaluate antioxidant effects on protein oxidation. They only studied the antioxidants effects on lipid and color stability of meat (Swanson et al., 1994; Sanchez-Escalante et al., 2001; Meilink et al., 2003; Djamel et al., 2004; Nam et al. 2006;
Hayes et al. 2010). Some studies (Lund et al., 2007a) have reported that rosemary extract and ascorbate/citrate inhibited lipid oxidation but not protein oxidation.

Perhaps a combination of antioxidants which are lipid and water soluble (or hydrophobic and hydrophilic, respectively) would have the potential to prevent both lipid and protein oxidations. Also, it seems that most of synthetic antioxidants (TBHQ, BHT, rosemary, and other phenolic compounds) added during processing had a significant protection against protein oxidation than other natural antioxidants incorporated to meat at the anti- or post-mortem stages. There are some synthetic antioxidants supplements available in the beef cattle feed industry. For example, AGRADO-PLUS (AG) is a beef cattle antioxidant supplement containing a very strong synthetic antioxidant mixture of ethoxyquin and TBHQ.

**Synthetic Dietary Antioxidant, AGRADO-PLUS**

Previous feeding trials practiced with AGRADO®PLUS supplementation shows both positive and neutral results on cattle performance and beef quality. Feeding AG (130 ppm/day) for last 28 d prior to harvest increases the lipid and color stability of ground beef and ribeye steaks during retail display compared to the control diet (Krumseik and Owens, 1998a). The same study showed a slight numerical improvement in feed efficiency and statistical significance in reducing carcass maturity and liver abscess incidence in feedlot cattle fed AG compared to control (Krumseik and Owens, 1998b). Another study (Walenciak et al., 1999) reported that feeding AG a long-term (123 d) did not have any significant improvement in color stability in top loin steaks and ground beef (shoulder clods) during retail display. However, the lipid oxidation was still remarkably
low in ground beef prepared from cattle fed AG. Both studies (Krumseik and Owens, 1998a; Walenciak et al., 1999) showed that AG feeding did not alter tenderness, juiciness, and overall desirability of longissimus steaks aged about 2 weeks. Choat et al. (2002) reported that mixed crossbred steers fed 150 ppm of Agrado daily for 25 days prior to harvest did not show any effect on gain, feed intake, feed efficiency, lean maturity or other quantitative carcass traits. They also reported that no treatment differences were detected for lipid oxidation levels of top loin steaks during retail display. Furthermore, Choat et al. (2002) reported that AG supplementation had no effect on serum levels of vitamin A, vitamin E, and beta-carotene compared with controls during last 25 d of feeding.

A case study, carried out by Stovall et al. (1999) and Kegley et al. (2002) stated that AG supplementation may improve the health of newly arrived feedlot cattle without altering the cattle performance during first 28 days of arrival. This health benefit was more prominent in heifer calves than bull and steer calves. Han et al. (1999) reported that transport-stressed heifers fed AG had higher blood tocopherol level compared to heifers received control diets during first 68 days of their arrival. Han et al. (2002) reported that AG supplementation significantly improved true ruminal organic matter digestion, reduced propionate and increased butyrate concentrations without altering acetate.

**Ethoxyquin.** Ethoxyquin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, a water-insoluble antioxidant, was first allowed for use in the U.K. for the prevention of scald in apples and pears (Madhavi and Salunkhe, 1996). It is known as an effective antioxidant in feeds rich in carotenoids (dehydrated alfalfa, fish meals, squalene, crawfish meal, chili powder and fish oils) for the stability of carotenoid pigments (Madhavi and Salunkhe,
In the animal body, ethoxyquin is readily absorbed, rapidly metabolized and eliminated though urine and feces. However, a study with rats fed a diet containing 0.005% of ethoxyquin for 10 – 11 days (daily intakes of ethoxyquin – 2.3 to 3.7 mg/kg body weight) showed that ethoxyquin was retained in the body. Retention of ethoxyquin in the animal body is tissue specific. The highest concentration of ethoxyquin retention was reported in the liver (2.9 ppm) and kidneys (2.4 ppm). Heart, skeletal muscles, and brain tissues had the least amount of ethoxyquin at 0.32, 0.16, and 0.09 ppm, respectively (Wilson et al., 1959). Some studies with animals and humans fed daily ethoxyquin doses ranging from 0.006 to 0.4% for a long-term reported some toxic effects on livers, kidneys, thyroid glands, colon and skin and the severity was higher in males (Wilson and DeEds, 1959; Tsuda, et al., 1984). Ethoxyquin is not permitted as an antioxidant for foods in Europe. However, it is permitted in the U.S under restricted levels (Mikova, K. 2001). The restricted level of ethoxyquin in uncooked meat is 0.5 ppm (FDA, 2010). King (1964) reported that feeding albino rats a vitamin E deficient diet containing ethoxyquin levels ranging from 0.0625 to 0.05% (of total diet) reduced the congenital malformations associated with vitamin E deficiency during gestation by either acting as a substitute for vitamin E or increasing absorption of available vitamin E in the diet.

**Tertiary-Butyl Hydroquinone or TBHQ.** Tertiary-butyl hydroquinone or TBHQ was approved for use as a food antioxidant early 1970s. A slight water soluble phenolic antioxidant, TBHQ is widely used in food oils and fat, confectionary products, and fried food, dry sausage, and raw or cooked meat products (Mikova, 2001). The antioxidant property of TBHQ is due to its two para-hydroxyl groups (Sherwin and Thompson, 1967). The permitted levels of TBHQ in food are < 0.02% of fat or oil content (FDA,
2010). It is permitted to use in the U.S. and some other countries but not in Europe and Japan. The antioxidant ability of TBHQ is equivalent to or greater than butylated hydroxyanisole (BHA), BHT, or propyl gallate (PG) in food systems (Sherwin and Thompson, 1967). Chastain et al. (1982) reported that a combination of BHA and TBHQ had a significant effect on reducing discoloration and flavor deterioration of restructured pork and beef steaks. Toxicology studies (Madhavi and Salunkhe, 1996) show that TBHQ is rapidly absorbed and progressively excreted in the urine. However, there are no adequate studies to prove tissue accumulation of TBHQ after oral administration. Therefore, there are no significant short- or long-term toxicities reported of TBHQ use (Madhavi and Salunkhe, 1996).

**Postmortem free calcium level and tenderness**

Enzymatic (Koohmaraie, 1988, 1992, 1994; Boehm et al., 1998) and non-enzymatic (Takahashi, 1992; Takahashi, 1996; Takahashi et al., 1999) calcium theories on meat tenderization were extensively studied. The non-enzymatic calcium-induced meat tenderization process indicates that the rise in free calcium in postmortem muscles causes tenderization through a direct effect of calcium on fragmentation on myofibrils due to weakening of Z-disks, weakening of linkage between actin and myosin, and disconnection of titin, nebulin, and desmin from sarcomere. This non-enzymatic calcium theory has less validity in meat aging than the calcium-induced enzymatic theory (fragmentation of myofibrils by calcium dependent calpain system) on postmortem meat tenderization. The functionality of the enzymatic system in postmortem muscles is influenced by several factors, mainly pH, free-calcium level, temperature, inhibitors
(calpastatin) and ionic strength (Huff-Lonergan et al., 2010). Among these factors affecting calpain activity, pH and free-calcium are notably important.

Higher pH and calcium levels are preferred for the optimum activity of calpains (Koohmaraie et al., 1986; Geesink et al., 1992; Koohmaraie, 1992; Kendall et al., 1993; Lee et al., 2000). After exsanguination of an animal, muscle pH gradually drops. Accelerated postmortem pH decline of muscles favors autolysis and activation of μ-calpains and proteolysis of myofibrillar proteins which speeds up tenderization (Melody et al., 2004; Carlin et al., 2006). However, too rapid pH decline negatively affects the calpain activity and degradation of myofibrillar protein substrates as it denatures calpains and enzyme substrates (Bee et al., 2007; Barbut et al., 2008). Lee et al. (2000) detects higher calpain activity in Biceps femoris and Semimembranosus muscles injected with sodium pyrophosphates plus sodium chlorides as sodium pyrophosphates and salt slowed down pH drop of postmortem muscles due to their buffering actions (Carpenter et al., 1961). In addition to the drop in pH, an increase in free Ca^{2+} concentration in postmortem muscles increases calcium depended protease (calpain) activity.

In living muscle cells, Ca^{2+} that is important for many cellular actions including muscle contraction is stored in sarcoplasmic or endoplasmic reticulum of muscle cells (Swartz et al., 2009). Ji and Takahashi (2006) reported that in rabbit psoas major muscle the free Ca^{2+} concentration increased from 33 μM to 230 μM at 30 hours of postmortem. Also, Ji and Takahashi (2006) reported that the free calcium concentration of beef and pork muscles increased from 16 μM (at 40 min postmortem) to 210 μM (at 3 days postmortem). Parrish et al. (1981) reported that free Ca^{2+} in beef longissimus muscle at 10-14 day aging was 638 – 970 μM. Ji and Takahashi (2006) reported that the
phospholipids in sarcoplasmic reticulum are degraded during postmortem aging. Depletion of phospholipids from sarcoplasmic reticulum weakens the sarcoplasmic reticulum and stored Ca^{2+} may leak into the sarcoplasm through channels formed by phospholipid liberation. This may be one of the main reasons for increasing free Ca^{2+} concentration in muscles during postmortem aging.

**Conclusion**

Modified atmosphere packages with 80% oxygen and 20% carbon dioxide (HiOx-MAP) are widely used in fresh beef retail markets as it holds up cherry-red color of meat longer compared to meat in regular overwrapped film packages. However, HiOx-MAP systems negatively affect eating quality of retail meat, including tenderness, juiciness, and flavor and also it reduce nutritional value by decreasing protein digestibility. The decrease in tenderness of beef in HiOx-MAP systems is mainly due to protein oxidation in myofibrillar proteins and postmortem muscle proteases. As proteins get oxidized they change the molecular structure and form intra/inter cross-links (di-sulfide or dityrosine). Cross-linking and chemical changes in proteins due to protein oxidation result in decreasing proteolytic activity of proteases and formation of protein aggregates. Most of studies regarding protein oxidation due to HiOx-MAP have been carried out for early postmortem muscles. In addition, information available on usage of antioxidants against protein oxidation of meat is quite lacking and ambiguous. Therefore, there is more to discover about protein oxidation in beef packaged in HiOx-MAP systems, especially its mechanism in toughening and control measures for beef. Little or no information is available on how WDGS feeding and aging times affect protein oxidation in beef
packaged in HiOx-MAP systems. Therefore, it is more to discover the effects of feeding WDGS, synthetic antioxidant supplementation (AGRADO®PLUS), and aging time on protein oxidation in beef packaged in HiOx-MAP related to beef toughening.
MATERIALS AND METHODS

Diets and feeding cattle. Crossbred yearlings for this study were a part of the feeding trial described by Justin et al. (2011). Crossbred (British × Continental) yearlings steers (n = 483; initial BW = 427 ± 37 kg) were acclimated to the feedlot for 5 or 6 d prior to initial processing. Initial BW was the average of weights collected on 2 consecutive d. On d 1, steers were implanted with Component TE-IS (Elanco Animal Health, Greenfield, IN). Cattle were re-implanted with Component TE-S with Tylan (Elanco Animal Health) on d 71. Cattle were stratified by BW, assigned to 8 weight blocks and assigned randomly to 32 pens. Steers in pens were randomly assigned to one of four dry-rolled corn based diets, containing 0, 30% (DM basis) wet distillers grains plus solubles (WDGS) with (150 mg/kg) or without (0 mg/kg) AGRADO®PLUS (AG) antioxidant supplements. Compositions of dietary treatments and formulated nutrients were listed in appendix I. Four treatment diets were assigned randomly to pen within each block, with 8 pens per treatment and 15 steers per pen. A 21 d adaption period consisted of three periods, each 7 d, where roughage was replaced with an equal amount of concentrate. Half of the weight blocks were fed 145 d and the other half for 160 d.

Slaughter and carcass characteristics. When approximately 60% of steers, within a block were expected to reach the USDA Choice grade and 1.2 cm of back fat, the steers were sent to a commercial abattoir (Tyson Fresh Meats, Inc., Lexington, NE). On the d of slaughter, HCW was recorded. Following a 48 h chill, 12th rib fat thickness, LM area, marbling score, USDA quality grade and USDA yield grade were recorded. To account for gut-fill, final live BW was adjusted using a common dressing percentage of 63%
calculated from HCW. The carcass adjusted final BW was used to calculate ADG and G:F.

**Carcass fabrication.** After grading, both sides of beef loin, short loins (IMPS # 174; NAMP, 2007) and beef chuck, shoulder clods (IMPS # 114; NAMP, 2007) from total of 80 (n = 20 per each dietary treatment) USDA Choice grade carcasses were vacuumed-packaged, and transported under refrigeration to Loeffel meat laboratory at the University of Nebraska-Lincoln. Left and right sides of each beef sub-primal were aged for either 8 or 29 d at 2°C.

**Muscle fabrication.** After 8 and 29 d aging, *m. longissimus lumborum* (strip loin; IMPS # 180; NAMP, 2007) and *m. psoas major* (tenderloin; IMPS # 190; NAMP, 2007) muscles from beef loin, short loins and *m. triceps brachii* (clod heart; IMPS # 114E; NAMP, 2007) muscles from beef chuck, shoulder clods were removed. The anterior end of each strip loin and the posterior end of each tenderloin were trimmed off to remove the surface exposed to the outside. Each strip loin was cut into one 1.25 cm-thick and five 2.54 cm-thick steaks from the anterior. Tenderloins were cut into three 2.54 cm-thick steaks from the posterior. Three 2.54 cm-thick steaks and a 14 cm long roast from the center of the clod heart muscles were removed.

**Packaging and assignment of steaks and roasts.** The first (for oxidation; 0 d retail displayed), and fourth (for tenderness testing; 0 d retail displayed) anterior steaks of strip loins and first anterior steaks of tenderloins and clod hearts (for oxidation; 0 d retail displayed) were immediately vacuum-packaged using vacuum pouches (3mil STD barrier, Prime Sources, St. Louis, OM) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas city, MO). Vacuum-packaged steaks were stored at -20°C to
until they were used. Second and third anterior strip loin steaks were split into halves and assigned for 4 and 7 d oxidation analysis either under oxygen-permeable polyvinyl chloride film packages (PVC-OW) or high oxygen modified atmosphere packages (HiOx-MAP; 80% O$_2$ and 20% CO$_2$). The last two strip loin steaks were allotted for d 7 tenderness testing and packaged either in PVC-OW or in HiOx-MAP. The last two tenderloin and clod heart steaks were assigned for d 4 and 7 retail displayed samples and packaged in PVC-OW. Clod heart roasts were assigned for 6 d retail displayed samples in HiOx-MAP system.

Strip loin steaks assigned for oxidation and tenderness testing in PVC-OW were packaged as four pieces per package and two steaks per package, respectively on Styrofoam trays (21.6 × 15.9 × 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen permeable polyvinyl chloride film. Tenderloin and clod heart steaks, assigned for oxidation testing in PVC-OW were arranged as two steaks per package on Styrofoam trays (13.3 × 25.6 × 1.4 cm, and 21.6 × 15.9 × 2.1 cm, Styro-Tech, Denver, CO). All the strip loin steaks, assigned for HiOx-MAP retail display were packaged (oxidation testing; as four pieces per tray and tenderness testing; as two steaks per tray) in high foam-barrier polypropylene trays (22.2 × 17.1 × 5.1 cm, 03200 series, Coextruded Plastic Technologies, Inc., Janesville, WI) with a gas mixture (80% O$_2$ and CO$_2$) and mechanically sealed with an oxygen-impermeable film. Clod heart roasts assigned for HiOx-MAP were packaged similar to strip loin steaks but in deeper foam-barrier polypropylene trays (22.2 × 17.1 × 7.6 cm, 03300 series, Coextruded Plastic Technologies, Inc., Janesville, WI).
Retail display. All the PVC-OW and HiOx-MAP packages, displayed on a table in a cooler (at 0 ± 2°C) were exposed to continuous 1000-1800 lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions. Packages were randomly rotated daily to avoid location effects. Steaks assigned for d 4 and 7 retail display were removed from retail display tables, immediately vacuum-packaged and stored at -20°C until they were used for testing. Packages containing clod heart roasts were displayed in two Tyler retail display cases (Model LNSC5, Tyler Refrigeration Corporation, Niles, MI) assembled to simulate retail display conditions as mentioned above.

Study I – Color and lipid oxidation of beef in case-ready packages during retail display.

Objective color evaluation. Steaks assigned for d 7 d retail displayed-tenderness testing in PVC-OW and HiOx-MAP (strip loins) and d 7 retail displayed-oxidation testing in PVC-OW (tenderloins and clod hearts) were used to evaluate objective color and subjective discoloration during retail display. Color of each steak was measured with a Hunter Lab® Mini Scan XE Plus (Model 45/0-L, Hunter Associates Laboratory, Inc., Reston, VA) portable colorimeter equipped with a 2.54 cm orifice and using illuminant D65 at 10° standard observer to determine CIE (1976) L* (measure of darkness to lightness; 0 = black and 100 = white, a* (measure of redness; positive values = reddish and negative values = greenish), and b* (measure of yellowness; positive values = yellowish and negative values = bluish) values. Every day before reading color
measurements, the colorimeter was standardized using a black tile and a white tile (X = 78.5, Y = 83.2, and Z = 88.7). The mean of three random readings of each color measurement on each steak during retail display at 24 h intervals were used for statistical analysis.

**Subjective discoloration evaluation.** A six-person trained panel containing graduate students in the Department of Animal Science at University of Nebraska-Lincoln subjectively evaluated discoloration of each steak as a percentage (0 – 100%) from total surface area according to a guide (Appendix II). Panelists were trained using a system of open discussion. Subjective discoloration evaluation was performed right after the objective color measurements.

**Lipid oxidation.** The 2-thiobarbutteric acid reactive substance (TBARS) assay (Appendix III) described by Ahn et al. (1998), which was a modification of the TBARS assay developed by Beuge and Aust (1978), was used to measure the oxidation status of 8 d and 29 d aged steaks displayed for 0, 4 and 7 d in simulated retail display.

Briefly, steaks assigned for oxidation analysis were diced and macerated using a Waring commercial blender (Model 51BL32, Waring Commercial, Torrington, CT), after dipping in liquid nitrogen. Powdered samples were stored at -80°C until they were used for testing (< 2 d). Fourteen mL of deionized, distilled water, 1 mL of butylated hydroxyanisole (10% BHA in 90% ethanol) were added to 5 g of pulverized sample. After homogenizing for 15 sec using a polytron (POLYTRON® Kinimatica CH-6010, Switzerland), the homogenate was centrifuged for 2000 × g for 5 min. One mL of homogenate was mixed with 2 mL of 2-thiobarbituric acid and trichloroacetic acid mixture (15% TCA (w/v) and 20 mM TBA in ddH₂O) and vortexed for 5 sec. The sample
mixture was incubated at 70°C in a water bath for 30 min to develop color. After samples were cooled in a cold-water bath for 10 min, the sample mixture was centrifuged at 2000 x g for 15 min. Finally, duplicate 200 µL aliquots of each sample were transferred into wells on a 96-well plate and the absorbance was read at 540 nm to calculate the mg of malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

**Determination of vitamin E levels.** Method described by Neirenberg and Nam (1992) was used with some modifications to quantify vitamin E levels in the pulverized strip loin steaks d 0 retail display samples (Appendix IV). A gram of pulverized meat samples and 3 mL of ethanol containing 6% pyrogallol were added into 50 mL centrifuge tubes. After vortexing for 1 min, 1 mL of saturated KOH was added and the mixture was flushed with nitrogen gas. Mixture was digested for 30 min at 70°C. After cooling to room temperature, 5 mL of water and 3 mL of hexane containing 0.05% BHT were added. The mixture was vortexed for 1 min and centrifuged at 10 000 rpm for 2 min. supernatant was collected and kept on ice in dark. Hexane extraction for the pellet was repeated for 3 times. Pooled supernatants were evaporated to dryness under nitrogen flush and the residue was resuspended in 200 µL of tetrahydrofuran. The volume was brought up to 300 µL by adding mobile phase (40:5:3:2 of acetonitrile, trtrahydrofuran containing 0.1% BHT and 0.05% triethylmine, methanol, and 1% ammonium acetate in HPLC grade water). After vortexing, 50 µL was injected into HPLC system and read at 292 nm for vitamin E.

**Statistical analysis.** Color and lipid oxidation data of strip loins were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as
the first split-plot treatment, packaging systems as the second split-plot treatment and retail display time (repeated measures) as the third split-plot treatment. Color and oxidation data of tenderloin and clod hearts were analyzed by ANOVA in the GLIMMIX procedure of SAS as a split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment and the retail display period (repeated measures) as the second split-plot treatment. Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, and No AG vs. AG.

**Study II – Mechanism of beef toughening in high oxygen packages during retail display.**

**Warner-Bratzler shear force (WBSF).** Mechanical tenderness of strip loin steaks were estimated using Warner-Bratzler shear force testing (WBSF). Steaks were thawed at 4°C for 24 h prior to grilling. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), turning over once at 35°C, until they reached an internal temperature of 71°C. All steaks from same animal were grilled in a single batch to avoid variation due to cooking session. Internal temperature during grilling was monitored by an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) with a ready-made insulated type T thermocouple attached to a miniature size spool caddy (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) inserted into the geometric center of each steak. Grilled steaks were cooled at 4°C for 24 h and then allowed to reach room temperature. Six cores with 1.27.
cm diameter were removed parallel to the muscle fibers using a drill press. Cores were sheared on a tabletop WBSF analyzer (3000, WBS 25 kg scale, 115 motor, ½ coring cutter, G-R Manufacturing Co., Manhattan, KS) with a triangular Warner-Bratzler shear attachment. An average of the peak shear force (kg) of 6 cores was calculated for each steak.

**Myofibrillar protein isolation.** Myofibrillar proteins, used for protein oxidation evaluations were isolated from strip loin steaks from 10 carcasses per dietary treatments, according to the method found in appendix V. Three grams of frozen muscle samples were knife-cut minced after trimming of visible fat and connective tissue. Minced meat was suspended in ice-cold 10 mL rigor buffer (0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, and 10 mM K₂HPO₄) at pH 7.4. Samples were homogenized using polytron (POLYTRON® Kinimatica CH-6010, Switzerland) at very low speed for 15 s. After homogenization, homogenate was passed thorough doubled layered cheese cloth to further remove connective and fat and connective tissue and filtrate was collected. Homogenate (1.4 mL) was placed in an Eppendorf tube and centrifuged at 4000 × g for 5 min. The supernatant was decanted and the pellet was broken after resuspending in 1 mL of ice-cold rigor buffer. Suspended pellet was vortexed for 10 s and centrifuged for 5 min at 4000 × g. Pellet washing step was repeated three times to remove as much myoglobin as possible. Supernatant was decanted and leftover-supernatant was removed using a pasture pipette. After suspending the pellet in 250 μL of ice-cold rigor buffer, mixture was vortexed thoroughly. Pellets containing eppendorfs were kept on ice at all the times.

**Determination of carbonyl content.** Carbonyls in myofibrillar proteins were measured according to the method described by Oliver et al. (1987) with slight
modifications (Appendix VI). Fifty microliters each of myofibrillar proteins were added to two eppendorf tubes (one for carbonyl estimation and other for protein estimation). Proteins in both were precipitated by adding 1 mL of 10% trichloroacetic acid (TCA) and centrifuged at 5000 × g for 5 min. Supernatants in both were decanted and all trichloroacetic acid were removed using a pasture pipette. Precipitated proteins in the eppendorf tube for carbonyl estimation were derivatized with 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl at room temperature for 40 min on a rocker and vortexed every 10 min. Precipitated proteins in the Eppendorf tube for protein estimation were mixed with 500 μL of 2 N HCl instead of DNPH. After incubation, 500 μL of 20% TCA were added and vortexed at 5000 × g for 5 min. After removing the supernatant, pellet was washed with three times with 1 mL ethanol:ethyl acetate (1:1) solution followed by centrifugation at 5000 × g for 5 min. During washing, the pellet was resuspended and vortexed for 20 s before centrifugation. After the last wash, the pellet was dissolved in 1 mL 6 M guanidine hydrochloride in 20 mM KH$_2$PO$_4$ (pH 2.3), vortexed for 10 s and left at 4°C for overnight. The final solution was centrifuged at 7000 × g for 5 min to remove any insoluble material. Two 200 μL aliquots of each sample were used to read absorbance at 370 nm to measure carbonyl contents using a plate microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California). Two 200 μL aliquots of each relevant protein samples were read at 280 nm to measure proteins contents and the actual protein levels were calculated using BSA in 6 M guanidine hydrochloride in 20 mM KH$_2$PO$_4$ as standard. Carbonyl contents were expressed as nmoles of DNPH incorporated per mg of proteins on the basis of molar
extinction coefficient of 22.0 mM$^{-1}$cm$^{-1}$ at 370 nm for protein hydrazones. The change (delta; $\Delta$; 4/7 d – 0 d) in carbonyls were calculated.

**Determination of free-thiol content.** Free-thiol contents in myofibrillar proteins were determined according to the method described by Ellman (1959) with slight modifications (Appendix VII). Two (for free-thiol and protein estimation) 25μL aliquots of myofibrillar proteins were dissolved in 1 mL of 0.1 M tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8). Twenty five μL of 10 mM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M tris-HCl at pH 8 was added to reaction mixtures for free-thiol estimation. For protein control for free-thiol estimation, 25 μL of 0.1 M tris-HCl (pH) was added. Both protein control and free-thiol mixtures were vortexed and kept at room temperature for 30 min. The final solutions were centrifuged at 5000 × g for 5 min. Two 200 μL aliquots from each free-thiol tube were used on micro plate and read absorbance at 412 nm against reference of 0.1 M tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) using a plate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California). Two 200 μL aliquots of each sample were read at 280 nm and calculated using BSA in 0.1 M tris-HCl, 1.25 mM EDTA, 5% SDS buffer as standard. Free thiol levels were calculated and expressed as nmoles of sulfhydryls per mg of proteins on the basis molar extinction coefficient of 13.6 mM$^{-1}$cm$^{-1}$ at 412 nm for protein sulfhydryls. The change (delta; $\Delta$; 4 or 7 d – 0 d) in free thiols were calculated.

**Determination of myofibrillar protein content.** The remaining amounts of myofibrillar stock samples used for carbonyls and free-thiol estimates were used for SDS-PAGE analysis. Samples from four carcasses per diet (including all aging times, packaging systems, retail display times; 0 and 7 d) were randomly selected for the SDS-
PAGE and immunoblotting analyses using Tris-HCl/glycine/SDS (pH 8.3) continuous buffer systems. Each myofibrillar proteins stock samples were dissolved in 1 mL of 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) and vortexed properly. Then, 100 μL of myofibrillar protein samples were further diluted with adding 100 μL of the same buffer. Total protein contents in the further-diluted protein samples was estimated using Pierce® bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL; Appendix VIII). A concentration series (20 - 2000 μg/mL) of bovine serum albumin (BSA) standards was prepared using 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) as diluents. The BCA working reagents (200 μL; 50:1 of Reagent A : Reagent B) were added to myofibrillar protein samples and BSA standards (25 μL) on a microwell plate and incubated at 37°C for 30 min. After cooling to the room temperature, absorbances were read at 562 nm and the protein concentrations were expressed as μg/mL.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).**

Protein content determined samples were used for the SDS-PAGE analysis using Tris-HCl/glycine/SDS (pH 8.3) continuous buffer systems (Appendix IX). Myofibrillar proteins were incubated for 10min at 95°C in a buffer (1:4; sample:buffer) containing 20% (v/v) of 10% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) of 0.05% (w/v) bromophenol blue and 55% double distilled deionized water under non-reducing conditions. For reducing conditions, the same conditions were applied together with sample buffer containing 5% (v/v) 2-mercaptoethanol. The SDS-PAGE was performed according to the method of Laemmli (1970) with 6% (6 × 8 cm, 1.5 mm thick) resolving and 4% stacking polyacrylamide gels using Mini-PROTEIN®2 Cells (Bio-Rad
Laboratories, Inc., Hercules, CA). The amount of protein load was adjusted to 50 μg per lane. Kaliedoscope prestained standards (10 μL, 161-0324, Bio-Rad Laboratories, Inc., Hercules, CA) were used. The electrophoresis was run at 120 V constant voltages for 90 min. Following electrophoresis, gels were stained (Coomassie brilliant blue R-250 staining solution; 161-0436, Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h on a rocker. Gels were destained in a solution containing 5% glacial acetic acid, 25% methanol, and 70% double distilled for overnight (12 h) at room temperature. After a deionized ddH₂O water rinse, gels were scanned using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE) at 700 nm. Protein band (myosin heavy chains; MHC, cross-linked proteins; CL-P) intensities were estimated as integrated intensities (K.pixels) using Odyssey application software version 1.1.

**Immunoblotting to evaluate myosin cross-linking.** The presence of myosin proteins in cross-linked proteins due to oxidation was evaluated by immunoblotting of myofibrillar proteins separated by 6% resolving and 4% stacking polyacrylamide gels in SDS-PAGE under non-reducing and reducing conditions (Appendix X). Proteins were transferred from gels to PVDF membranes (IPFL20200; 0.45 μm, Immobilon-FL transfer membrane, Millipore Corporation, Billerica MA) for 120 min at a constant voltage of 100 V and 180 mM current using Bio-Rad Mini-Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA) with ice-cold transfer buffer (48 mM tris-base, 39 mM glycine, 20% methanol, 0.0375% SDS at pH 9.2). Membranes were removed and blocked with 10 mL of Odyssey blocking buffer (927-40100; LI-COR, Lincoln, NE) for 120 min at room temperature. Membranes were incubated with primary anti-myosin skeletal muscle monoclonal (MY-32; MA5-11748; Pierce Biotechnology, Rockford, IL)
antibody at a dilution of 1:2000 in Odyssey blocking buffer containing 0.2% Tween-20. Primary antibody incubation was carried out for 1 h at room temperature and then for overnight at 4°C while gently shaking. Following primary antibody incubation, membranes were washed 3 times with freshly made TBS with 0.1% Tween-20. Membranes were incubated for 1 h at room temperature with IRDye-680LT conjugated goat anti-mouse IgG1 (926-68050, LI-COR, Lincoln, NE) at a dilution of 1:5000 in Odyssey blocking buffer containing 0.2% Tween-20 and 0.02% SDS under dark condition. Membranes were then rinsed 3 times with TBS with 0.1% Tween-20 and scanned using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE) at 700 nm.

**Immunoblotting to evaluate troponin-T degradation.** The degree of troponin-T degradation was evaluated by quantifying protein bands at 30 kDa or less. Myofibrillar proteins (100 μg per lane) were separated by 15% resolving and 4% stacking polyacrylamide gels in SDS-PAGE under reducing conditions using Tris-HCl/glycine/SDS (pH 8.3) continuous buffer system (Appendix X). The SDS-PAGE was ran at constant voltage of 100 V and 50 mA current for 150 min. Proteins in the gels were transferred to PVDF membranes for 90 min at a constant voltage of 100 V and 180 mM current using Bio-Rad Mini-Trans-Blot Electrophoretic transfer cell with ice-cold transfer buffer (48 mM Tris-base, 39 mM glycine, 20% methanol, 0.0375% SDS at pH 9.2). Membranes were incubated with primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a dilution of 1:10000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h at room temperature and then for overnight at 4°C while gently shaking. Membranes were washed 3 times with freshly prepared TBS containing 0.1%
Tween-20. Then, they were incubated with secondary antibody, rinsed, and scanned as mentioned under myosin cross-linking detection. Troponin-T degraded products at 30 kDa and less on the gels were measured by quantifying band intensities (k. pixels) using Odyssey application software version 1.1 (Appendix X).

**Statistical analysis.** Data were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment, packaging systems as the second split-plot treatment and retail display time (repeated measures) as the third split-plot treatment. Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, and No AG vs. AG.

**Study III – Protein oxidation gradient in beef packaged in high oxygen packages during retail display.** In this study, myofibrillar protein oxidation and mechanical tenderness were evaluated at different depths (outer most, middle and inner most layers), and muscle fiber directions (parallel and perpendicular) from 8 and 29 d aged *m. triceps brachii* packaged in HiOx-MAP during 6 d of retail display period. In addition, effects of feeding WDGS or corn diets with or without AG antioxidant supplementation on protein oxidation were studied.

**Steak cutting and sample collection.** After 6 d of retail display, 2.45cm-thick steaks were cut perpendicular to muscle fibers (Fig. 1b), vacuum-packaged and stored at -
20°C until tested. From each steak, samples were removed for protein oxidation analyses perpendicular and parallel to muscle fibers at various depths (outer most, middle and inner most) in the roast (Fig. 1c, Appendix XI).

**Figure 1.** Diagram showing the location of steaks and muscle strips removed from the clod heart (*m. triceps brachii*) roasts after retail display. A. clod heart roast; B. steaks cut perpendicular to the muscle fibers; and C. muscle strips removed both parallel and perpendicular to the muscle fibers from all three steaks.

1 perpendicular- oxygen exposure to the longitudinal surface of the muscle fibers.
parallel-oxygen exposure to the cross sectional surface of the muscle fibers.

**Myofibrillar protein isolation.** Myofibrillar proteins in the samples at different depths and muscle fiber orientations were isolated using the same protocol mentioned under study II.

**Determination of carbonyls content.** Carbonyl contents in the myofibrillar protein samples were evaluated using the DNPH method as mentioned under study II.

**Determination of free thiol content.** Myofibrillar free-thiol contents were quantified using DTNB method mentioned in the study II.

**Determination of myofibrillar protein content.** Myofibrillar protein samples from four animals per dietary treatment were randomly selected and used for SDS-PAGE. Prior to SDS-PAGE, protein concentrations in each sample were estimated using Pierce bicinchoninic acid (BCA) protein assay kit as described in the study II.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).** Cross linked myofibrillar proteins identified and estimated using SDS-PAGE as described in the study II.

**Warner-Bratzler shear force (WBSF).** Frozen steaks were thawed at 4°C for 24 h and grilled to an internal temperature of 71°C on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), turning over once at 35°C. During grilling, internal temperature at the geometric center of each steak was monitor using an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) with a ready-made insulated type T thermocouple attached to a miniature size spool caddy (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT). After grilling,
steaks were cooled at 4°C for 24 h. About 2 – 5 cores with 1.27 cm diameter were removed from each layer of the steak parallel to the muscle fibers. Cores were sheared on a tabletop WBSF analyzer (3000, WBS 25 kg scale, 115 motor, ½ coring cutter, G-R Manufacturing Co., Manhattan, KS) attached with a triangular Warner-Bratzler shear attachment. An average of the peak shear force (kg) of each layer perpendicular or parallel to muscle fibers of each steak was used for statistical analysis.

**Statistical analysis.** Data were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment, muscle fiber orientation as the second split-plot treatment and depth in the roast as the third split-plot treatment. Separation of means was conducted using LSMEANS procedure with DIFF and SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare the effects of feeding Corn vs. WDGS, and No AG vs. AG.

**Study IV - Lipid oxidation rate and tenderness of beef.**

**Lipid oxidation.** Steaks lipid oxidation was evaluated using modified 2-thiobarbuteric acid reactive substances (TBARS) assay as mentioned in the study I.

**Warner-Bratzler shear force (WBSF).** Warner-Bratzler shear force test (WBSF) was performed to measure instrumental tenderness of steaks as described in study II.

**Determination of free-calcium level.** The protocol described by Parrish et al. (1981) with some modifications was used to quantify free-calcium levels of each steak (Appendix XII). Each steak was evaluated for free-calcium contents in duplicates.
Briefly, 3 g of steaks, minced by a stainless-steel knife were placed in a thickwall polyallomer ultracentrifuge tubes (13 × 55 mm) and centrifuged at 196 000 × g at 4ºC for 30 min. Seven hundred μL of the supernatant was placed in an eppendorf tube, treated with 0.1 mL of 27.5% trichloroacetic acid and vortexed for 15 s. After standing for 10 min at room temperature, eppendorf tubes were centrifuged for 10 min. Five hundred μL of supernatant was pipetted into plastic tubes and brought up to 5 mL with double distilled deionized water. Prepared samples were filtered through 13 mm diameter Millex-LG 0.20 μm syringe filters (Millipore, Bedford, MA). Calcium concentration of samples was quantified using inductively coupled plasma spectroscopy using appropriate calcium concentration standards (Appendix XII).

**Statistical analysis.** Data were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009) as a split-split-split-plot design with dietary treatments as the whole-plot, aging period and packaging system as sub-plots and retail display time as the repeated measures. Separation of means was conducted using LSMEANS procedure with PDIF or SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, and No AG vs. AG.
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Color and lipid oxidation in case-ready beef from cattle fed wet distillers grains plus solubles and supplemented with antioxidants (AGRADO-PLUS)\textsuperscript{1 2}

L. S. Senaratne-Lenagala,* J. P. Moore,* C. R. Calkins,*\textsuperscript{3} G. E. Erickson,* M. A. Andersen,‡ S. A. Furman,† A. S. de Mello Jr.,* S. Pokharel,* K. A. Varnold,* J. B. Hinkle,* and J. E. Hergenreder*

*Department of Animal Science, University of Nebraska, Lincoln, NE 68583; ‡Novus International, Inc. St. Louis, MO 63304; and †Panhandle Research & Extension Center, University of Nebraska, Scottsbluff, NE 69361

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\textsuperscript{3}Corresponding author: ccalkins1@unl.edu
ABSTRACT

This study was conducted to evaluate the effects of feeding a synthetic antioxidant blend (ethoxyquin and tertiary butyl hydroquinone; AGRADO-PLUS; AG) and high oxygen modified packaging (HiOx-MAP; 80% O₂ and 20% CO₂) on shelf life of beef fed wet distillers grains plus solubles (WDGS). Effects of feeding AG on cattle performance and carcass characteristics also were evaluated. Crossbred steers (n =483) were fed dry-rolled corn based finishing diets containing 0 or 30% WDGS with AG (0 or 150 mg/kg/steer/day for last 145 - 160 d). Beef short loins and beef chuck shoulder clods from 80 USDA Choice carcasses (20 per treatment) were collected, vacuum-packaged, and aged for 8 and 29 d at 2°C. The m. longissimus lumborum, m. psoas major, and m. triceps brachii muscles were fabricated and cut into 2.54 cm-thick steaks. A steak from each muscle type (for d 0 lipid oxidation) was vacuumed packaged and frozen. Two additional steaks from each tenderloin and clod heart were (PVC-OW) packaged and displayed for 4 and 7 d for lipid oxidation. Two strip loin steaks were split into halves, packaged in PVC-OW or under HiOx-MAP, and displayed for 4 or 7 d for lipid oxidation. Percentage surface discoloration and objective color of steaks were evaluated daily. Lipid oxidation of steaks displayed for 0, 4, and 7 d was tested by thiobarbituric acid reactive substances (TBARS) analysis. There were no interactions between feeding AG and WDGS (P > 0.32) on performance or carcass traits. Feeding AG had no effect on performance (P > 0.31) or carcass characteristics (P > 0.25). Feeding 30% WDGS increased (P < 0.01) final BW, DMI, ADG and G:F compared to corn. Cattle fed WDGS had increased (P < 0.01) HCW, 12th rib fat, and USDA YG. Discoloration and TBARS increased during retail display time in both aging periods and in both packaging systems.
The PVC-OW strip loin steaks were significantly more discolored than HiO$_2$-MAP.

Steaks from corn + AG-supplemented cattle had ($P < 0.05$) less discoloration and lower TBARS at the end of retail display than other dietary treatments. The anti-discooloration and antioxidant effects of AG could not be seen when feeding WDGS. Dietary supplementation of AG to cattle fed only corn diets appears to be a viable means to increase lipid and color stability of case-ready beef with no detrimental effects on feedlot performance and carcass characteristics.

**Key Words:** antioxidant, beef, shelf-life, packaging, wet distillers grains
INTRODUCTION

Inclusion of 10% to 40% (DM basis) wet distillers grains plus soluble (WDGS) in the corn-based finishing diets increases performance and carcass characteristics of feedlot cattle (Larson et al., 1993; Corrigan et al., 2009; Luebbe et al., 2012). However, Senaratne et al. (2009) and de Mello Jr. et al. (2010) have shown that feeding WDGS to feedlot cattle linearly increases polyunsaturated fatty acid (PUFA) levels in beef and detrimentally affects beef case-life by compromising its color and lipid stability.

Supplementation of vitamin E to the feedlot steers, fed WDGS rations, has been proven to be a promising strategy to mitigate the negative effects of WDGS feeding on retail shelf life of beef (Senaratne, 2009). Westcott et al. (2000) showed that vitamin E supplementation for 100 d could increase the feeding cost by about $4.00 per animal. Therefore, it is important to investigate the effects of other dietary antioxidant supplements on case-life of beef from cattle fed WDGS containing finishing diets.

Previous feedlot studies, practiced with a commercial synthetic antioxidant blend named, AGRADO-PLUS (AG; ethoxyquin and tertiary-butyl hydroquinone or TBHQ) as a supplement, have shown both positive and neutral results on cattle performance, carcass characteristics and retail beef quality (Krumbsiek and Owens, 1998a,b; Walenciak et al., 1999; Choat et al., 2002). Han et al. (1999) reported that AG supplementation increased blood tocopherol levels. Therefore, it is probable to assume that dietary AG supplementation could deposit more tocopherols in muscles and thereby reduce color and lipid deterioration of beef during retail display. Another means of extending the case-life of beef is packaging beef steaks in high oxygen modified atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂) as elevated oxygen levels used in high HiOx-MAP
stabilize cherry-red, oxymyoglobin pigments in meats and reduce brown metmyoglobin formation (Jakobsen and Bertelsen, 2000; Behrends et al., 2003; Cornforth and Hunt, 2008).

Therefore, this study primarily focused on investigating the effects of AGRADO-PLUS dietary antioxidant supplementation and the HiOx-MAP system on color and lipid stability of case-ready beef from WDGS fed cattle. In addition, effects of AG supplementation in the WDGS containing finisher rations on cattle performance and carcass characteristics were evaluated.

MATERIALS AND METHODS

All procedures related to live animals for this study were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Diets and feeding cattle

Crossbred (British × Continental) yearling steers (n = 483; initial BW = 427 ± 37 kg) were acclimated to the feedlot for 5 or 6 d prior to initial processing. Upon arrival and prior to the start of the experiment, steers were weighed. Initial BW was calculated by averaging weights collected on two consecutive d. On d 1, steers were implanted with Component TE-IS (Elanco Animal Health, Greenfield, IN). Then, cattle were re-implanted with Component TE-S with Tylan (Elanco Animal Health) on d 71. Cattle were stratified by BW, assigned to 8 weight-blocks and assigned randomly to 32 pens. Four treatment diets (Table 1) were assigned randomly to pen within each block, with 8 pens per treatment and 15 steers per pen. Briefly, four dietary treatments were dry-rolled corn-based feedlot diets, containing 0 or 30% (DM basis) wet distillers grains plus solubles (WDGS) with (150 mg/kg) or without (0 mg/kg) AGRADO-PLUS (AG; a blend
of ethoxyquin and tertiary-butyl hydroquinone or TBHQ) per steer per d. The total adaption period consisted of three periods, each 7 d, where roughage was replaced with an equal amount of concentrate. Half of the weight blocks were fed 145 d and the other half for 160 d.

**Slaughter and carcass characteristics**

When approximately 60% of steers, within a block were expected to reach the USDA Choice grade and 1.2 cm of back fat, the steers were sent to a commercial abattoir (Tyson Fresh Meats, Inc., Lexington, NE). On the d of slaughter, HWC was recorded. Following a 48 h chill, 12\textsuperscript{th} rib fat thickness, LM area, marbling score, USDA quality grade and USDA yield grade were recorded. To account for gut-fill, final live BW was adjusted using a common dressing percentage of 63% calculated from HCW. The carcass adjusted final BW was used to calculate ADG and G:F.

**Carcass fabrication**

After grading, both sides of beef loin, short loins (IMPS # 174; NAMP, 2007) and beef chuck, shoulder clods (IMPS # 114; NAMP, 2007) from total of 80 (n = 20 per each dietary treatment) USDA Choice grade carcasses were vacuumed-packaged and transported under refrigeration to Loeffel Meat Laboratory at the University of Nebraska-Lincoln. Left and right sides of each beef sub-primal were aged for either 8 or 29 d at 2°C.

**Muscle fabrication**

After 8 and 29 d aging, *m. longissimus lumborum* (strip loin; IMPS # 180; NAMP, 2007) and *m. psoas major* (tenderloin; IMPS # 190 A; NAMP, 2007) muscles from beef loin, short loins and *m. triceps brachii* (clod heart; IMPS # 114E; NAMP, 2007) muscles
from beef chuck, shoulder clods were removed. The anterior end of each strip loin and the posterior end of each tenderloin were trimmed off to remove the surface exposed to the outside. Each strip loin was cut into one 1.25 cm-thick and five 2.54 cm-thick steaks from the anterior end of the muscle. Tenderloins were cut into three 2.54 cm-thick steaks from the posterior end of the muscle. From the center of the clod heart muscles, three 2.54 cm-thick steaks were removed.

**Assignment of steaks and packaging**

The first steaks of all three muscles were assigned for d 0 lipid oxidation evaluation, immediately vacuum-packaged in pouches (3mil STD barrier, Prime Sources, St. Louis, MO) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas City, MO) and stored at -20°C until they were used for testing. Second and third anterior strip loin steaks were split into halves and assigned for d 4 and 7 oxidation analysis, either under oxygen-permeable polyvinyl chloride film packages (PVC-OW) or under high oxygen modified atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂). The last two strip loin steaks were displayed under simulated retail conditions, after packaging either in PVC-OW or in HiOx-MAP. The last two tenderloin and clod heart steaks were assigned for d 4 and 7 retail displayed samples for lipid oxidation and packaged in PVC-OW.

Strip loin, tenderloin and clod heart steaks assigned for oxidation testing under PVC-OW were packaged in Styrofoam trays (Styro-Tech, Denver, CO) and overwrapped with oxygen permeable polyvinyl chloride film. All strip loin steaks assigned for HiOx-MAP retail display were packaged in high foam-barrier polypropylene trays (22.2 × 17.1 × 5.1
cm, 03200 series, Coextruded Plastic Technologies, Inc., Janesville, WI) with a gas mixture (80% \( \text{O}_2 \) and 20% \( \text{CO}_2 \)) and mechanically sealed with oxygen-impermeable film.

**Retail display**

The PVC-OW and HiOx-MAP packages were displayed on a table in a cooler (at 0 ± 2°C) and exposed to a continuous 1000-1800 lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions. All the packages were randomly rotated daily during the retail display period to avoid location effects.

Steaks assigned for d 4 and 7 retail display lipid oxidation tests were removed from tables, immediately vacuum-packaged and stored at -20°C until they were used for testing.

**Objective color evaluation**

Whole strip loin steaks assigned for d 7 retail display in PVC-OW and HiOx-MAP and other two muscle steaks allotted for d 7 retail display oxidation testing in PVC-OW packages were used to evaluate objective color and subjective discoloration scores during retail display period.

Color of each steak was measured with a Hunter Lab Mini Scan XE Plus (Model 45/0-L, Hunter Associates Laboratory, Inc., Reston, VA) portable colorimeter equipped with a 2.54 cm orifice and using illuminant D65 at 10° standard observer to determine CIE (1976) \( \text{L}^* \) (measure of darkness to lightness; 0 = black and 100 = white), \( \text{a}^* \) (measure of redness; positive values = reddish and negative values = greenish), and \( \text{b}^* \) (measure of yellowness; positive values = yellowish and negative values = bluish) values. Every day before reading color measurements, the colorimeter was standardized using a
black tile and a white tile (X = 78.5, Y = 83.2, and Z = 88.7). The mean of three random readings of each color measurement on each steak during retail display at 24 h intervals were used for statistical analysis.

Subjective discoloration evaluation

A six-person trained panel containing graduate students in the Department of Animal Science at the University of Nebraska-Lincoln subjectively evaluated discoloration of each steak as a percentage (0 – 100%) from total surface area. Panelists were trained using a system of open discussion. Subjective discoloration evaluation occurred right after the objective color measurements.

Lipid oxidation evaluation

The 2-thiobarbituric acid reactive substance (TBARS) assay described by Ahn et al. (1998), which was a modification of the TBARS assay developed by Beuge and Aust (1978), was used to measure oxidation levels of 8 and 29 d aged steaks displayed for 0, 4 and 7 d. Briefly, steaks were diced and macerated after dipping in liquid nitrogen using a Waring commercial blender (Model 51BL32, Waring Commercial, Torrington, CT). Powdered samples were stored at -80°C until they were analyzed. Fourteen mL of deionized-ddH₂O, 1 mL of butylated hydroxyanisole (10% BHA in 90% ethanol) were added to 5-g pulverized sample. After homogenizing for 15 s using a polytron (POLYTRON, Kinimatica CH-6010, Switzerland), the homogenate was centrifuged for 3000 × g for 5 min. One mL homogenate was mixed with 2 mL of 2-thiobarbituric acid and trichloroacetic acid mixture (15% TCA (w/v) and 20 mM TBA in deionized-ddH₂O) and vortexed for 5 s. The sample mixture was incubated at 70°C in a water bath for 30 min to develop color. After samples were cooled in a cold-water bath for 10 min, the
sample mixture was centrifuged at 3000 × g for 15 min. Finally, duplicate 200 µL aliquots of each sample were transferred into wells on a 96-well plate and the absorbance was read at 540 nm to calculate the mg of malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

**Vitamin E concentration evaluation**

Vitamin E concentrations in 8 and 29 d aged, and d 0 retail displayed strip loin steaks were quantified by using the method described by Neirenberg and Nam (1992) with modifications. Briefly, a gram of pulverized meat sample and 3 mL of ethanol containing 6% pyrogallol were mixed in a 50 mL centrifuge tube. After vortexing for 1 min, 1 mL of saturated KOH was added and the mixture was purged with nitrogen. The mixture was digested for 30 min at 70°C and cooled to the room temperature. Then, 5 mL deionized-ddH2O and 3 mL hexane containing 0.05% BHT were added. After vortexing for 1 min, the mixture was centrifuged at 10 000 rpm for 2 min. The supernatant was collected and kept on ice in the dark. For the remaining pellet, hexane extraction was repeated for 3 times. Collected supernatants were pooled and dried under nitrogen flush. The residue was resuspended in 200 µL of tetrahydrofuran. The volume was brought up to 300 µL by adding the mobile phase (40:5:3:2 of acetonitrile, tetrahydrofuran containing 0.1% BHT and 0.05% triethylamine, methanol, and 1% ammonium acetate in HPLC grade water).

After vortexing, 50 µL was injected into a HPLC system, attached with a reversed phase Microsorb-MV C18 column (5 µm, 250 × 4.6 mm; Rainin, Woburn, MA) and a C18 guard column (3 cm × 4.6 mm; packed with 5 µm sphere-5-C18). The column was eluted with the mobile phase under isocratic conditions and absorbance at 292 nm was read for
vitamin E. The HPLC system used was equipped with Waters Associates instruments; 600E solvent delivery system, Pheodyne 484 UV detector, and 74SB integrator (Milford, MA).

**Statistical analysis**

Performance and carcass characteristics data were analyzed as a $2 \times 2$ factorial using the PROC MIXED procedure of SAS (Version 9.2, SAS Inc., Cary, N.C.) with pen used as the experimental unit. The factors included in the model were WDGS inclusion and dietary antioxidant inclusion, with weight block as a fixed variable and initial BW as a covariate due to a small, yet statistical difference in initial BW. The PROC FREQ procedure was used in the Chi-square analyses of USDA quality grade distribution.

Color and lipid oxidation of strip loins were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment, packaging systems as the second split-plot treatment and retail display time (repeated measures) as the third split-plot treatment. Color and oxidation data of tenderloin and clod hearts were analyzed by ANOVA in the GLIMMIX procedure of SAS as a split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment and the retail display period (repeated measures) as the second split-plot treatment. In addition, vitamin E data of strip loins were analyzed by ANOVA in the GLIMMIX procedure as a completely randomized design. Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare the effects of feeding Corn vs. WDGS, and No AG vs. AG on each attribute.
RESULTS AND DISCUSSION

Performance and carcass characteristics

No WDGS level × AG level interaction was observed for performance ($P > 0.32$) or carcass characteristics ($P > 0.34$); therefore, only main effects were evaluated. The effect of dietary AG level are reported in Table 2 and were not significantly different for performance ($P > 0.24$) traits, including DMI, ADG, or G:F. Similar results were reported by Krumseik and Owens (1998b) and Choat et al. (2002), each of which found that AG supplementation for 25 d prior to slaughter did not show any improvements in gain, feed intake, feed efficiency, or other quantitative carcass traits. Fernandez-Duenas (2009) also mentioned that a blend of ethoxyquin and TBHQ as a dietary supplement did not have any effect on animal performance of finishing pigs. However, Krumseik and Owens (1998b) found that AG supplementation significantly reduced carcass maturity and liver abscess incidence in feedlot cattle, compared to the control. Moreover, a case study, carried out by Kegley et al. (2002) and Stovall et al. (1999) showed that AG supplementation could improve the health of newly arrived feedlot cattle without altering the cattle performance during first 28 days of arrival. These health benefits were more prominent in heifer calves than bull or steer calves. Also, Han et al. (2002) reported that AG supplementation significantly improved true ruminal organic matter digestion, reduced propionate and increased butyrate concentrations without altering acetate.

Performance and carcass characteristics for WDGS main effects are presented in Table 2. Initial BW was slightly lighter ($P < 0.01$) for steers receiving the 30% WDGS. Final BW increased ($P < 0.01$) with WDGS inclusion. Daily intake increased ($P < 0.01$) with WDGS inclusion. Gain increased ($P < 0.01$) when including WDGS in the diet.
Feeding WDGS at 30% inclusion increased \( P < 0.01 \) G:F compared to feeding corn diets. Carcasses were heavier \( P < 0.01 \), 12\textsuperscript{th} rib fat increased \( P < 0.01 \), and USDA YG increased \( P < 0.01 \) with WDGS inclusion. Percentage USDA Choice and above tended to increase \( P = 0.14 \), and percentage USDA Select tended to decrease \( P = 0.13 \) when including WDGS. No differences were observed between diets for LM area \( P = 0.21 \) or marbling score \( P = 0.35 \). Similar results for cattle performance and carcass characteristics have been reported by numerous feedlot studies. Vander Pol et al. (2006) showed that feeding 0, 10, 20, 30, 40 and 50% WDGS (DM basis) to feedlot cattle quadratically increased ADG, G:F, DMI, and BW. Klopfenstein et al. (2008) showed that ADG and DMI were maximized at about 30% WDGS inclusion level and G:F was highest at 30 to 50% WDGS inclusion. According to the study conducted by Vander Pol et al. (2009), WDGS has greater energy value compared to corn due to more propionate production, greater fat digestibility and more unsaturated fatty acids reaching the duodenum.

**Subjective discoloration**

Overall, clod heart and tenderloin steaks had more discoloration than strip loin steaks (Tables 3, 4, and 5). Many studies have shown that color stability of tenderloins is inferior to strip loins (O’Keefe and Hood, 1982; Chen et al., 1996; Liu et al., 1996). This is due to compositional, metabolic and physiochemical differences existing in different muscles. Jeong et al. (2009), McKenna et al. (2005), O’Keeffe and Hood (1982) and Renerre and Labas (1987) reported that muscles having more red muscle fibers, more lipid, greater oxygen consumption rates, and lower metmyoglobin reducing activities discolor rapidly.
Significant ($P < .0001$; Table 3) four-way interaction effects of diet $\times$ packaging $\times$ aging $\times$ retail display on percentage surface discoloration of strip loin steaks and significant ($P < .0001$; Table 4 and 5) three-way interaction effects of diet $\times$ aging $\times$ retail display on percentage discoloration of tenderloin and clod heart steaks were observed. Among strip loin steaks, steaks packaged in PVC-OW were significantly ($P < 0.05$; Table 3) more discolored than steaks in HiOx-MAP, which had less than 20% discoloration of the steak surface. Ordonez and Ledward (1977) and Cornforth and Hunt (2008) reported similar results in studies where meat pigments existed more as oxymyoglobins (cherry-red color), when they were packaged in HiOx-MAP systems than in PVC-OW packages. Giddings (1977) stated that formation of metmyoglobin (brown color) from deoxymyoglobin was faster than from oxymyoglobin during oxidation. Therefore, it is plausible to conclude that rate of formation of metmyoglobins is slow in steaks packaged under HiOx-MAP, as much of the pigment exists in the stable oxymyoglobin form under the high oxygen levels in the packages. Many other studies on HiOx-MAP (Daun et al., 1971; Taylor and MacDougall, 1973; Lopez-Lorenzo et al., 1980; and Okayama, 1987; Gill, 1996; Jakobsen and Bertelsen, 2000; Behrends et al. 2003) also reported that elevated levels of oxygen in the packages prolonged color shelf life of meats. Faustman and Cassens (1990) further clarified that oxygen-enriched environments in packages could satisfy the oxygen demand of residual mitochondrial activity of meat without sacrificing already oxygenated pigments and other residual pigments from being oxygenated thereby stabilizing oxygenated pigments and extending the color shelf life of meats. Bekhit et al. (2005) and Fasutman et al. (2010) have suggested that maintaining ferrous forms of myoglobin in meats (either as deoxymyoglobins or oxymyoglobin) by
providing a complete vacuum or oxygen saturation conditions, respectively, could delay discoloration of meat.

All muscle steaks (except 8 d aged and HiOx-MAP packaged strip loin steaks; \( P > 0.10 \)) discolored at an increasing rate (\( P < 0.0001 \)) during retail display period (Table 3, 4, and 5). After 29 d aging, all the steaks, except cloid heart steaks, had greater (\( P < 0.05 \); Table 3, 4 and 5) discoloration compared to 8 d aged steaks during retail display. Similar results were reported by Senaratne (2009) in a study where 28 d aged strip loin and tenderloin steaks highly discolored during retail display than 7 d aged steaks.

Eight d aged strip loin steaks in HiOx-MAP did not show (\( P > 0.05 \); Table 3) any discoloration differences during retail display due to diet. Significant dietary effects on steak discoloration were observed only at the last d of retail display for 29 d aged steaks in HiOx-MAP (\( P = 0.004 \); Table 3) and 8 d aged steaks in PVC-OW (\( P < .0001 \); Table 3). However, steaks aged longer and packaged in PVC-OW showed discoloration differences due to diets after d 5 of retail display (\( P < 0.003 \); Table 3). In all cases, strip loin steaks from corn + AG diets fed cattle discolored more slowly (\( P < 0.05 \); Table 3) compared to steaks from other diet fed cattle. A significant reduction in steak discoloration rates due to AG supplementation was observed only when cattle were on non-WDGS diets (\( P < 0.004 \); Table 3). Antioxidant effects of AG supplementation in reducing discoloration were not noticeable in strip loin steaks from WDGS fed cattle (\( P > 0.05 \); Table 3).

Numerous feedlot studies have reported that feeding WDGS greatly increases polyunsaturated fatty acid (PUFA; Gill et al. 2008; Kinman et al. 2011; de Mello Jr., 2010; Koger et al., 2010; Senaratne, 2009) levels in meat. Polyunsaturated fatty acids are
more prone to oxidize and so compromise color and lipid stability (de Mello Jr., 2010; Senaratne, 2009; Kinman et al. 2011). The current study showed that AG supplementation was not sufficient to maintain color stability of strip loin steaks when cattle were fed WDGS diets. The possible reason could be due to the increase in PUFA levels in steaks by feeding WDGS.

No significant discoloration differences among tenderloin steaks due to diets ($P > 0.05$; Table 4) were observed until d 4. Feeding AG helped to lower ($P < .0003$; Table 4) surface discoloration in short-term aged tenderloin steaks from both corn and WDGS fed cattle. However, after longer-term aging, AG supplementation reduced discoloration only in steaks from corn diets ($P < 0.05$; Table 4) and at the last 2 d of retail display. In addition, feeding WDGS significantly increased ($P < 0.05$; Table 4) surface discoloration of tenderloin steaks compared to steaks from corn diets.

Until the last few days (5-7 d) of retail display, no significant discoloration differences ($P > 0.05$; Table 5) in clod heart steaks due to diet were detected. All clod heart steaks from corn + AG diets had the least ($P < 0.05$; Table 5) discoloration at the end of retail display compared to steaks from other diets. Feeding WDGS significantly ($P < 0.05$; Table 5) increased surface discoloration of long-term aged clod heart steaks. In tenderloin and clod heart steaks also, AG supplementation in reducing discoloration was more noticeable when cattle were fed corn diets than WDGS diets. This could be due to the increase in PUFA levels in muscle from WDGS diets as discussed under discoloration of strip loin steaks.

The AGRADO-PLUS is a commercial antioxidant supplement containing a blend of ethoxyquin; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline and tertiary-butyl
hydroquinone or TBHQ (Novus International, Inc. St. Charles, MO). Previous feeding trials with AG supplementation have shown both positive and neutral results in decreasing retail beef discoloration. Krumseik and Owens (1998 a) reported that feeding AGRADO-PLUS (130 mg/kg/day) for the last 28 d prior to harvest increased color stability of ground beef and ribeye steaks during retail display compared to the control diet. However, Walenciak et al. (1999) reported that feeding AG for 123 d did not provide any significant improvement in color stability in strip loin steaks and ground beef (shoulder clods) during retail display.

Objective color

The main effects of diet on L* (lightness), and a* (redness) values of all muscles steaks were significant ($P < 0.05$; Table 6). Diets did not significantly interact with aging time, retail display d (except a* of strip loin steaks; diets × d, $P < 0.0001$; Table 6) or packaging systems for any objective color parameter ($P > 0.05$; Table 6, 7 and 8). There were no significant ($P > 0.05$; Table 6) dietary effect on b* (yellowness) values for any steak type.

All steaks types from cattle fed WDGS + no-AG were ($P < 0.05$; Table 6) lighter in color than steaks from other diets. Supplementation of AG to WDGS-fed cattle reduced ($P < 0.07$; Table 6) L* values of all steaks; however, similar effects of AG supplementation were not ($P > 0.05$; Table 6) observed among steaks from cattle fed corn diets.

Tenderloin and clod heart steaks from corn diets had greater ($P < 0.05$; Table 6) a* values than steaks from WDGS diets. Also, tenderloin and clod heart steaks from AG-supplemented diets tended to have greater ($P = 0.06$; Table 6) a* values than steaks from
non-AG diets. There were significant \( P = 0.02; \) Table 6) interaction effects of diet \( \times \) retail display d on a* values of strip loin steaks. During the retail display period, a* values of strip loin steaks from all cattle decreased \( P < 0.0001; \) Table 6. Significant \( P < 0.05; \) Table 6) differences in a* of strip loin steaks due to diet were observed from d 3 of retail display and onwards. Strip loin steaks from AG supplemented cattle on Corn or WDGS diets had greater \( P < 0.01; \) Table 6) a* values on the last 2 d of retail display compared to steaks from non-AG supplemented cattle.

The three-way interaction effects of packaging \( \times \) aging \( \times \) retail display d on L*, a* and b* values of strip loin steaks were significant \( P < .0001; \) Table 7). Strip loin steaks in HiOx-MAP had higher \( P <.0001; \) Table 7) L* values and lower \( P < 0.03; \) Table 7) and b* values than steaks in PVC-OW packages. Behrends et al. (2003), Gunderson et al. (2009) and de Mello Jr. (2010) also reported similar results when steaks were packaged in HiOx-MAP systems compared to PVC-OW packages.

Eight d aged strip loin steaks in HiOx-MAP packages had lower \( P < 0.05; \) Table 7) a* values than steaks in PVC-OW during retail display period. Gunderson et al. (2009) and de Mello Jr. (2010) also observed that steaks in PVC-OW were redder than steaks in PVC-OW packages. With long-term aging, a* values of strip loin steaks in both packaging systems were similar \( P > 0.05; \) Table 7). However, at the end of the retail display period, both short- and long-term aged steaks in HiOx-MAP packages were redder \( P < 0.002; \) Table 7) than steaks in PVC-OW packages. This indicates that HiOx-MAP packaging systems can hold steak redness for longer retail display times than PVC-OW packages. The possible reason could be due to a delay in metmyoglobin formation in
steaks in HiOx-MAP packages compared to PVC-OW packages as explained under steak discoloration.

Two-way interaction effects of aging × retail display d on L*, a* and b* values of tenderloin and clod heart steaks were significant ($P < .0001$; Table 8). Eight d aged tenderloin and clod steaks had greater ($P < 0.05$; Table 8) L* (for clod heart steaks- first 4 d only), a* and b* values than steaks aged 29 d.

*Lipid oxidation*

Lipid oxidation levels in all steak types increased with longer aging and retail display times ($P < 0.05$; Table 9 and 10). Three-way interaction effects of diet × aging × retail display d on lipid oxidation (TBARS) values for strip loin steaks were significant ($P = 0.05$; Table 9). There were no significant packaging effects on lipid oxidation levels of strip loin steaks. This might be due to the dilution effect of oxidized lipid on the surface of the thick steaks when prepared for TBARS analysis. Other studies carried out by Jayasingh et al. (2002; for ground beef) and de Mello, Jr. (2010; for strip loin steaks) reported that HiOx-MAP systems increased lipid oxidation levels in meats, compared to PVC-OW packages. As aging and retail display time increased, lipid oxidation levels in strip loin steaks also increased. However, there were no significant differences in oxidation levels among 8 d aged steaks due to diet. The only significant ($P < .0001$; Table 9) difference in lipid oxidation levels in 29 d aged strip loin steaks due to diet could be seen at the end of retail display period. Similar to discoloration results of strip loins, AG supplementation was more effective ($P < 0.05$; Table 9) in reducing lipid oxidation levels when cattle were fed Corn diets than WDGS diets.
Two-way interaction effects of diet × retail display d ($P < 0.03$; Table 9) and aging × retail display d ($P < .03$; Table 10) on lipid oxidation in tenderloin and clod heart steaks were significant. Significant differences ($P < 0.05$; Table 9) in lipid oxidation levels among tenderloin and clod heart steaks due to diet appeared with longer retail display time. Supplementation with AG decreased ($P < 0.10$; Table 9) lipid oxidation levels of steaks compared to non-AG supplemented diets. Clod heart steaks from WDGS diets tended ($P = 0.10$; Table 9) to have higher lipid oxidation levels compared to steaks from Corn diets at the end of the retail display time. However, there were no significant ($P > 0.05$; Table 9) effects of feeding WDGS diets on lipid oxidation levels in tenderloin steaks during retail display. In addition, two-way interaction effects of aging × retail display d on lipid oxidation levels of tenderloin and clod heart steaks were significant ($P < .0001$; Table 10). Longer aging and retail display times increased levels of lipid oxidation in tenderloin and clod heart steaks.

Overall, AG supplementation increased lipid stability in all muscle steaks especially when cattle were fed Corn diets. Similar results were reported by Krumseik and Owens. (1998 a) and Walenciak et al. (1999) in retail case-life studies where ground beef and ribeye steaks were obtained from cattle supplemented with AG. However, Choat et al. (2002) reported that AG supplementation prior to harvest did not show any effect on lipid oxidation levels in beef top sirloin steaks during retail display. The current study did not statistically support that feeding 30% WDGS (DM basis) significantly ($P > 0.05$; Table 9) increased lipid oxidation levels compared to Corn diets. However, numerous feedlot studies have shown that feeding WDGS greatly increases lipid oxidation levels in retail
displayed meats (Gill et al. 2008; Senaratne, 2009; de Mello Jr., 2010; Koger et al., 2010; and Kinman et al. 2011).

**Vitamin E concentrations**

Diet did not affect \( P = 0.25 \); Table 9) vitamin E levels of strip loin steaks. Han et al. (1999) reported that feeding AG (a mixture of ethoxyquin and TBHQ) to transport-stressed heifers increased blood vitamin E levels during first 68 d of their arrival, compared to the control diets. Also, King (1984) reported that feeding albino rats with a vitamin E-deficient diet containing ethoxyquin levels (0.0625 to 0.05% of total diet) reduced the congenital malformations related to vitamin E deficiency during gestation. King assumed that ethoxyquin could act as a substitute for vitamin E or improve absorption of available vitamin E in the diet to reduce the disease. Conversely, Choat et al. (2002) reported that AG supplementation had no effect on serum levels of vitamin A, vitamin E, and beta-carotene compared with controls during last 25 d of feeding. Based on available literature, it is obvious that a positive influence of feeding AG or ethoxyquin along in increasing vitamin E levels in serum can be seen when animals are deficient in vitamin E or immunologically depressed. All the diets in our study were balanced for vitamin E levels (Table 1) and steers were healthy during the study which could be plausible reasons for the absence of an increase in vitamin E levels in strip loins due to AG supplementation.

Toxicology studies have reported that TBHQ is rapidly absorbed and progressively excreted in the urine; therefore, there is no evidence to prove the accumulation of TBHQ in animal body after oral administration (Madhavi and Salunkhe, 1996). On the other hand, according to Wilson et al. (1959), ethoxyquin can be retained in tissues and its
retention level is tissue specific (liver > kidneys > heart > skeletal muscles > brain).
Bohne et al. (2008) also reported that ethoxyquin or ethoxyquin derivatives could be seen in the muscles of Atlantic salmon after feeding ethoxyquin-supplemented fish meal. Therefore, we hypothesize that the antioxidant effect of AG on color and lipid stability would be due to the accumulation of ethoxyquin in muscles. However, further research would be needed to confirm the levels of ethoxyquin accretion in muscle tissues after feeding AG.

CONCLUSIONS

Supplementation of AGRADO-PLUS has no effect on feedlot cattle performance or carcasses characteristics. Conversely, inclusion of wet distillers grains in the corn-based finisher diet positively influences cattle performance and carcass characteristics. High oxygen modified atmosphere packages with 80% oxygen and 20% carbon dioxide extends color shelf life of strip loin steaks, compared to oxygen permeable film packages. Feeding feedlot cattle with the bend of synthetic antioxidants (ethoxyquin and tertiary butyl hydroquinone) contained in AGRADO-PLUS shows positive antioxidant effects against color and lipid oxidations of strip loin, tenderloin, and clod heart steaks during retail display. However, the antioxidant effect of AGRADO-PLUS in reducing lipid and color oxidation of beef steaks is diminished when feeding wet distillers grains plus solubles diets due to an increase of polyunsaturated fatty acids in beef. The antioxidant effect of AGRADO-PLUS in reducing lipid and color oxidation of beef is far more noticeable with longer aging and retail display.
LITERATURE CITED


Stillwater.


Table 1. Composition of finishing diets and formulated nutrient analysis.

<table>
<thead>
<tr>
<th>Item</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn</td>
</tr>
<tr>
<td>Ingredients, DM%¹</td>
<td></td>
</tr>
<tr>
<td>Dry-rolled corn</td>
<td>78.00</td>
</tr>
<tr>
<td>WDGS</td>
<td>0.00</td>
</tr>
<tr>
<td>Corn silage</td>
<td>12.00</td>
</tr>
<tr>
<td>Pellet⁴</td>
<td>4.00</td>
</tr>
<tr>
<td>Liquid supplementation⁵</td>
<td>6.00</td>
</tr>
<tr>
<td>Nutrient composition</td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>78.90</td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>13.40</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>3.88</td>
</tr>
<tr>
<td>NEₘ, Mcal/kg</td>
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</tr>
<tr>
<td>NEₜ, Mcal/kg</td>
<td>1.44</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.70</td>
</tr>
<tr>
<td>Phosphorus, %</td>
<td>0.32</td>
</tr>
<tr>
<td>Potassium, %</td>
<td>0.72</td>
</tr>
<tr>
<td>Sulfur, %</td>
<td>0.17</td>
</tr>
<tr>
<td>Vitamin A, IU/kg</td>
<td>2656.40</td>
</tr>
<tr>
<td>Vitamin D, IU/kg</td>
<td>266.50</td>
</tr>
<tr>
<td>Vitamin E, IU/kg</td>
<td>8.81</td>
</tr>
<tr>
<td>Calcium : Phosphorus</td>
<td>2.19</td>
</tr>
</tbody>
</table>

¹Values presented on a percentage DM per animal basis.
²WDGS = wet distillers grains plus solubles.
³AG = AGRADO-PLUS.
⁴Soybean:urea;1:86:1
⁵Contained Rumensin (345 mg/steer/d; Elanco Animal Health, Greenfield, IN), Tylan (90 mg/steer/d; Elanco Animal Health, Greenfield, IN) with or without AGRADO-PLUS (150 mg/kg/steer/d; Novus International, Inc., St. Louis, MO).
Table 2. Main effects of feeding \(^1\)AGRADO-PLUS (0 or 150 mg/kg) and \(^2\)wet distillers grains plus solubles (0 and 30%) on live performance and carcass characteristics

<table>
<thead>
<tr>
<th>Item</th>
<th>AG(^1) levels</th>
<th>WDGS(^2) levels</th>
<th>SEM(^6)</th>
<th>P- value</th>
<th>SEM(^6)</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No AG (0 mg/kg)</td>
<td>AG (150 mg/kg)</td>
<td>SEM</td>
<td></td>
<td>Corn</td>
<td>30% WDGS</td>
</tr>
<tr>
<td>Cattle performance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td>354</td>
<td>354</td>
<td>0.3</td>
<td>0.94</td>
<td>355</td>
<td>354</td>
</tr>
<tr>
<td>Final BW, kg (^3)</td>
<td>652</td>
<td>653</td>
<td>2.4</td>
<td>0.84</td>
<td>630</td>
<td>674</td>
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<tr>
<td>DMI, kg/day</td>
<td>11.0</td>
<td>11.1</td>
<td>0.04</td>
<td>0.31</td>
<td>10.9</td>
<td>11.2</td>
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<tr>
<td>ADG, kg</td>
<td>1.94</td>
<td>1.95</td>
<td>0.02</td>
<td>0.90</td>
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<td>2.09</td>
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<tr>
<td>G:F</td>
<td>0.175</td>
<td>1.74</td>
<td>0.04</td>
<td>0.70</td>
<td>0.164</td>
<td>0.186</td>
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<tr>
<td>Carcass characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HCW, kg</td>
<td>410</td>
<td>411</td>
<td>1.5</td>
<td>0.81</td>
<td>397</td>
<td>425</td>
</tr>
<tr>
<td>12(^{\text{th}}) rib fat, cm</td>
<td>1.52</td>
<td>1.52</td>
<td>0.03</td>
<td>0.68</td>
<td>1.32</td>
<td>1.73</td>
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<tr>
<td>LM area, cm(^3)</td>
<td>91.6</td>
<td>91.6</td>
<td>0.84</td>
<td>0.70</td>
<td>92.3</td>
<td>91.0</td>
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<tr>
<td>USDA YG (^4)</td>
<td>3.32</td>
<td>3.28</td>
<td>0.05</td>
<td>0.60</td>
<td>2.93</td>
<td>3.67</td>
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<tr>
<td>Marbling (^5)</td>
<td>550</td>
<td>542</td>
<td>4.8</td>
<td>0.25</td>
<td>545</td>
<td>550</td>
</tr>
</tbody>
</table>

\(^3\)HCW / 63% average dressing
\(^4\)Calculated Yield Grade = 2.5 + (2.5×12\(^{\text{th}}\) rib fat, in) + (0.0038×HCW, lb) – (0.32×LM area, in\(^2\)) + (0.2×KPH, %)
\(^5\)Marbling Score: 400 = slight 0; 500 = small 0
\(^6\)SEM = standard error of means
Table 3. Least square means of percentage discoloration of 8 and 29 d aged strip loin steaks (*m. longissimus lumborum*) packaged in high oxygen (80% O\textsubscript{2}; 20% CO\textsubscript{2}) modified atmosphere packages (HiOx-MAP) and oxygen permeable polyvinyl chloride film packages (PVC-OW) during retail display (diet x packaging x aging x retail display d, *P* <.0001).

<table>
<thead>
<tr>
<th>Packaging</th>
<th>HiOx-MAP\textsuperscript{1}</th>
<th>Contrasts</th>
<th>PVC-OW\textsuperscript{2}</th>
<th>Contrasts</th>
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<tr>
<td></td>
<td>No AG\textsuperscript{3} (0 mg/kg)</td>
<td>AG (150 mg/kg)</td>
<td>Pooled SEM\textsuperscript{4}</td>
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<tr>
<td>Retail Display, d</td>
<td>8 d aged</td>
<td>29 d aged</td>
<td>8 d aged</td>
<td>29 d aged</td>
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<tr>
<td>0 d</td>
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<td>1 d</td>
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<td>7 d</td>
<td>3.12</td>
<td>2.23</td>
<td>0.76</td>
<td>0.28</td>
</tr>
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</table>

\textsuperscript{1}HiOx-MAP = high oxygen (80% O\textsubscript{2}; 20% CO\textsubscript{2}) modified atmosphere packages.

\textsuperscript{2}PVC-OW = oxygen permeable polyvinyl chloride film packages.

\textsuperscript{3}WDGS = wet distillers grains plus solubles (30% DM).

\textsuperscript{4}AG = AGRADO-PLUS (0 or 150 mg/kg/steer/d).

\textsuperscript{5}Pooled SEM = pooled standard error of means.

\textsuperscript{a,c}Within a diet, means without a common superscript differ at *P* ≤.05.

\textsuperscript{a,b,c}Within a row, means without a common superscript differ at *P* ≤.05.
Table 4. Least square means of percentage discoloration of 8 and 29 d aged tenderloin (*m. psoas major*) steaks packaged in oxygen permeable polyvinyl chloride film packages (PVC-OW) during retail display (diet × aging × retail display d, *P* <.0001).

<table>
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<tr>
<th>Retail display, d</th>
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<th>Pooled SEM*</th>
<th><em>P</em>-Value</th>
<th>Contrasts <em>P</em>- Value</th>
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<td>AG (150 mg/kg)</td>
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<td>Corn</td>
<td>WDGS</td>
<td>Corn</td>
<td>WDGS</td>
</tr>
<tr>
<td>8 d aged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>0.55e</td>
<td>0.71f</td>
<td>0.20e</td>
<td>0.40f</td>
</tr>
<tr>
<td>1 d</td>
<td>0.92e</td>
<td>1.15f</td>
<td>0.26e</td>
<td>0.36f</td>
</tr>
<tr>
<td>2 d</td>
<td>1.48e</td>
<td>3.17f</td>
<td>0.91e</td>
<td>1.12ef</td>
</tr>
<tr>
<td>3 d</td>
<td>5.07e</td>
<td>11.98ef</td>
<td>4.24e</td>
<td>6.16f</td>
</tr>
<tr>
<td>4 d</td>
<td>11.50dy</td>
<td>26.55dx</td>
<td>9.05dy</td>
<td>13.46dy</td>
</tr>
<tr>
<td>5 d</td>
<td>25.05xc,y</td>
<td>49.34cv,x</td>
<td>18.20xc,y</td>
<td>28.40yc,v</td>
</tr>
<tr>
<td>6 d</td>
<td>43.16bc,y</td>
<td>76.92cx,h,x</td>
<td>31.61bc,y</td>
<td>46.18bc,y</td>
</tr>
<tr>
<td>7 d</td>
<td>61.12bac,y</td>
<td>87.40cx,h,x</td>
<td>44.25bac,y</td>
<td>63.84bac,y</td>
</tr>
<tr>
<td>29 d aged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>0e</td>
<td>0f</td>
<td>0e</td>
<td>0f</td>
</tr>
<tr>
<td>1 d</td>
<td>0e</td>
<td>0f</td>
<td>0e</td>
<td>0f</td>
</tr>
<tr>
<td>2 d</td>
<td>0.33e</td>
<td>2.88f</td>
<td>0.53e</td>
<td>0.79ef</td>
</tr>
<tr>
<td>3 d</td>
<td>2.21e</td>
<td>9.63f</td>
<td>3.05e</td>
<td>5.74e</td>
</tr>
<tr>
<td>4 d</td>
<td>18.71d,y</td>
<td>34.51d,x</td>
<td>19.79d,y</td>
<td>29.93d,x</td>
</tr>
<tr>
<td>5 d</td>
<td>39.23c,y</td>
<td>54.68c,x</td>
<td>32.29c,y</td>
<td>52.49c,x</td>
</tr>
<tr>
<td>6 d</td>
<td>58.80abc,y</td>
<td>75.65bc,x</td>
<td>50.00abc,y</td>
<td>69.42bc,x</td>
</tr>
<tr>
<td>7 d</td>
<td>72.03abc,y</td>
<td>81.18abc,x</td>
<td>62.77abc,y</td>
<td>80.62abc,x</td>
</tr>
</tbody>
</table>

*1* WDGS = wet distillers grains plus solubles (30% DM).

*2* AG = AGRADO-PLUS (0 or 150 mg/kg/steer/d).

*3* Pooled SEM = pooled standard error of means.

*4* Within a diet, means without a common superscript differ at *P* ≤ 0.05.

*5* Within a row, means without a common superscript differ at *P* ≤ 0.05.
Table 5. Least square means of percentage discoloration of 8 and 29 d aged clod heart (*m. triceps brachii*) steaks packaged in oxygen permeable polyvinyl chloride film packages (PVC-OW) during retail display (diet × aging × d, *P* < .0001).

<table>
<thead>
<tr>
<th>Retail display, d</th>
<th>Diet</th>
<th>Contrasts * P*-Value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No AG(^1) (0 mg/kg)</td>
<td>AG (150 mg/kg)</td>
<td>Pooled SEM(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>WDGS(^2)</td>
<td>Corn</td>
<td>WDGS</td>
</tr>
<tr>
<td>8d aged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.02(\text{g})</td>
<td>0.09(\text{g})</td>
<td>0(\text{g})</td>
<td>0.04(\text{f})</td>
</tr>
<tr>
<td>1</td>
<td>0.32(\text{g})</td>
<td>0.36(\text{g})</td>
<td>0.41(\text{g})</td>
<td>0.26(\text{f})</td>
</tr>
<tr>
<td>2</td>
<td>4.18(\text{f})</td>
<td>5.15(\text{f})</td>
<td>5.02(\text{f})</td>
<td>4.16(\text{f})</td>
</tr>
<tr>
<td>3</td>
<td>11.61(\text{e})</td>
<td>13.56(\text{e})</td>
<td>12.10(\text{e})</td>
<td>11.66(\text{e})</td>
</tr>
<tr>
<td>4</td>
<td>21.70(\text{d})</td>
<td>25.33(\text{d})</td>
<td>19.78(\text{d})</td>
<td>19.78(\text{d})</td>
</tr>
<tr>
<td>5</td>
<td>36.32(\text{e})</td>
<td>42.48(\text{e})</td>
<td>33.75(\text{c})</td>
<td>35.75(\text{c})</td>
</tr>
<tr>
<td>6</td>
<td>54.41(\text{b,xy})</td>
<td>60.81(\text{b,x})</td>
<td>50.97(\text{b,y})</td>
<td>54.80(\text{b,xy})</td>
</tr>
<tr>
<td>7</td>
<td>72.19(\text{a,xy})</td>
<td>71.08(\text{a,x})</td>
<td>64.13(\text{a,xy})</td>
<td>72.60(\text{a,xy})</td>
</tr>
</tbody>
</table>

*P*-Value: <.0001 <.0001 <.0001 <.0001

29 d aged

| 0 | 0\(\text{g}\) | 0\(\text{f}\) | 0\(\text{g}\) | 0\(\text{e}\) | 2.99 | 1.00 | - | - |
| 1 | 0.00\(\text{e}\) | 0.00\(\text{f}\) | 0\(\text{e}\) | 0\(\text{e}\) | 2.99 | 1.00 | - | - |
| 2 | 0.17\(\text{e}\) | 0.74\(\text{d}\) | 0.33\(\text{e}\) | 0.36\(\text{e}\) | 2.99 | 1.00 | 0.01 | 0.35 |
| 3 | 2.79\(\text{e}\) | 5.51\(\text{e}\) | 2.91\(\text{e}\) | 3.66\(\text{e}\) | 2.99 | 0.85 | 0.15 | 0.44 |
| 4 | 16.48\(\text{d}\) | 23.67\(\text{d}\) | 15.39\(\text{d}\) | 22.09\(\text{d}\) | 2.99 | 0.07 | 0.05 | 0.70 |
| 5 | 35.94\(\text{c,xy}\) | 43.03\(\text{c,x}\) | 32.10\(\text{c,xy}\) | 43.86\(\text{c,x}\) | 2.99 | 0.003 | 0.14 | 0.81 |
| 6 | 50.93\(\text{b,y}\) | 69.51\(\text{b,x}\) | 46.89\(\text{b,xy}\) | 64.53\(\text{b,xy}\) | 2.99 | <.0001 | 0.001 | 0.42 |
| 7 | 68.21\(\text{a,xy}\) | 82.41\(\text{a,x}\) | 60.58\(\text{a,z}\) | 76.18\(\text{a,xy}\) | 2.99 | <.0001 | 0.002 | 0.15 |

*P*-Value: <.0001 <.0001 <.0001 <.0001

\(^1\)AG = AGRADO PLUS (0 or 150 mg/kg/steer/d).

\(^2\)WDGS = wet distillers grains plus solubles (30% DM).

\(^3\)Pooled SEM = pooled standard error of means.

\(^{\text{a-g}}\) Within a diet, means without a common superscript differ at *P* ≤ 0.05.

\(^{\text{x-z}}\) Within a row, means without a common superscript differ at *P* ≤ 0.05.
Table 6. Least square means of L*(lightness), a*(redness) and b* (yellowness) values of strip loin (m. longissimus lumborum), tenderloin (m. psoas major), and clod heart (m. triceps brachii) steaks during retail display.

<table>
<thead>
<tr>
<th>Diet</th>
<th>L*</th>
<th>a* (lightness)</th>
<th>b* (yellowness)</th>
<th>P - Value</th>
<th>Contrasts P - Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No AG1 (0 mg/kg)</td>
<td>AG (150 mg/kg)</td>
<td>Pooled SEM2</td>
<td>Corn vs. WDGS</td>
<td>No AG vs. AG</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>WDGS2</td>
<td>Corn</td>
<td>WDGS</td>
<td></td>
</tr>
<tr>
<td>Strip loin</td>
<td>40.0y</td>
<td>40.9x</td>
<td>39.9y</td>
<td>39.9y</td>
<td>3.63</td>
</tr>
<tr>
<td>Tenderloin</td>
<td>39.7y</td>
<td>41.2x</td>
<td>40.0y</td>
<td>40.2y</td>
<td>2.29</td>
</tr>
<tr>
<td>Clod heart</td>
<td>35.1y</td>
<td>37.1x</td>
<td>35.1y</td>
<td>35.4y</td>
<td>2.67</td>
</tr>
<tr>
<td>a*4</td>
<td>Tenderloin</td>
<td>16.2x</td>
<td>15.1y</td>
<td>16.7x</td>
<td>15.9xy</td>
</tr>
<tr>
<td></td>
<td>Clod heart</td>
<td>20.4y</td>
<td>19.4z</td>
<td>21.0x</td>
<td>20.0yz</td>
</tr>
<tr>
<td></td>
<td>Strip loin</td>
<td>27.0a</td>
<td>26.8a</td>
<td>26.5a</td>
<td>27.0a</td>
</tr>
<tr>
<td></td>
<td>1 d</td>
<td>24.5b</td>
<td>24.5b</td>
<td>25.0b</td>
<td>24.6b</td>
</tr>
<tr>
<td></td>
<td>2 d</td>
<td>24.3b</td>
<td>23.5c</td>
<td>24.6bc</td>
<td>24.3bc</td>
</tr>
<tr>
<td></td>
<td>3 d</td>
<td>23.3c,xy</td>
<td>22.3c,xy</td>
<td>24.0c,xy</td>
<td>23.7c,xy</td>
</tr>
<tr>
<td></td>
<td>4 d</td>
<td>22.5c,cd</td>
<td>21.8de</td>
<td>23.2de</td>
<td>22.7d</td>
</tr>
<tr>
<td></td>
<td>5 d</td>
<td>22.1de,xy</td>
<td>21.3c,xy</td>
<td>23.1c,xy</td>
<td>21.9d,xy</td>
</tr>
<tr>
<td></td>
<td>6 d</td>
<td>21.4c,xy</td>
<td>20.2fx,xy</td>
<td>22.8e,xy</td>
<td>21.8d,xy</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>18.7f,xy</td>
<td>17.7e,xy</td>
<td>20.4f,xy</td>
<td>19.4be,xy</td>
</tr>
</tbody>
</table>

1AG = AGRADO-PLUS (0 or 150 mg/kg/steer/d).
2WDGS = wet distillers grains plus solubles (30% DM).
3Pooled SEM = pooled standard error of means.
4a*of strip loin; diet x d, P = 0.02.
5Within a diet, means without a common superscript differ at P ≤ 0.05.
6Within a row, means without a common superscript differ at P ≤ 0.05.
Table 7. Least square means of L*(lightness), a*(redness) and b* (yellowness) values of 8 and 29 d aged strip loin steaks (*m. longissimus lumborum*) packaged in high oxygen (80% O\(_2\): 20% CO\(_2\)) modified atmosphere packages (HiOx-MAP) and oxygen permeable polyvinyl chloride film packages (PVC-OV) during retail display (packaging × aging × d, \(P < .0001\)).

<table>
<thead>
<tr>
<th></th>
<th>L*</th>
<th>P-Value</th>
<th>a*</th>
<th>P-Value</th>
<th>b*</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PVC-OV(^1)</td>
<td>HiOx-MAP(^2)</td>
<td>Pooled SEM</td>
<td></td>
<td>PVC-OV(^1)</td>
<td>HiOx-MAP(^2)</td>
</tr>
<tr>
<td>8 d aged</td>
<td></td>
<td></td>
<td>&lt;.0001</td>
<td></td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40.2(^{a,y})</td>
<td>42.8(^{b,x})</td>
<td>0.45</td>
<td>28.5(^a)</td>
<td>28.0(^a)</td>
<td>0.55</td>
</tr>
<tr>
<td>1</td>
<td>39.6(^{b,y})</td>
<td>41.2(^{a,x})</td>
<td>0.45</td>
<td>28.0(^{b,x})</td>
<td>21.6(^{c,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>2</td>
<td>40.7(^{a,y})</td>
<td>43.0(^{a,b,x})</td>
<td>0.45</td>
<td>27.1(^{d,x})</td>
<td>24.6(^{b,c,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>3</td>
<td>39.9(^{b,y})</td>
<td>42.0(^{a,d,x})</td>
<td>0.45</td>
<td>27.5(^{c,x})</td>
<td>24.6(^{b,c,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>4</td>
<td>38.9(^{c,y})</td>
<td>42.5(^{b,c,x})</td>
<td>0.45</td>
<td>26.7(^{d,x})</td>
<td>23.9(^{d,c,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>38.8(^{d,y})</td>
<td>41.5(^{b,d,x})</td>
<td>0.45</td>
<td>26.5(^{d,x})</td>
<td>24.9(^{b,c,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>6</td>
<td>38.7(^{a,y})</td>
<td>43.6(^{c,d,x})</td>
<td>0.45</td>
<td>25.5(^{e,x})</td>
<td>23.5(^{d,y})</td>
<td>0.55</td>
</tr>
<tr>
<td>7</td>
<td>38.3(^{b,y})</td>
<td>41.8(^{a,d,x})</td>
<td>0.45</td>
<td>22.3(^{f,y})</td>
<td>24.1(^{b,c,x})</td>
<td>0.55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>P-Value</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>29 d aged</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

\(^1\)PVC-OV = oxygen permeable polyvinyl chloride film packages.
\(^2\)HiOx-MAP = high oxygen (80% O\(_2\): 20% CO\(_2\)) modified atmosphere packages.
\(^3\)Pooled SEM = pooled standard error of means.
\(^a,y\) Within a packaging system, means without a common superscript differ at \(P \leq 0.05\).
\(^x,y\) Within a row, means without a common superscript differ at \(P \leq 0.05\).
Table 8. Least square means of $L^*$ (lightness), $a^*$ (redness) and $b^*$ (yellowness) values of 8 and 29 d aged tenderloin (*m. psoas major*) and clod heart (*m. triceps brachii*) steaks packaged in oxygen permeable polyvinyl chloride film packages (PVC-OH) during retail display (tenderloin and clod heart- aging × d, $P < .0001$).

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Retail display, d</th>
<th>$L^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
<th>$a^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
<th>$b^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenderloin</td>
<td>0</td>
<td>43.3$^{a,x}$</td>
<td>0.41</td>
<td>&lt;.0001</td>
<td>27.9$^{a,x}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>18.8$^{a,x}$</td>
<td>0.37</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>42.6$^{b,x}$</td>
<td>0.41</td>
<td>&lt;.0001</td>
<td>22.9$^{b,x}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>19.9$^{b,x}$</td>
<td>0.37</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>42.0$^{c,y}$</td>
<td>0.41</td>
<td>&lt;.0001</td>
<td>18.3$^{c,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>16.9$^{c,y}$</td>
<td>0.37</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>40.8$^{d,y}$</td>
<td>0.41</td>
<td>&lt;.0001</td>
<td>18.6$^{d,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>18.7$^{d,y}$</td>
<td>0.37</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.7$^{e,y}$</td>
<td>0.41</td>
<td>0.0002</td>
<td>17.6$^{e,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>18.5$^{e,y}$</td>
<td>0.37</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>40.6$^{f,y}$</td>
<td>0.41</td>
<td>0.0002</td>
<td>14.9$^{f,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>17.0$^{f,y}$</td>
<td>0.37</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>41.8$^{g,y}$</td>
<td>0.41</td>
<td>&lt;.0001</td>
<td>13.2$^{g,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>16.6$^{g,y}$</td>
<td>0.37</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>41.1$^{h,y}$</td>
<td>0.41</td>
<td>0.004</td>
<td>12.4$^{h,y}$</td>
<td>0.54</td>
<td>&lt;.0001</td>
<td>16.6$^{h,y}$</td>
<td>0.37</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

$P$-Value $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$

Clod heart

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Retail display, d</th>
<th>$L^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
<th>$a^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
<th>$b^*$</th>
<th>Pooled SEM</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>39.2$^{a,z}$</td>
<td>0.47</td>
<td>&lt;.0001</td>
<td>28.7$^{a,z}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>18.7$^{a,z}$</td>
<td>0.46</td>
<td>0.0422</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>38.2$^{b,z}$</td>
<td>0.47</td>
<td>&lt;.0001</td>
<td>28.6$^{b,z}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>23.7$^{b,z}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>36.4$^{c,z}$</td>
<td>0.47</td>
<td>0.004</td>
<td>24.2$^{c,z}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>20.9$^{c,z}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.7$^{d,z}$</td>
<td>0.47</td>
<td>0.002</td>
<td>23.1$^{d,z}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>21.0$^{d,z}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>34.2$^{e}$</td>
<td>0.47</td>
<td>0.72</td>
<td>22.8$^{e}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>22.4$^{e}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35.0$^{f}$</td>
<td>0.47</td>
<td>0.86</td>
<td>20.6$^{f}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>20.6$^{f}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>34.9$^{g}$</td>
<td>0.47</td>
<td>0.10</td>
<td>18.2$^{g}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>20.7$^{g}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>34.7$^{h}$</td>
<td>0.47</td>
<td>0.09</td>
<td>16.8$^{h}$</td>
<td>0.56</td>
<td>&lt;.0001</td>
<td>19.3$^{h}$</td>
<td>0.46</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

$P$-Value $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$ $< .0001$

$^1$Pooled SEM = pooled standard error of means.

$^a$-$f$ Within a column, means without a common superscript differ at $P \leq 0.05$.

$x$-$z$ Within a row, means without a common superscript differ at $P \leq 0.05$. 

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Table 9. Least square means of thiobarbituric reactive substances (TBARS; mg malonaldehyde/kg of tissue) value and vitamin E concentration (mg/100 g of tissues) of 8 and 29 d aged strip loin (*m. longissimus lumborum*), tenderloin (*m. psoas major*) and clod heart (*m. triceps brachii*) steaks during retail display (strip loin- diet × aging × d, \( P = 0.05 \); tenderloin- diet × d, \( P = 0.03 \); clod heart- diet × retail display d, \( P = 0.001 \)).

<table>
<thead>
<tr>
<th>Retail display, d</th>
<th>Diet</th>
<th>Pooled SEM</th>
<th>( P )-Value</th>
<th>Contrasts ( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No AG(^1) (0 mg/kg)</td>
<td>AG (150 mg/kg)</td>
<td>Corn</td>
<td>WDGS</td>
</tr>
<tr>
<td>Strip loin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 d aged 0</td>
<td>0.09(^b)</td>
<td>0.02(^b)</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.10(^b)</td>
<td>0.22(^b)</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>7</td>
<td>0.44(^a)</td>
<td>0.67(^a)</td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>( P )-Value</td>
<td>0.03</td>
<td>0.0001</td>
<td>0.19</td>
<td>0.10</td>
</tr>
<tr>
<td>29 d aged 0</td>
<td>0.01(^c)</td>
<td>0.32(^c)</td>
<td>0.16</td>
<td>0.18(^c)</td>
</tr>
<tr>
<td>4</td>
<td>1.00(^b)</td>
<td>1.06(^b)</td>
<td>0.66(^b)</td>
<td>0.69(^b)</td>
</tr>
<tr>
<td>7</td>
<td>2.07(^x)(^x)</td>
<td>2.17(^x)(^x)</td>
<td>1.11(^x)(^y)</td>
<td>1.62(^x)(^y)</td>
</tr>
<tr>
<td>( P )-Value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Tenderloin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.04(^c)</td>
<td>0.07(^c)</td>
<td>0.01(^c)</td>
<td>0.01(^c)</td>
</tr>
<tr>
<td>4</td>
<td>1.16(^x)(^y)</td>
<td>1.38(^x)(^z)</td>
<td>0.59(^h)(^x)</td>
<td>0.88(^h)(^y)</td>
</tr>
<tr>
<td>7</td>
<td>1.81(^x)(^y)</td>
<td>2.06(^x)(^z)</td>
<td>1.40(^x)(^y)</td>
<td>1.64(^x)(^y)</td>
</tr>
<tr>
<td>( P )-Value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Clod heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.23(^c)</td>
<td>0.10(^c)</td>
<td>0.08(^c)</td>
<td>0.03(^c)</td>
</tr>
<tr>
<td>4</td>
<td>1.84(^b)</td>
<td>2.00(^b)</td>
<td>1.42(^b)</td>
<td>1.66(^b)</td>
</tr>
<tr>
<td>7</td>
<td>3.27(^x)(^y)</td>
<td>3.78(^x)(^z)</td>
<td>2.46(^x)(^y)</td>
<td>3.05(^x)(^y)</td>
</tr>
<tr>
<td>( P )-Value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

\(^1\)AG = AGRADO\(^®\)PLUS (0 or 150 mg/kg/steer/d).
\(^2\)WDGS = wet distillers grains plus solubles (30% DM).
\(^3\)Pooled SEM = pooled standard error of means.
\(^a\)\(^-\)\(^c\) Within a diet, means without a common superscript differ at \( P \leq 0.05 \).
\(^x\)\(^-\)\(^z\) Within a row, means without a common superscript differ at \( P \leq 0.05 \).
Table 10. Least square means of thiobarbituric reactive substances (TBARS; mg malonaldehyde per kg of tissue) value of 8 and 29 d aged tenderloin (*m. psoas major*) and clod heart (*m. triceps brachii*) steaks during retail display (tenderloin and clod heart- aging x d, $P < .0001$)

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Retail display, d</th>
<th>Aging, d</th>
<th>Pooled SEM$^1$</th>
<th>$P$ -Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Tenderloin</td>
<td>0</td>
<td>0.03$^c$</td>
<td>0.03$^c$</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.39$^{b,y}$</td>
<td>1.62$^{b,x}$</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.82$^{a,y}$</td>
<td>2.64$^{a,x}$</td>
<td>0.13</td>
</tr>
<tr>
<td>Clod heart</td>
<td>0</td>
<td>0.10$^c$</td>
<td>0.12$^c$</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.94$^{b,y}$</td>
<td>2.52$^{b,x}$</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.96$^{a,y}$</td>
<td>4.31$^{a,x}$</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$P$ -Value <.0001 <.0001

$^1$Pooled SEM = pooled standard error of means.

$^{a-c}$ Within a column, means without a common superscript differ at $P \leq 0.05$.

$^{x-y}$ Within a row, means without a common superscript differ at $P \leq 0.05$. 
Mechanism and control of beef toughening during retail display in high oxygen modified atmosphere packages $^{12}$

L. S. Senaratne-Lenagala,* C. R. Calkins,*$^3$ S. Pokharel,* A. S. de Mello Jr.,* K. A. Varnold,* J. B. Hinkle,* and J. E. Hergenreder*

*Department of Animal Science, University of Nebraska, Lincoln, NE 68583

$^1$A contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE, 68583

$^2$This project was funded by Novus International, Inc. (St. Louis, MO).

$^3$Corresponding author: ccalkins1@unl.edu
ABSTRACT

This research was conducted to study factors affecting protein oxidation-related toughening of beef during retail display in high oxygen packages (80% O2/20%CO2; HiOx-MAP). Steers were fed diets containing 0 or 30% wet distiller grains (WDGS) supplemented with (150 mg/kg) or without (0 mg/kg) AGRADO-PLUS for the last 160 d. After 48 h postmortem, USDA Choice short loins (n=20/diet) were collected and wet-aged for 8 and 29 d. Each m. longissimus lumborum was cut into steaks, assigned for protein oxidation and instrumental tenderness tests either under oxygen permeable film (PVC-OW) or HiOx-MAP packaging and displayed for 7 d. Myofibrillar protein oxidation was quantified by measuring carbonyls and free thiol levels while myofibrillar protein cross-linking was determined by SDS-PAGE. Proteolysis was measured by quantifying troponin-T degradation and tenderness was determined by the Warner-Bratzler shear force test. Packaging system did not significantly interact with aging or diet for any parameter. Steaks in HiOx-MAP were less (P < 0.0001) tender than steaks in PVC-OW during display. Compared to PVC-OW, HiOx-MAP tended to increase accumulation of carbonyls (P = 0.067), and loss of free thiols (P = 0.092) in beef during retail display. The HiOx-MAP also increased formation of cross-linked myosin aggregates (P = 0.004) and reduced troponin-T degraded fragments (P = 0.041) during retail display. At the end of retail display, 29 d aged steaks had increased carbonyls (P = 0.0002), loss of free thiols (P < 0.05), and aggregates (P = 0.019), and tended to increase shear force values (P = 0.067) compared to steaks aged 8 d. Feeding AG increased (P = 0.04) steak shear force values and loss of free thiols (P < 0.04), and reduced troponin-T degraded products (P = 0.0005). In addition, feeding WDGS increased carbonyls (P =
0.002), cross-linked proteins ($P < 0.07$), and tenderness ($P = 0.06$). Retail display of steaks aged long-term increased protein oxidation ($P < .0001$) and polymerization ($P < .0007$), thereby making beef tougher ($P < 0.06$). Feeding WDGS tends to increase beef tenderness while increasing protein oxidation. Dietary AGRADO-PLUS acts as a prooxidant towards protein oxidation; therefore, it is not an effective method to impede protein oxidation in beef.

**Key words:** beef, packaging, protein oxidation, toughening
INTRODUCTION

Meat packages containing high levels of oxygen (80% O\textsubscript{2} and 20% CO\textsubscript{2}; HiOx-MAP) negatively affect tenderness (Lund et al., 2007a, b; Lagerstedt et al., 2011), juiciness (Lund et al., 2007 a, b; Rosconi et al., 2012) and flavor (Grobbel et al., 2008; Bingol and Ergum, 2011) of meats. The available literature (Lund et al., 2007a, b; Kim et al., 2010) hints that decrease in tenderness of meat packaged in HiO\textsubscript{2}-MAP is due to protein oxidation. Little information is available on protein oxidation-related toughening, especially the mechanism of toughening and factors to control the phenomenon.

According to Lund et al. (2011), radicals formed from lipid peroxidation intact or denature ferric hemochromogen (metmyoglobins) produced from oxidation of color pigments can initiate protein oxidation in postmortem muscles. However, under HiOx-MAP, myoglobin molecules are available more as oxymyoglobin – the non-catalytic form which would be expected to have minimal impact on lipid and protein oxidation. Numerous studies (Viljanen et al., 2004; Vuorela et al., 2005; Park et al. 2006; Estevez et al., 2008;) have also shown that lipid-derived radicals and hydroperoxides rapidly promote protein oxidation to a greater extent than active hemochromogens formed from myoglobin oxidation. Thus, protein oxidation in muscle foods due to HiOx-MAP would be expected to depend more on the rate of lipid oxidation than oxidation of color pigments. Lipid oxidation levels in muscle foods depend mainly on the balance between polyunsaturated fatty acid and antioxidant levels. Feeding wet distillers grains plus solubles (WDGS; Senaratne, 2009; de Mello Jr., 2010), low antioxidant status of meat (Estevez and Cava, 2004; Baron et al., 2009; Senaratne, 2009), and longer postmortem aging time (Martinaud et al., 1997; Senaratne, 2009) are known to boost lipid oxidation.
levels in meats. Therefore, it is hypothesized that factors which promote lipid oxidation in meat could also increase protein oxidation.

The current study was designed to study factors affecting toughening of beef *m. longissimus lumborum* steaks during retail display in high oxygen (80% oxygen and 20% carbon dioxide) packages.

**MATERIALS AND METHODS**

All procedures related to live animals for this study were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

*Diets and feeding cattle*

Cattle for this study were a part of the feeding trial described by Senaratne-Lenagala (2012). Briefly, crossbred yearlings (*n* = 483; initial BW = 427 ± 37 kg) were fed one of four corn-based finishing diets containing 0 or 30% (DM basis) wet distillers grains plus solubles (*WDGS*) with (150 mg/kg) and without (0 mg/kg) AGRADO-PLUS (*AG*; a blend of ethoxyquin and tertiary-butyl hydroquinone or TBHQ; Novus International, Inc., St. Louis, MO). Yearlings were fed a total of 160 d prior to slaughter.

*Slaughter and carcass fabrication*

After approximately 60% of steers were estimated to reach USDA Choice grade and 1.2 cm of back fat, the steers were harvested at a commercial abattoir (Tyson Fresh Meats, Inc., Lexington, NE). Following 48 h of postmortem chilling, both sides of beef loins, short loins (IMPS # 174; NAMP, 2007) from a total of 80 (*n* = 20 per dietary treatment) USDA Choice grade carcasses were vacuumed-packaged, and transported under refrigeration to the Loeffel meat laboratory at the University of Nebraska-Lincoln. Both sides of beef sub-primals were aged for either 8 or 29 d at 2°C.
Muscle fabrication

After aging, *m. longissimus lumborum* (strip loin; IMPS # 180; NAMP, 2007) muscles were removed from sub-primals. The anterior end of strip loins was trimmed off to remove the surface exposed to the outside. Each strip loin was cut into one 1.25 cm-thick and five 2.54 cm-thick steaks from the anterior end of the muscle.

Assignment of steaks and packaging

The 1.25 cm-thick steaks were assigned for d 0 oxidation (lipid and protein) evaluations, vacuum-packaged in pouches (3 mil STD barrier, Prime Source, St Louis, MO) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas City, MO) and stored at -20ºC until they were used. The second and third anterior steaks were split into halves and assigned for d 4 or d 7 oxidation evaluation, either under high oxygen modified atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂) or oxygen-permeable polyvinyl chloride film packages (PVC-OW). The fourth anterior steaks were assigned for d 0 Warner-Bratzler shear force (WBSF) evaluation, immediately vacuum-packaged, and stored at -20ºC until they were used. The last two steaks were allotted for d 7 WBSF evaluations and packaged in PVC-OW and HiOx-MAP, respectively.

Steaks assigned for oxidation analysis and WBSF testing were packaged as four pieces and two steaks per tray, respectively. Steaks assigned for PVC-OW were packaged on Styrofoam trays (21.6 × 15.9 × 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen permeable transparent film. All the steaks allotted for HiOx-MAP were packaged in high foam-barrier polypropylene trays (22.2 × 17.1 × 5.1 cm, 03200 series, Coextruded Plastic Technologies, Inc., Janesville, WI) with a gas mixture (80% O₂ and 20% CO₂) and mechanically sealed with oxygen-impermeable transparent film.
Retail display

All the packages were displayed on a table in a cooler (at 0 ± 2°C) and exposed to continuous 1000-1800 lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions. Steaks allotted for d 4 and 7 retail display times were removed from tables on each designated d, immediately vacuum-packaged and stored at -20°C until they were used.

Warner-Bratzler shear force

Instrumental tenderness of steaks was evaluated by using WBSF test. Frozen steaks were thawed at 4°C for 24 h before grilling. All steaks from the same animal were grilled in a single batch to avoid variation due to cooking session. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC) to an internal temperature of 71°C turning over once at 35°C. During grilling, steak internal temperature was monitored by using an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) connected to a ready-made insulated type T thermocouple (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT). The thermocouples were inserted into the geometric center of the steak prior to grilling. After cooling at 4°C for 24 h, 6 cores with 1.27 cm diameter were removed parallel to the muscle fibers using a drill press. Prepared cores were sheared on a tabletop WBSF analyzer (3000, WBS 25 kg scale, 115 motor, ½ coring cutter, G-R Manufacturing Co., Manhattan, KS) with attached triangular Warner-Bratzler shear blade. An average of the peak shear force (kg) of 6 cores was calculated for each steak. In addition, the change in the peak WBSF between d 7 and d 0 steaks (delta; Δ; 4/7 d – 0 d) was calculated.
Myofibrillar protein isolation

Myofibrillar proteins, used for protein oxidation evaluations were isolated from steaks from 10 carcasses per dietary treatments according to the method described by Pietrazak et al. (1997) with slight modifications. Three grams of frozen steaks were knife-minced after trimming off fat and connective tissue. Minced meat was suspended in 15 mL of ice-cold rigor buffer (0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, and 10 mM K₂HPO₄, pH 7.4) and homogenized (POLYTRON® Kinimatica CH-6010, Switzerland) at a very low speed for 15 s. Homogenate was passed through doubled layered cheese cloth to further remove fat and connective tissue. The homogenate (1.4 mL) was centrifuged at 4000 × g for 5 min and the supernatant was decanted. The protein pellet was resuspended in 1 mL of ice-cold rigor buffer, vortexed for 10 s, and centrifuged for 5 min at 4000 × g. The pellet washing step was repeated three times to remove as much myoglobin as possible. The washed myofibrillar protein pellet was resuspended in 250 μL of ice-cold rigor buffer. Myofibrillar protein pellets were kept on ice throughout the isolation process to avoid further oxidation.

Determination of carbonyl content

Carbonyl contents in myofibrillar proteins were quantified according to the method described by Oliver et al. (1987) with some modifications. Briefly, two 50 μL aliquots of each myofibrillar protein preparation were used for carbonyl and protein estimations, respectively. Proteins in both aliquots were precipitated with 1 mL of 10% trichloroacetic acids (TAC) followed by centrifugation at 5000 × g for 5 min. Precipitated proteins for the carbonyl estimation was derivatized with 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl at room temperature for 40 min on a rocker
and vortexed every 10 min. Instead of DNPH, precipitated proteins for the protein estimation were incubated with 2 N HCl. After incubation, 500 μL of 20% TCA was added, vortexed and centrifuged at 5000 × g for 5 min to precipitate proteins. Protein pellets were washed three times with 1 mL ethanol:ethyl acetate (1:1) solution followed by centrifugation at 5000 × g for 5 min. During each wash, protein pellets were resuspended and vortexed for 20 s prior to centrifugation. After the last wash, protein pellets were dissolved in 1 mL 6 M guanidine hydrochloride in 20 mM KH₂PO₄ (pH 2.3) and vortexed for 10 s followed by overnight incubation at 4ºC. The final solution was centrifuged at 7000 × g for 5 min to remove any insoluble matter. Absorbance in carbonyl and protein preparations were read at 370 nm to measure carbonyl contents using a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California). Absorbance in protein preparations were read at 280 nm to quantify protein contents using a protein concentration standard, prepared by dissolving bovine serum albumin in 6 M guanidine hydrochloride buffer (pH 2.3). Carbonyl contents were calculated and expressed as nmoles of DNPH incorporated per mg of proteins on the basis of molar extinction coefficient of 22.0 mM⁻¹cm⁻¹ at 370 nm for protein hydrazones. The production of carbonyls during retail display was calculated by subtracting d₀ carbonyl contents from d₄ or 7 carbonyl values (delta; Δ; 4/7 d – 0 d).

**Determination of free-thiol contents**

Free-thiol contents in myofibrillar proteins were determined using the protocol described by Ellman (1959) with some modifications. Briefly, two 25 μL aliquots of each myofibrillar protein preparation were used to estimate free-thiols and protein levels in each sample separately. Myofibrillar proteins were dissolved in 1 mL of a buffer (pH 8)
containing 0.1 M tris-HCl, 1.25 mM EDTA, and 5% SDS. Protein preparations for free-thiol estimation were mixed with 25 μL of 10 mM 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) in containing 0.1 M tris-HCl buffer (pH 8). Instead of DTNB, proteins in the preparation for protein estimation were mixed with 25 μL of 0.1 M tris-HCl buffer (pH 8). After vortexing, both preparations were incubated at room temperature for 30 min followed by centrifugation at 5000 × g for 5 min. Absorbance in both preparations was read at 412 nm on a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California) for free-thiol estimation. The preparations for protein estimation were again read at 280 nm to estimate protein levels. The actual sulfhydryl level in each was calculated using a molar extinction coefficient of 13.6 mM⁻¹ cm⁻¹ at 412 nm and expressed as nmoles/mg of proteins. The loss of free-thiols in each sample during retail display was calculated by subtracting free-thiol levels in d 4 or d7 samples from d 0 samples (delta; Δ; 4/7 d – 0 d).

**Determination of myofibrillar protein content**

The remaining amounts of myofibrillar proteins used for carbonyl and free-thiol estimations were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting evaluations. The SDS-PAGE and immunoblotting analyses were performed for samples from four carcasses randomly selected from each dietary treatment. Before estimating protein content in each by Pierce® bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL), myofibrillar proteins were dissolved in 1 mL of buffer (pH 8) containing 0.1 M Tris-HCl, 1.25 mM EDTA, 5% and SDS buffer.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**
Myofibrillar proteins were incubated for 10 min at 95°C in a buffer (1:4; sample:buffer) containing 20% (v/v) of 10% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) of 0.05% (w/v) bromophenol blue and 55% double distilled deionized water under non-reducing conditions. For reducing conditions, the same conditions were applied together with the buffer containing 5% (v/v) 2-mercaptoethanol. The SDS-PAGE was performed according to the method of Laemmli (1970) with 6% (6 × 8 cm, 1.5 mm thick) resolving and 4% stacking polyacrylamide gels using Mini-PROTEIN®2 Cells (Bio-Rad Laboratories, Inc., Hercules, CA). The amount of protein loaded per lane was 50 μg. Kaliedoscope prestained standards (10 μL, 161-0324, Bio-Rad Laboratories, Inc., Hercules, CA) were used. The electrophoresis was performed at a constant voltage of 120 V for 90 min using Tris-HCl/glycine/SDS (pH 8.3) continuous buffer systems. Gels were stained (Coomassie brilliant blue R-250 staining solution; 161-0436, Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h on a rocker and destained for overnight (12 h) at room temperature in a solution containing 5% glacial acetic acid, 25% methanol, and 70% deionized ddH₂O. After a deionized-ddH₂O rinse, gels were scanned on the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE) at 700 nm. Band intensities of cross-linked protein aggregates (CL-P) were estimated using Odyssey application software version 1.1.

**Immunoblotting to evaluate cross-linked proteins**

The presence of myosin proteins in cross-linked proteins due to oxidation was assessed by immunoblotting after separation of myofibrillar proteins on 6% resolving and 4% stacking polyacrylamide gels in the SDS-PAGE under non-reducing and reducing conditions. Proteins were transferred from gels to polyvinylidene fluoride (PVDF)
membranes (IPFL20200; 0.45 μm, Immobilon-FL transfer membrane, Millipore Corporation, Billerica MA) for 120 min at a constant voltage of 100 V and 180 mM current using Bio-Rad Mini-Trans-Blot Electrophoretic transfer cells (Bio-Rad Laboratories, Inc., Hercules, CA) with ice-cold transfer buffer (48 mM tris-base, 39 mM glycine, 20% methanol, and 0.0375% SDS at pH 9.2). Membranes were removed and blocked with 10 mL of Odyssey blocking buffer (927-40100; LI-COR, Lincoln, NE) for 120 min at room temperature on a rocker. Then, membranes were incubated with primary anti-myosin skeletal muscle monoclonal (MY-32; MA5-11748; Pierce Biotechnology, Rockford, IL) antibodies at a dilution of 1:2000 in Odyssey blocking buffer containing 0.2% Tween-20. Membranes were incubated with primary antibodies for 1 h at room temperature and then for overnight at 4°C while gently shaking. After probing, membranes were washed 3 times with freshly made tris-buffered saline (TBS) containing 0.1% Tween-20. Then, membranes were incubated for 1 h at room temperature with IRDye-680LT conjugated goat anti-mouse IgG1 (926-68050, LI-COR, Lincoln, NE) at a dilution of 1:5000 in Odyssey blocking buffer containing 0.2% Tween-20 and 0.02% SDS under dark condition. Membranes were then rinsed 3 times with TBS with 0.1% Tween-20 and scanned using Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln NE) at 700 nm.

**Immunoblotting to evaluate troponin-T degradation**

The degree of troponin-T degradation was evaluated by quantifying protein bands at 30 kDa or less. Myofibrillar proteins (100 μg per lane) were separated by 15% resolving and 4% stacking polyacrylamide gels in SDS-PAGE under reducing conditions using a tris-HCl/glycine/SDS (pH 8.3) continuous buffer system. The SDS-PAGE was performed...
at a constant voltage of 100 V and 50 mA current for 150 min. Proteins in the acrylamide gels were transferred to PVDF membranes for 90 min at a constant voltage of 100 V and 180 mM current using Bio-Rad Mini-Trans-Blot transfer cells with ice-cold transfer buffer (48 mM Tris-base, 39 mM glycine, 20% methanol, 0.0375% SDS at pH 9.2).

Protein-transferred membranes were blocked with 10 mL of Odyssey blocking buffer (927-40100; LI-COR, Lincoln, NE) for 120 min at room temperature. Then, membranes were incubated with primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibodies at a dilution of 1:10000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h at room temperature and then for overnight at 4ºC while gently shaking. Membranes were washed 3 times with freshly prepared TBS containing 0.1% Tween-20. Then, they were incubated with secondary antibody, rinsed, and scanned as mentioned under myosin cross-linking detection. Troponin-T degraded products at 30 kDa and less on the gels were measured by quantifying band intensities using Odyssey application software version 1.1.

**Cook loss**

Frozen steaks allotted for WBSF were used to calculate cook loss. Frozen steaks were thawed for 24 h at 4ºC. Thawed steaks were wiped with paper towels and weights were recorded. Ready-made insulated type T thermocouples attached to a miniature size spool caddy (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) were inserted into the geometric center of the steak prior to grilling. Then, steaks were grilled on a Hamilton Beach Indoor-Outdoor Grill (Model 31605A, Proctor-Silex Inc., Washington, NC), turning over once at 35ºC, until they reached an internal temperature of 71ºC. During grilling, temperature was monitored using an OMEGA 450-ATT thermometer (OMEGA
Engineering, Inc., Stamford, CT) connected to thermocouples. Weights of cooked steaks were recorded immediately after grilling. The percentage cook loss was calculated by 

\[
\frac{\text{raw steak weight} - \text{cooked steak weight}}{\text{raw steak}} \times 100.
\]

Also, the change in the cook loss between d 7 and d 0 steaks of each (delta; Δ; 4/7 d – 0 d) was calculated.

Statistical analysis

Data were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment, packaging systems as the second split-plot treatment and retail display time (repeated measures) as the third split-plot treatment. To determine the correct degrees of freedom for the estimates, the Kenward-Rogers approximation method was used. Separation of means was conducted using LSMEANS procedure with DIFF, LINES, SLICE or SLICEDIFF options in SAS at \( P \leq 0.05 \). In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, and No AG vs. AG.

RESULTS

Warner-Bratzler shear force

Four-way interaction effects of diet × aging time × packaging system × retail display d on steak shear force values were not significant \( (P = 0.34) \). Two-way interaction effects of packaging system × retail display d on shear force values were significant \( (P < 0.0001; \text{Table 1}) \). As shown in the Table 1, steaks packaged in HiOx-MAP increased \( (P < 0.0001) \) in shear force values during retail display whereas shear force values of steaks in PVC-OW decreased \( (P = 0.001) \). The change (Δ) in steak WBSF
values during retail display due to packaging systems was significant ($P < 0.0001$; Table 1).

In addition, as shown in Table 2, interaction effects of aging time × retail display d on steak WBSF values were significant ($P = 0.03$). For all steaks, those aged 29 d were more tender ($P < 0.0001$; Table 2) than those aged 8 d. During retail display, there was a slight increase ($P = 0.06$; Table 2) in shear force values in steaks from 29 d aged strip loins. The same results were observed when calculating the change (Δ) of WBSF values during retail display ($P = 0.03$; Table 2).

The main effects of diet on steak shear force values were significant ($P = 0.02$; Table 3). Steaks from cattle fed corn + AG were less ($P = 0.02$) tender than steaks from the other diets. Also, feeding AG significantly ($P = 0.04$) increased WBSF values of steaks than non-AG supplementation. Steaks from WDGS diets had lower ($P = 0.06$) shear force values compared to steak from corn diets.

**Carbonyl contents**

Four-way interaction effects of diet × aging time × packaging system × retail display d on Δcarbonyl values were not significant ($P = 0.78$). During the retail display period, there was a trend ($P = 0.07$; Table 1) to increase carbonyl levels when steaks were packed in HiOx-MAP, compared to PVC-OW packages. Significant ($P = 0.0002$; Table 2) two-way interaction effects of aging time × retail display d on carbonyl levels showed that steaks from strip loins aged 29 d had greater ($P < 0.004$) carbonyl levels than steaks from strip loins aged 8 d.

Moreover, interaction effects of diet × aging d on carbonyl levels were significant ($P < 0.0001$; Table 3). Eight d aged steaks from animals fed WDGS + no AG diets had
lower \( (P = 0.01; \text{Table 3}) \) carbonyl levels than steaks from other diets. Steaks (aged 8 d) from AG fed cattle had higher \( (P = 0.004; \text{Table 3}) \) carbonyl levels compared to steaks from non-AG supplemented cattle. However, the same trend could not be seen among steaks after 29 d aged. The data clearly show that feeding WDGS boosted \( (P = 0.002) \) carbonyl levels during retail display more than corn diets after long-term aging.

**Free-thiol contents**

Lower values indicate greater loss of free-thiols or more protein oxidation. Four-way interaction effects of diet \( \times \) aging time \( \times \) packaging system \( \times \) retail display d on \( \Delta \text{free-thiols} \) were not significant \( (P = 0.48) \). As shown in Table 1, steaks packaged in HiOx-MAP had a tendency \( (P = 0.09) \) to deplete free-thiols more, compared to steaks in PVC-OW packages. Two-way interaction effects of diet \( \times \) aging d on loss of free-thiols were significant \( (P < 0.0001; \text{Table 3}) \). Loss of free-thiols among 8 d aged steaks from corn diets showed that AG supplementation reduced \( (P < 0.05; \text{Table 3}) \) loss of free-thiols during retail display whereas feeding AG with WDGS increased \( (P < 0.05) \) loss of free-thiols. Overall, AG supplementation increased loss of free-thiols in 8 d \( (P = 0.04; \text{Table 3}) \) and 29 d \( (P = 0.001; \text{Table 3}) \) aged steaks.

**Cross-linked protein aggregates**

An increase in cross-linked proteins is an indication of protein oxidation. Four-way interaction effects of diet \( \times \) aging time \( \times \) packaging system \( \times \) retail display d on formation of cross-linked protein aggregates were not significant \( (P = 0.99) \). As shown in Table 1, there were significant \( (P = 0.0004) \) interaction effects of packaging system \( \times \) retail display d on formation of cross-linked protein aggregates. Cross-linked proteins levels of steaks packaged in both packaging systems increased \( (P < 0.0001; \text{Table 1 and Figure 1a}) \)
during retail display period. However, at the end of the retail display period, steaks in HiOx-MAP had greater ($P = 0.01$; Table 1 and Figure 1a) levels of cross-linked proteins compared to steaks in PVC-OW packages. Table 2 shows that there were significant ($P = 0.02$) two-way interaction effects of aging time $\times$ retail display $d$ on cross-linked protein levels in steaks. Steaks from strip loins aged 8 and 29 d increased in protein aggregates ($P < 0.0001$; Table 2 and Figure 1a) during retail display. This increase was higher ($P = 0.007$; Table 2) for steaks from loins aged longer (29 d).

Also, two-way interaction effects of diet $\times$ retail display $d$ on protein cross-linking were significant ($P = 0.05$; Table 3). Steaks from corn + AG diets had the lowest ($P = 0.002$; Table 3) levels of protein aggregates than steaks from all the other diets at the end of retail display period. Steaks from all cattle increased ($P < 0.0001$; Table 3) in protein aggregation during retail display. However, steaks from WDGS diets had higher levels of protein aggregation compared to steaks from corn diets at the beginning ($P = 0.07$; Table 3) and the end of retail display period ($P = 0.03$; Table 3). No significant ($P > 0.49$; Table 3) effect of AG supplementation on formation of protein aggregates were observed.

Composition of cross-linked protein aggregates

As shown in Figure 1a, heavy molecular weight protein aggregates could be separated from myofibrillar proteins isolated from steaks packaged in either packaging system. Cross-linked protein band intensities increased with packaging under HiOx-MAP, longer aging or retail display $d$ (Figure 1a). When the intensities of cross-linked protein bands increased the intensities of myosin heavy chain bands decreased (Figure 1a). Figure 1b shows separation of myofibrillar proteins after incubation with 2-
mercaptoethanol (a reducing reagent). Almost all the cross-linked protein aggregate bands disappeared and myosin heavy chain band intensities increased.

Immunoblotting was performed with anti-myosin skeletal muscle monoclonal (MY-32) antibodies to confirm further which proteins were involved in the formation of protein aggregates during oxidation. Figure 2a and b clearly show that proteins involved in formation of protein aggregates were mainly myosin heavy chain proteins. Also, myosin heavy chains were cross-linked by mainly disulfide bonds; however, some other bonds were also involved in cross-linking of myosin heavy chain proteins as some aggregates remained after incubation under reducing conditions.

**Troponin-T degradation**

Troponin T degradation was quantified by measuring troponin-T degraded products at less than 30 kDa to get an indirect measurement of the postmortem tenderization process. Four-way interaction effects of diet × aging time × packaging system × retail display d on troponin-T degradation were not significant \((P = 0.99)\). There were significant \((P = 0.04; \text{Table 1})\) two-way interaction effects of packaging × retail display d on accumulation of troponin-T degraded products. Troponin-T degraded products increased in steaks in both packaging systems during retail display. However, at the end of retail display, steaks in PVC-OW packages had higher \((P = 0.001; \text{Table 1})\) levels of troponin-T degraded products than steaks packaged in HiOx-MAP.

As shown in Table 2, troponin-T degraded products in steaks increased \((P = 0.0001)\) with longer aging time. Main effects of diet on accumulation of troponin-T degraded products were significant \((P = 0.002; \text{Table 3})\). Feeding AG reduced \((P = 0.001)\) accumulation of troponin-T degraded products, compared to non-AG supplementation.
Percentage cook loss

Four-way interaction effects of diet × aging time × packaging system × retail display on percentage cook loss were not significant ($P = 0.54$). As shown in Table 1, two-way interaction effects of packaging system × retail display were significant ($P = 0.001$). Steaks packaged in HiOx-MAP had higher ($P = 0.04$; Table 1) cook loss at the end of retail display period whereas cook loss of steak in PVC-OW packages decreased ($P = 0.09$).

Two-way interaction effects of aging time × retail display were significant ($P = 0.02$; Table 2). At the beginning of the retail display, long-term aged steaks had more ($P = 0.003$; Table 2) cook loss than short term aged steaks. Also, at the end of retail display, short-term aged steaks had more ($P = 0.03$; Table 2) cook loss; however, cook loss of longer-term aged steaks did not differ ($P = 0.29$). There was no dietary effect on steak cook loss.

**DISCUSSION**

Packaging systems containing high oxygen levels negatively affect instrumental and sensory tenderness of muscle foods (Seyfert et al., 2005; Lund et al., 2007b; Linares et al., 2008; Bornez et al., 2010; Lagerstedt et al., 2011). Zakrys et al. (2009) stated that degree of reduction in tenderness due to high oxygen packaging systems mainly depend on the levels of oxygen in the packages. They showed that $>40\%$ oxygen inclusion in MAP significantly increases WBSF valves and sensory toughness in beef. The current study also showed that strip loin steaks in HiOx-MAP (80% $\text{O}_2$ and $\text{CO}_2$) tended to have higher shear force values, compared to steaks in PVC-OW packages during retail display. The decrease in shear force value of steaks packaged in PVC-OW packages during retail display.
display indicated that postmortem tenderization process still occurred. According to Koohmaraie (1992), Huff-Lonergan et al. (1996), and Huff-Lonergan and Lonergan (1999) calpain enzymes are the major enzymes responsible for pre- and post-rigor proteolysis in muscles. According to Taylor et al. (1995), μ-calpain is known to be activated and responsible for tenderization of meat in the early postmortem period. In long-term aging, m-calpains are recognized as the key enzyme responsible for meat tenderization (Sensky et al., 1998; Boehm et al., 1998; Geesink et al., 2006). However, Alarcon-Rojo and Dransfield (1995) Senatandreu et al. (2002) and Ouali et al. (2006) reported that other than calpains, other enzymes (cathepsin, caspase, and 20S proteasome) are also involved in the tenderizing process of meat.

According to Ouali et al. (2006) and Lametsch et al. (2008) all these enzymes, collectively recognized as enzymes accountable for postmortem tenderization, belong to cysteine proteases which have free-thiol containing residues at their active sites; therefore they are prone to be oxidized and inactivated easily.

Other reasons for increasing shear force values of steaks packaged in HiOx-MAP could be due to oxidation of myofibrillar proteins and formation of protein aggregates during retail display. According to Amici et al. (1989) and Stadtman and Berlett (1997) side chains of some amino acids (cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine, and methionine) are more susceptible to oxidation and thus form intra- or intermolecular molecular cross-links which lead to formation of protein aggregates. In general, sulfhydryl or free-thiol groups in cysteine residues (Bloksma et al. 1963; Stadtman and Berlett, 1997; Davies, 2003) and tyrosyl radical formed from tyrosine residues (Stadtman and Berlett, 1997; Lund et al. 2011;) form disulfide and
covalent dityrosine cross-links upon oxidation, respectively. Extensive reduction of sulphydryl levels in muscle foods packaged in high oxygen-containing packages were also reported by Jongberg et al. (2011) and Zakryswaliander et al. (2012). The present study also showed that that HiOx-MAP system increased carbonyls, loss of free-thiols, and protein aggregate levels in myofibrillar proteins during retail display (Table 1). As shown in Figure 1a, steaks in HiOx-MAP packages had higher levels of cross-linked proteins at the end of the retail display period compared to steaks in PVC-OW packages. Similar results were reported by Lund et al. (2007a, b, 2008 a, b) and Kim et al. (2010). In addition, the current study showed that the majority of cross-linked proteins were due to disulfide bonds rather than covalent bonds (Figure 1a and b). This was confirmed by SDS-PAGE separation of myofibrillar proteins under reducing conditions; heavy molecular weight cross-linked protein bands disappeared after adding 2-mercaptoethanol (reducing agent) in to the myofibrillar protein containing sample buffer. Xiong et al. (2009) also reported that disulfide cross-linking was responsible for the cross-linking in porcine myofibrillar proteins exposed to different oxidative environments (iron-catalyzed-, linoleic acid-, and metmyoglobin-oxidizing systems). Immunoblotting (Figure 2a and b) proved that those cross-linked proteins were composed mainly of myosin heavy chain proteins. However, Western blots also showed slightly thin bands at the cross-linked protein band location even after adding 2-mercaptoethanol. Those cross-linked myosin proteins could be due to formation of covalent bonds in between myosin heavy chains. Those cross-links could be speculated as dityrosine bonds which were covalent bonds between tyrosine residues; however, further testing is required for confirmation of this hypothesis. All these chemical modifications in myofibrillar proteins due to protein
oxidation could make the proteins resistant to proteolysis by proteolytic enzymes. Rowe et al. (2004) also stated that chemical modifications in myofibrillar proteins due to oxidants caused a reduction in proteolysis.

The current study showed that strip loin steaks packaged in HiOx-MAP had greater cook loss at the end of retail display. Previous studies also showed that muscle foods packaged in high oxygen containing systems had more drip loss (Wicklund et al., 2006; Linares et al., 2008; Waliwander et al., 2012) and cook loss (Zakrys et al., 2009; Resconi et al., 2012). Lund et al. (2007a, b) also reported that chemical alterations in muscle microstructure due to protein oxidation through HiOx-MAP systems increased purge or drip loss of meat during retail display and cook loss during cooking. Lui et al. (2009) showed that reduction in myofibrillar hydration capacity after exposing to hydroxyl radicals was due to myosin oxidation as it decreased transverse expansion through myosin cross-linking by disulfide bonds and thereby decreasing capillarity. Liu et al. (2010) also reported that oxidation of myofibrillar proteins reduced water holding capacity and thereby increased the cook loss.

The present study showed that aging time of short loins significantly affected steak shear force values during retail display. At the beginning and end of the retail display period steaks from long-term short loins were more tender than steaks from short-term aged loins. However, at the end of retail display period, long-term aged steaks tended to have greater shear force values than at the beginning of retail display. A possible reason for increasing shear force values in long-term aged steaks during the retail display period could be due to an increase in steak protein oxidation levels with longer-aging. This was supported by steak carbonyl and cross-linked protein aggregate levels, as they increased
with longer aging (Table 2). Similar observations of increasing toughness with longer aging time were reported by Novakofski and Brewer (2006).

Numerous studies (Viljanen et al., 2004; Vuorela et al., 2005; Park et al., 2006; Estevez et al., 2008) have shown that lipid-derived radicals, hydroperoxides, and active heamocromogens rapidly promote oxidation of proteins in muscle foods. In this study also lipid and color oxidation levels of 29 d aged steaks (previously published; Senaratne-Lenagala, 2012) were greater than 8 d aged steaks during retail display period. Therefore, such compounds could contribute to increased protein oxidation levels in steaks and eventually increased shear force values during retail display.

Oxidative stability of muscle foods depends on the fatty acid composition and antioxidant status in the feed given to animals. de Mello Jr. (2010) and Senaratne-Lenagala (2009) reported that feeding corn wet distillers grains increased polyunsaturated fatty acids (PUFA) levels thereby decrease oxidative stability (increase lipid oxidation) of beef. The increase in PUFA was believed to increase lipid-derived radicals and thereby increase protein oxidation levels in beef. The current study also clearly showed that carbonyl and cross-linked protein levels in steaks from WDGS fed cattle increased, compared to those in steaks from corn diets (Table 3).

Prooxidants effects of feeding AG on protein oxidation levels and toughening in beef were demonstrated. Feeding AG significantly increased loss of free thiols, carbonyl and WBSF values and decreased troponin-T degradation levels. Previous studies have shown that AG supplementation shows neutral (Choat et al., 2002) and positive (Krumseik and Owens, 1998a; Walenciak et al., 1999; Senaratne-Lenagala, 2012) effects on reducing lipid oxidation levels in beef; however, no any literature is available on protein oxidation
levels in beef from antioxidant-fed cattle. The AGRADO-PLUS feed supplement is a blend of antioxidants - ethoxyquin and tertiary-butyl hydroquinone (TBHQ). Ethoxyquin is water insoluble and lipid miscible antioxidant whereas TBHQ is slightly soluble in water. Also, ethoxyquin can be retained in body tissues, including skeletal muscles (Wilson et al. 1959) and no information of tissue accumulation of TBHQ after oral administration was reported (Madhavi and Salunkhe, 1996). Therefore, we speculate that the effects of feeding AG were mainly due to ethoxyquin. However, further research is needed for conformation. In addition, data clearly showed that AG supplementation reduced troponin-T degradation and increased in shear force values of steaks. As ethoxyquin is a lipid soluble antioxidant, it could be located close to the lipid membranes and/or lipid depots in muscle tissue. Protection of lipid membranes by antioxidants could reduce release of Ca$^{+2}$ from sarcoplasmic reticulum in to sarcoplasm for calpain activity. Therefore, we speculated that AG supplementation could influence postmortem tenderization of beef. Senaratne-Lenagala (2012) reported that AG supplementation reduces free Ca$^{+2}$ levels in muscle tissues. However, further research would be needed for conformation.

**CONCLUSIONS**

In the present study, high oxygen modified atmosphere packaging decreases instrumental tenderness of beef strip loin steaks by increasing myofibrillar protein oxidation (increasing carbonyls, loss of free thiols, protein aggregates) and decreasing proteolysis (troponin-T degradation) over retail display time. Regardless of high oxygen packaging, longer aging time of subprimals and feeding wet distillers grains decreases tenderness in steaks by increasing carbonyls and protein aggregates of myofibrillar
proteins. In addition, dietary AGRADO-PLUS antioxidant supplement acts as a prooxidant towards protein oxidation and reducing proteolysis; therefore, it is not an effective method to impede protein oxidation in beef strip loins steaks. However, AGRADO-PLUS supplementation could negatively influence proteolysis by influencing calcium release from sarcoplasmic reticulum.

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Table 1. Effects of packaging system on Warner-Bratzler shear force (WBSF), ΔWBSF, Δ carbonyls, ΔFree-thiols, protein aggregates, troponin-T degradation % cook loss, and Δ% cook loss of strip loin (m. longissimus lumborum) steaks during retail display.

<table>
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<tr>
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<td>PVC-OW&lt;sup&gt;2&lt;/sup&gt;</td>
<td>HiOx-MAP&lt;sup&gt;3&lt;/sup&gt;</td>
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<td></td>
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<tr>
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<sup>1</sup>WBSF = Warner-Bratzler shear force.
<sup>2</sup>PVC-OW = Oxygen permeable overwrap film package.
<sup>3</sup>HiOx-MAP = High oxygen (80% O<sub>2</sub> and 20% CO<sub>2</sub>) modified atmosphere packages.
<sup>4</sup>SEM = pooled standard error of means.
<sup>5</sup>Δcarbonyls/free-thiols/WBSF/cook loss = (values in d 4/7 retail display – value in d 0).
<sup>6</sup>% cook loss = [(raw steak weight – cooked steak weight)/raw steak weight] × 100
<sup>a</sup>-<sup>b</sup> Within a row, means without a common superscript differ at P ≤ 0.05.
<sup>x</sup>-<sup>y</sup> Within a packaging system, means without a common superscript differ at P ≤ 0.05.
Table 2. Effects of aging time on Warner-Bratzler shear force (WBSF), ΔWBSF, Δ carbonyls, protein aggregates, troponin-T degradation, % cook loss, and Δ % cook loss of strip loin (m. longissimus lumborum) steaks during retail display.

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<td>Δ WBSF³, kg</td>
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<td>1.03x,x</td>
<td>0.12</td>
</tr>
<tr>
<td>Cross-linked protein aggregates, Au</td>
<td>0 d</td>
<td>2.70y</td>
<td>2.90b</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>3.97x,y</td>
<td>4.69a,x</td>
<td>0.26</td>
</tr>
<tr>
<td>P -Value</td>
<td>0.84</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cook loss⁴, %</td>
<td>0 d</td>
<td>18.32b,y</td>
<td>19.86a</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>19.38a</td>
<td>19.36</td>
<td>0.51</td>
</tr>
<tr>
<td>Δ Cook loss⁵, %</td>
<td></td>
<td>1.06x</td>
<td>-0.47y</td>
<td>0.67</td>
</tr>
<tr>
<td>Troponin T degraded products, Au</td>
<td></td>
<td>89.73y</td>
<td>107.84a</td>
<td>4.34</td>
</tr>
</tbody>
</table>

¹WBSF = Warner-Bratzler shear force.
²SEM = pooled standard error of means.
³Δcarbonyls/WBSF/cook loss = (values in d 4/7 retail display – value in d 0).
⁴% cook loss = [(raw steak weight – cooked steak weight)/raw steak weight] × 100
⁵Within a row, means without a common superscript differ at P ≤ 0.05.
⁶Within a column, means without a common superscript differ at P ≤ 0.05.
Table 3. Effects of diets on Warner-Bratzler shear force (WBSF), Δ carbonyls, Δ free-thiols, protein aggregates, and troponin-T degradation in 8 and 29 d aged strip loin (m. longissimus lumborum) steaks during retail display.

<table>
<thead>
<tr>
<th>Diets</th>
<th>No AG (^2) (0 mg/kg)</th>
<th>AG (150 mg/kg)</th>
<th>SEM(^4)</th>
<th>P-value</th>
<th>Corn vs. WDGS</th>
<th>No AG vs. AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBSF(^1), kg</td>
<td>2.77(^y)</td>
<td>2.75(^y)</td>
<td>2.98(^a)</td>
<td>2.79(^y)</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>Δ Carbonyls(^3), nmoles/mg</td>
<td>8 d aging</td>
<td>0.26(^b)</td>
<td>-0.15(^b)</td>
<td>0.25(^b)</td>
<td>0.51(^b)</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>29 d aging</td>
<td>0.61(^a)</td>
<td>0.98(^a)</td>
<td>0.57(^a)</td>
<td>0.88(^a)</td>
<td>0.27</td>
</tr>
<tr>
<td>P-value</td>
<td>0.02</td>
<td>&lt;.0001</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ Free-thiols(^3), nmoles/mg</td>
<td>8 d aging</td>
<td>-10.31(^b)</td>
<td>1.18(^a)</td>
<td>-1.59(^b)</td>
<td>-14.84(^y)</td>
<td>3.93</td>
</tr>
<tr>
<td></td>
<td>29 d aging</td>
<td>-6.88(^a)</td>
<td>-8.44(^b)</td>
<td>-12.73(^b)</td>
<td>-12.16</td>
<td>3.89</td>
</tr>
<tr>
<td>P-value</td>
<td>0.05</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-linked protein aggregates, Au</td>
<td>d 0 retail display</td>
<td>2.79(^b)</td>
<td>2.89(^b)</td>
<td>2.45(^b)</td>
<td>3.08(^b)</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>d 7 retail display</td>
<td>4.49(^a)</td>
<td>4.37(^a)</td>
<td>3.50(^a)</td>
<td>4.95(^a)</td>
<td>0.51</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Troponin T degraded products, Au</td>
<td>105.43(^x)</td>
<td>108.36(^x)</td>
<td>96.42(^y)</td>
<td>84.94(^y)</td>
<td>8.68</td>
<td>0.002</td>
</tr>
</tbody>
</table>

---

\(^1\)WBSF = Warner-Bratzler shear force.
\(^2\)AG: AGRADO-PLUS.
\(^3\)WDGS: wet distillers grains plus solubles.
\(^4\)SEM = pooled standard error of means.
\(^5\)Δ carbonyls / free-thiols = (values in d 4/7 retail display – value in d 0).
\(^a\)\(^y\) Within a row, means without a common superscript differ at \(P \leq 0.05\).
\(^ab\) Within a diet, means without a common superscript differ at \(P \leq 0.05\).
**Figure 1.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis separation of myofibrillar proteins (50 µg per lane) on 6% polyacrylamide gel under A. no-reducing and B. reducing conditions. Each gel was loaded with myofibrillar proteins isolated from strip loin (*m. longissimus lumborum*) steaks, aged 8 or 29 d, packaged in ¹oxygen permeable film packages (PVC-OW) or in ²high oxygen (80% O₂ and 20% CO₂) modified atmosphere packages (HiOx-MAP) during retail display (0 or 7 d).

³CL-P: cross-linked protein aggregates.
⁴MHC: myosin heavy chains.
⁵MS = Kaleidoscope™ Prestained Standards (Myosin, 200 kDa; β-galactosidase, 122 kDa; Bovine serum albumin, 82 kDa; Carbonic anhydrase, 37 kDa; Soybean trypsin inhibitor, 31 kDa; Lysozyme, 16 kD; and Aprotinin, 6 kDa).

**Figure 2.** Western blot depicting myosin protein aggregation of 8 and 29 d aged beef strip loin (*m. longissimus lumborum*) steaks packaged in packaged in ¹oxygen permeable film packages (PVC-OW) or in ²high oxygen (80% O₂ and 20% CO₂) modified atmosphere packages (HiOx-MAP) during retail display (0 or 7 d) run on 6% gels under A. no-reducing and B. reducing conditions. All lanes were loaded with 50 µg of myofibrillar proteins.

³CL-P: cross-linked protein aggregates.
⁴MHC: myosin heavy chains.
⁵MS = Kaleidoscope™ Prestained Standards (Myosin, 200 kDa; β-galactosidase, 122 kDa; Bovine serum albumin, 82 kDa; Carbonic anhydrase, 37 kDa; Soybean trypsin inhibitor, 31 kDa; Lysozyme, 16 kD; and Aprotinin, 6 kDa).
Figure 1.

A. Non-reducing condition

B. Reducing condition
Figure 2.
Depth of protein oxidation in beef *m. triceps brachii* roasts packaged in high oxygen atmosphere

L. S. Senaratne-Lenagala,* C. R. Calkins,* S. Pokharel,* A. S. de Mello Jr.,* K. A. Varnold,* J. B. Hinkle,* and J. E. Hergenreder*

*Department of Animal Science, University of Nebraska, Lincoln, NE 68583

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2. This project was funded, in part, by Novus International, Inc. (St. Louis, MO).

3. Corresponding author: ccalkins1@unl.edu
ABSTRACT

This study investigated the effects of feeding wet distillers grains plus soluble supplemented with antioxidant and postmortem aging on tenderness and myofibrillar protein oxidation at different depths and muscle fiber orientations in beef clod heart (*m. triceps brachii*) muscle cuts packaged in high oxygen (80% O$_2$: 20% CO$_2$) modified atmosphere packages (HiOx-MAP). Crossbred steers (n = 483; initial BW = 427 ± 37 kg) were fed corn-based finishing diets containing 0 (corn) or 30% (DM basis) wet distillers grains plus solubles (WDGS) supplemented with (150 ppm) or without (0 ppm) antioxidant, AGRADO®PLUS (AG), for the last 160 d. Following 48 h postmortem chilling, 40 USDA Choice beef shoulder clods (n = 10 per diet) were collected and aged 8 and 29 d at 0 ± 2°C. Beef clod hearts cuts were packaged in HiOx-MAP and displayed under retail display conditions for 6 d. After display, three 2.54 cm-thick steaks, from the outside to the inside (labeled as outermost, middle, and innermost steaks) were removed, vacuumed-packaged and stored at -20°C. From those steaks, myofibrillar proteins were isolated both parallel and perpendicular to the muscle fibers from the outside to the inside of roasts. The rest of the steaks were used for Warner-Bratzler shear force testing. Oxidation in myofibrillar protein samples was measured by quantifying carbonyls, free-thiols, and protein aggregates. More carbonyls, fewer free-thiols and more protein aggregates indicate more protein oxidation. Protein oxidation was higher (*P* < .0001) in the outermost layer than subsequent inner layers in the roast. Tenderness also decreased (*P* < .0001) in the outermost later compared to the inner layers of the roast. Moreover, longer aging greatly increased protein oxidation only in the outermost layer of the roast compared to short-term aging (*P* < 0.03). There were no meaningful effects of feeding
WDGS or AG and muscle fiber direction on protein oxidation and tenderness in beef clod heart roasts. Overall, protein oxidation and toughening in beef clod heart roasts, due to HiOx-MAP, happens at a descending gradient and is increased by long-term aging. Also, there are no meaningful effects of muscle fiber directions, feeding wet distiller grain plus solubles or antioxidant supplementation on protein oxidation and toughening due to high oxygen packaging in beef clod hearts.

**Key words:** antioxidant, packaging, protein oxidation, roasts, toughing
INTRODUCTION

Modified atmosphere packaging with 80% oxygen and 20% carbon dioxide (HiOx-MAP) is widely used in fresh beef retail markets as it holds cherry-red color of beef for a longer time (Jakobson and Bertelsen, 1999; Cornforth and Hunt, 2008). However, Clausen (2004), and Sorheim et al. (2004) showed that HiOx-MAP increased off-flavors in meats. In addition, Huff-Lonergan et al. (2010) and Lund et al. (2011) explained that HiOx-MAP packaging negatively affected tenderness, juiciness and nutritional value of muscle foods by oxidizing myofibrillar proteins and postmortem protease. It has been shown that alterations in meat proteins occurred during storage in HiOx-MAP due to changing molecular structure and protein aggregates formed via intra- and intermolecular cross-linking.

Estevez et al. (2008) and Park et al. (2006) showed that lipid-derived radicals and hydroperoxides, produced from lipid oxidation, also rapidly promoted protein oxidation in muscle foods in HiOx-MAP. Therefore, any factor which increases lipid oxidation levels in muscle foods would also boost protein oxidation, such as feeding wet distillers grains plus solubles (WDGS; Senaratne, 2009; de Mello Jr., 2010), low antioxidant status of meat (Estevez and Cava, 2004; Sante-Lhoutellier et al., 2008; Baron et al., 2009; Senaratne, 2009), and longer postmortem aging time (Martinaud et al., 1997; Senaratne, 2009).

Previously available literature on protein oxidation due to HiOx-MAP was mainly performed on small meat cuts, such as steaks or minced meat. Lindahl et al. (2010) showed that the thickness of the oxygenated layer due to oxygen penetration was about 2 cm deep from the surface, when a 10 cm-long beef strip loin piece was packaged in HiOx-MAP and retail displayed for about 15 d. Thus, we hypothesized that degree of protein oxidation due
to HiOx-MAP would not be equally distributed within the muscle when packaging as roasts. Muscle fiber orientation (parallel of perpendicular) to the oxygen exposure could also exert an effect on protein oxidation rate of muscles in high oxygen packages.

The current study was designed to investigate the effects of wet distillers grain feeding, antioxidant supplementation, postmortem aging time, muscle fiber orientation, and depth in the muscle cut on degree of protein oxidation related toughing in beef clod heart (m. triceps brachii) roasts in modified atmosphere packages with 80% oxygen and 20% carbon dioxide during retail display.

**MATERIALS AND METHODS**

All procedures related to live animals for this study were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

*Diets and feeding cattle*

Crossbred (British × Continental) yearlings (n = 483; initial BW = 427 ± 37 kg) for this study were a part of the feeding trial described by Senaratne-Lenagala (2012). Briefly, yearling steers were randomly assigned to one of four dry-rolled corn based diets, containing 0 or 30% (DM basis) wet distillers grains plus solubles (WDGS) with (150 ppm) and without (0 ppm) AGRADO®PLUS (AG; a blend of ethoxyquin and tertiary-butyl hydroquinone or TBHQ; Novus International, Inc., St. Louis, MO) for the last 160 d.

*Slaughter and carcass fabrication*

When approximately 60% of steers was expected to be graded USDA Choice grade and possess 1.2 cm of back fat, the steers were transported to a commercial abattoir (Tyson Fresh Meats, Inc., Lexington, NE). After 48 h postmortem chilling, USDA
Choice beef chuck, shoulder clods (IMPS # 114; NAMP 2007) from 10 carcasses (both sides) per dietary treatment were collected, vacuumed packaged, and transported under refrigeration to the Loeffel meat laboratory at the University of Nebraska-Lincoln. Vacuumed-packaged beef sub-primals were aged a total of 8 and 29 d at 2ºC.

*Muscle fabrication and packaging*

After aging, *m. triceps brachii* (clod heart; IMPS # 114E; NAMP, 2007) muscles from beef chuck, shoulder clods were removed. From the center of the clod heart muscles, 13 cm-long roasts were removed, packaged in high foam-barrier polypropylene trays (22.2 × 17.1 × 7.6 cm, 03300 series, Coextruded Plastic Technologies, Inc., Janesville, WI) with a gas mixture (80% O<sub>2</sub> and CO<sub>2</sub>) and mechanically sealed with oxygen-impermeable transparent film.

*Retail display*

All the HiOx-MAP packages were displayed at 0 ± 2ºC for 6 d in Tyler retail display cases (Model LNSC5, Tyler Refrigeration Corporation, Niles, MI) under continuous 1000-1800 lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands). During retail display period, packages were rotated within the retail display cases to avoid location effects.

*Steak cutting and sample preparation*

After the retail display period, roasts were cut into three 2.54 cm-thick steaks perpendicular to the muscle fibers from the outside to the inside of the roast and labeled as outermost, middle and innermost steaks (Figure 1). All the steaks and the remaining
pieces of roasts were immediately vacuum-packaged and stored at -20°C until they were used.

**Myofibrillar protein isolation**

Myofibrillar proteins were isolated from muscle strips (< 4 g) cut both parallel and perpendicular to the muscle fibers from each layer outside to the inside of the same frozen steaks as shown in the Figure 1. Myofibrillar protein isolation occurred according to the method described by Pietrazak et al. (1997) with some modifications. Briefly, after trimming off visible fat and connective tissue, three grams of frozen muscle samples were knife-cut and minced, suspended in 15 mL ice-cold rigor buffer (0.1 M KCl, 2 mM MgCl$_2$, 1 mM EGTA, and 10 mM K$_2$HPO$_4$; pH 7.4) and homogenized at very low speed for 15 s using the polytron homogenizer (POLYTRON® Kinimatica CH-6010, Switzerland). The homogenate was passed through doubled –layered cheese cloth for further removal of connective tissues and fats and filtrate was collected. From the filtrate, 1.4 mL aliquot was pipetted in to an Eppendorf tube and centrifuged at 4000 × g for 5 min. The supernatant was decanted and the pellet was resuspended in 1 mL of ice-cold rigor buffer. Suspended pellet was vortexed for 10 s and centrifuged for 5 min at 4000 × g. The pellet washing step was repeated three times to remove as much myoglobin as possible. The pellet was resuspended in 250 μL of ice-cold rigor buffer and vortexed thoroughly. Eppendorfs containing myofibrillar protein pellets were kept on ice throughout the preparation process to avoid further oxidation of proteins.

**Determination of carbonyl content**

Carbonyl content in each sample was measured according to the method described by Oliver et al. (1987) with slight modifications. Briefly, two 50 μL aliquots of
myofibrillar protein preparations were used to quantify carbonyl and protein amounts, respectively. Myofibrillar proteins in both aliquots were precipitated by adding 1 mL of 10% trichloroacetic acid (TCA) and centrifuged at 5000 × g for 5 min. For carbonyl estimation, precipitated proteins were derivatized with 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl at room temperature for 40 min on a rocker and vortexed every 10 min. Precipitated myofibrillar proteins for protein estimation were mixed with 500 μL of 2 N HCl instead of DNPH. After incubation, 500 μL of 20% TCA was added, vortexed, and centrifuged at 5000 × g for 5 min. After removing the supernatant, the pellets were washed three times with 1 mL ethanol:ethyl acetate (1:1) solution followed by centrifugation at 5000 × g for 5 min. During washing, the protein pellets were dismantled and vortexed for 20 s before centrifugation. After the last wash, the protein pellets were dissolved in 1 mL 6 M guanidine hydrochloride in 20 mM KH₂PO₄ (pH 2.3) and incubated at 4ºC for overnight. The final protein solutions were centrifuged at 7000 × g for 5 min to remove any insoluble material before reading absorbance at 370 nm to measure carbonyl contents using a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California). The relevant myofibrillar proteins samples were read at 280 nm to quantify protein contents. The actual protein levels were calculated using a standard curve produced by dissolving bovine serum albumin (BSA) in 20 mM KH₂PO₄ contained 6 M guanidine hydrochloride. The carbonyl content in each sample was expressed as nmoles of DNPH incorporated per mg of proteins on the basis of molar extinction coefficient of 22.0 mM⁻¹cm⁻¹ at 370 nm for hydrazones.

* Determination of free-thiol content *
Free-thiol contents in myofibrillar proteins were determined by measuring sulfhydryls according to the method described by Ellman (1959) with slight modifications. Two 25 μL aliquots of myofibrillar proteins were used to measure free-thiol and protein contents. Myofibrillar protein aliquots were dissolved in 1 mL of 0.1 M tris-HCl, 1.25 mM ethylenediaminetetraacetic acid (EDTA), and 5% sodium dodecyl sulfate (SDS) containing buffer (pH 8). Twenty five μL of 10 mM 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) in 0.1 M tris-HCl at pH 8 was added to the reaction mixture for free-thiol estimation. For protein control, only 25 μL of 0.1 M tris-HCl (pH 8) was added. Both protein control and free-thiol mixtures were vortexed and incubated at room temperature for 30 min. After incubation, the final solutions were centrifuged at 5000 × g for 5 min before reading absorbance at 412 nm to measure free-thiol contents using a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California). The relevant myofibrillar protein samples for protein estimation were read at 280 nm. The actual protein levels were calculated using a standard curve produced by dissolving BSA in 0.1 M tris-HCl, 1.25 mM EDTA, 5% SDS buffer. Free thiol levels were calculated and expressed as nmoles of sulfhydryls per mg of proteins on the basis of molar extinction coefficient of 13.6 mM⁻¹ cm⁻¹ at 412 nm for sulfhydryls.

**Determination of myofibrillar protein content**

After estimating carbonyl and free thiol contents, the remaining myofibrillar proteins were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Myofibrillar protein samples from four animals per dietary treatment were randomly selected and used for SDS-PAGE analysis. Prior to the electrophoresis, protein concentration in each sample was determined using the Pierce® bicinchoninic acid (BCA)
protein assay kit (Pierce Biotechnology, Rockford, IL). The buffer used for free-thiol
determination (0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS; pH 8) was used as the diluent
to dissolve myofibrillar proteins in the protein sample.

* Determination of cross-linked protein aggregates *

Cross-linked myofibrillar protein aggregates were measured using sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method
described by Laemmli (1970) using tris-HCl/glycine/SDS continuous buffer (pH 8.3)
system. Myofibrillar proteins were incubated for 10 min at 95ºC in buffer at 1:4
(sample:buffer) ratio under non-reducing and reducing conditions. The buffer used to
provide non-reducing condition contained 20% (v/v) of 10% (w/v) SDS, 10% (v/v)
glycerol, 2.5% (v/v) of 0.05% (w/v) bromophenol blue and 55% deionized-ddH2O. For
reducing condition, same buffer composition was used with 5% (v/v) β-mercaptoethanol.
The SDS-PAGE was performed with 6% (6 × 8, 1.5 m-thick) resolving and 4% stacking
polyacrylamide gels using Mini-PROTEIN®2 Cells (Bio-Rad laboratories, Inc., Hercules,
CA). The amount of myofibrillar protein loaded per lane was 50 μg. Kaliedoscope
prestained standards (10 μL per lane, 161-0324, Bio-Rad Laboratories, Inc., Hercules,
CA) was used. The electrophoresis was run at constant 120 V for 90 min. After
electrophoresis, gels were stained in the staining solution (Coomassie brilliant blue R-250
staining solution; 161-0436, Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h on a
rocker. Gels were destained overnight (12 h) at room temperature in a solution containing
5% glacial acetic acid, 25% methanol, and 70% deionized-ddH2O. After deionized-
ddH2O rinse, gels were scanned on Odyssey Infrared Imaging system (Li-COR
Biosciences, Lincoln, NE) at 700 nm. Intensities of protein band (myosin heavy chains;
MHC, cross-linked proteins; CL-P) were quantified using Odyssey application software version 1.1.

**Warner-Bratzler shear force (WBSF)**

Frozen steaks were thawed at 4°C for 24 h and grilled to an internal temperature of 71°C on a Hamilton Beach Indoor-outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), turning over once at 35°C. During grilling, internal temperature at the geometric center of the steaks was monitoring using an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) with a ready-made insulated type T thermocouple attached to a miniature size spool caddy (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT). After grilling, steaks were cooled at 4°C for 24 h. About 2 - 5 cores with 1.27 cm diameter were removed from each layer of the steak parallel to the muscle fibers. Cores were sheared on a tabletop WBSF analyzer (3000, WBS 25 kg scale, 115 motor, ½ coring cutter, G-R Manufacturing Co., Manhattan, KS) attached with a triangular Warner-Bratzler shear attachment. An average of the peak shear force (kg) of each layer perpendicular or parallel to muscle fibers of each steak was used for statistical analysis.

**Statistical analysis**

Data were analyzed by ANOVA in the GLIMMIX procedure of SAS (version 9.2, Cary, NC., 2009) as a split-split-split-plot design with dietary treatments as the whole-plot treatment, aging period as the first split-plot treatment, muscle fiber orientation as the second split-plot treatment and depth in the roast as the third split-plot treatment. Separation of means was conducted using LSMEANS procedure with DIFF and
SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare the effects of feeding Corn vs. WDGS, and No AG vs. AG.

RESULTS AND DISCUSSION

Carbonyl content

According to Amici et al. (1989) and Stadman and Berlett (1997), production of carbonyl derivatives during protein oxidation is mainly due to the direct oxidation of amino acid side chains of lysine, proline, arginine, and threonine residues. In addition, some byproducts (4-hydroxy-2-nonenal; HNE) of lipid peroxidation can directly react with ϵ-amino group of lysine, the imidazole moiety of histidine, or the sulfhydryl group of cysteine residues in other proteins to introduce carbonyl groups in them (Uchida and Stadtman, 1993; Friguet et al., 1994; Nadkarni and Sayre, 1994). Carbonyls formed during protein oxidation are chemically stable and can be detected easily (Shacter, 2000). Therefore, higher level of carbonyls present in meats is an indication of more protein oxidation. Lund et al. (2007b), Leygonie et al. (2011), Senaratne-Lenagala (2012), and Zakrys-Waliwander et al. (2012) reported that HiOx-MAP systems increase formation of carbonyls in meats, compared to vacuum, oxygen permeable film, and 100% N₂-MAP packaging systems. However, some studies showed that packaging systems with or without oxygen did not affect myofibrillar carbonyl levels in meat (Sante-Lhoutellier et al., 2008; Jongberg et al., 2011; Lagerstedt et al., 2011).

In the current study, the four-way interaction effects of diet × aging time × muscle fiber orientation × location of the layer (depth in the roast) on carbonyl levels in clod heart roasts were not significant ($P = 0.84$). Also, main or interaction effects of dietary treatments did not significantly ($P > 0.05$) affect carbonyl levels in clod heart roasts.
during retail display. However, Sante-Lhoutellier et al. (2008) reported that protein stability depends on the balance between PUFA and antioxidant levels. They observed that lamb fed concentrate diets had higher carbonyl contents than lambs on pasture. They further showed that meat from pasture diets had higher levels of vitamin E than meat from concentrate diets. In the current study, a higher level of carbonyls in beef from WDGS fed cattle and fewer carbonyls in beef from corn + AG diets was anticipated as feeding WDGS significantly increases PUFA levels in meats compared to corn diets (Senaratne, 2009; de Mello Jr., 2010) and AG, an antioxidant, helps to reduce lipid oxidation rates in beef. For example, AG reduced lipid oxidation in ground beef (Krumseik and Owens, 1998a), ribeye steaks (Walenciak et al., 1999), and strip loin, tenderloin and clod heart steaks (Senaratne-Lenagala, 2012) in oxygen permeable film packages. However, Choat et al. (2002) reported that AG supplementation did not show any effect on lipid oxidation levels in beef sirloin steaks during retail display.

Two-way interaction effects of aging time × depth in the roast on carbonyl levels were significant ($P < 0.0001$; Table 1). The outermost layers of roasts from both aging periods had higher ($P < 0.0001$) carbonyl contents compared to the subsequent layers. However, carbonyl contents in the outermost layers were also affected by the aging time; long-term aging (29 d) increased ($P < 0.0001$; Table 1) carbonyl levels compared to short-term aging of clod hearts. Murphy and Kehrer (1989), Liu and Xiong (1996), Martinaud et al. (1997), and Lindahl et al. (2010) also found that increases in postmortem aging time of muscles significantly increased carbonyl formation compared to short-term aged muscles. The increase in carbonyl levels in clod hearts with long-term aging could be due to increase in lipid oxidation levels. Uchida and Stadtman (1993), Friguet et al.
(1994) and Nadkarni and Sayre (1994) have showed that some products (4-hydroxy-2-
nonenal; HNE) from lipid oxidation can react with amino acids to produce carbonyl
groups in proteins.

Two-way interaction effects of muscle fiber orientation × depth in the roast were
significant ($P < 0.03$; Table 2). The outer-most layer of both muscle fiber directions had
higher ($P < 0.0001$; Table 2) levels of carbonyls than subsequent inner layers. In addition,
the outer-most layer, cut perpendicular to the muscle fibers, had higher ($P = 0.0002$;
Table 2) carbonyl levels than the outer layer cut parallel to the fibers. The same trend
could not be found in subsequent inner layers ($P > 0.05$; Table 2). The probable reason
for the increase in carbonyls in the outermost layer cut perpendicular to the muscle fibers
could be due to an increase of myofibril area exposed directly to oxygen as oxygen was
exposed to the longitudinal axis of the muscle fibers.

_Free-thiol content_

According to Stadtman and Berlett (1997), Bloksma et al. (1963) and Davies (2003),
sulphhydral or free-thiol groups in cysteine amino acids are highly prone to oxidation, in
the presence of H$_2$O$_2$ or molecular oxygen. Oxidation of free-thiol groups forms various
oxidized products such as sulfenic acid, sulfinic acid, and disulfide cross-links
(Winterbourn et al., 1999; Davies 2003). Jongberg et al. (2011) and Zakrys-waliwander et
al. (2012) have shown that high oxygen-containing packaging systems extensively
decrease sulphhydral contents in muscle foods compared to vacuum, oxygen permeable
film, or other packages containing N$_2$ or CO$_2$. Therefore, quantification of loss of free
thiol contents in meats packaged in HiOx-MAP is one of the methods of measuring
protein oxidation levels in muscle foods.
In this study, two-way interaction effects of aging time × depth in the roast on free-thiol levels in the clod heart roasts were significant ($P < 0.0001$; Table 1). The outer-most layer of both 8 and 29 d aged roasts had lower ($P < 0.0001$; Table 1) levels of free-thiols compared to subsequent inner layers, indicating that the outermost layer had higher protein oxidation than inner layers. Similar to the carbonyl levels, free-thiol levels in the outermost layers significantly ($P = 0.0002$; Table 1) decreased with longer aging (29 d) compared to the short-term aging of clod hearts.

The main effect of dietary treatment on free-thiol levels was significant ($P = 0.05$; Table 3). Clod hearts from WDGS + no AG fed cattle had higher ($P = 0.05$; Table 3) free-thiol levels compared to clod heart roast from other diets fed cattle. Also, feeding corn diets tended to increase ($P = 0.06$; Table 3) the loss of free thiols than feeding WDGS diets. In addition, data did not show that feeding AG would help to reduce protein oxidation in clod heart roasts ($P = 0.52$; Table 3). Therefore, there was no meaningful dietary effect on free-thiol levels in beef clod heart muscles.

**Cross-linked protein aggregates**

Stadtman and Berlett (1997), Lund et al. (2011), Senaratne-Lenagala (2012) showed that formation of intra- and inter-molecular cross-links of muscle proteins leads to aggregation or polymerization of proteins. They have further explained that aggregation of proteins mainly occurred due to formation of disulfide (formed by oxidation of cysteine) and dityrosine cross-links (due to oxidation of tyrosine) in proteins. However, Xiong et al. (2009) have shown that disulfide cross-linking is more responsible for myofibrillar aggregation. In this study, the myofibrillar protein aggregates were identified using SDS-PAGE under non-reducing conditions (without β-mercaptoethanol). Two-way
interaction effects of aging time × depth in the roast were significant \((P = 0.05)\). The outermost layers of roasts had higher levels of cross-linked proteins as shown in the Table 1 and Figure 2a, compared to inner layers. After 29 d of postmortem aging, band intensities of crossed-linked proteins isolated from the outermost layer was higher than those extracted from 8 d aged muscles (Table 1 and Figure 2a). When cross-linked protein band intensities increased, the band intensities of intact myosin heavy chain proteins decreased (Figure 2a). Similar cross-linked protein aggregates were found on electrophoresis gels by Martinaud et al. (1997), Lund et al. (2007b, 2008a, b), Kim et al. (2010), Senaratne-Lenagala (2012) in studies where muscle foods were packaged in HiOx-MAP systems. As shown in Figure 2b, after applying reducing conditions (with β-mercaptoethanol) to the myofibrillar proteins, cross-linked protein bands disappeared and intact myosin heavy chain protein band intensities increased on gel electrophoresis. This indicated that most of the cross-linked proteins were composed of myosin heavy chains. Therefore, it is plausible to presume that myosin heavy chain proteins are more susceptible to oxidation and formation of cross-linked proteins than other myofibrillar proteins. The credible reasons for higher oxidation susceptibility of myosin molecules could be due to being the most abundant (about 43% of total myofibrillar proteins) myofibrillar protein type in a muscle cell (Swartz et al., 2009) and also consisting of more oxidation susceptible amino acids. Senaratne-Lenagala (2012) also showed that protein aggregates isolated from muscles in HiOx-MAP consisted of myosin proteins. In addition to myosin heavy chain proteins, Kim et al. (2010) found titin in cross-linked proteins isolated from beef packaged in HiOx-MAP systems.
Dietary treatment also significantly ($P = 0.006$; Table 3) affected formation of cross-linked protein aggregates. However, there were no meaningful effects of feeding AG or WDGS on formation of protein aggregates.

**Warner-Bratzler shear force**

The development of tenderness in muscle foods depends on the integrity of the skeletal muscle cell structure and the activity of endogenous proteases (Huff-Lonergan et al., 1995; Koohmaraie and Geesink, 2006; McCormick, 2009). Any process which interrupts the postmortem aging process could negatively impact the final tenderness of meat. Oxidative changes in amino acids, especially in histidine, cysteine, and tyrosine, cause alterations in proteins, such as protein fragmentation, cross-linking, and aggregation thereby reducing activities of proteolytic enzymes and increasing resistance of myofibrillar proteins to proteolytic enzyme degradation (Levine et al., 1994; Martinaud et al., 1997; Xiong, 2000). Many studies (Seyfert et al., 2005; Lund et al., 2008a, b; Zakrys et al., 2009; Bornez et al., 2010; Lagerstedt et al., 2011; Senaratne-Lenagala, 2012) reported that HiOx-MAP systems reduce instrumental and sensory tenderness of muscle foods compared to other packaging systems.

The current study showed that two-way interaction effects of muscle fiber orientation $\times$ depth in the roast on WBSF values of clod heart roasts were significant ($P = 0.02$; Table 2). The outermost layer had the highest ($P < 0.0002$; Table 2) shear force values compared to the inner layers of the roasts. Similar results were reported by Lindahl et al. (2010) in a study where strip loin roasts were packed in HiOx-MAP systems for 15 d. They showed that oxygen penetration in the strip loin roasts were only up to 2 cm deep from the surface and the WBSF values further inside were not affected.
by high oxygen conditions. Also, they showed that samples taken closer to the oxygenated layer of the strip loin roast had lower m-calpain activity but no WBSF values from the same region were reported.

As shown in the Table 3, two-way interaction effects of diet × aging time on WBSF values of clod heart roast were significant ($P = 0.006$). Feeding WDGS diets increased ($P = 0.02$) tenderness in 8 d aged steaks, compared to corn diets. However, after 29 d of aging, steaks from WDGS diets became less tender ($P = 0.04$) than steaks from cattle fed corn. A plausible reason could be due to the occurrence of excessive oxidation in longer aged muscles due to WDGS diets.

**CONCLUSIONS**

Collectively, the outermost layer had the highest carbonyl levels, cross-linked proteins contents, loss of free-thiols, and Warner-Bratzler shear force values compared to subsequent inner layers. Thus, the highest protein oxidization and the lowest tenderness occurred in the outermost layer, which had the highest oxygen penetration when beef clod hearts were in modified atmosphere packages containing 80% oxygen and 20% carbon dioxide. Also, protein oxidation measured by carbonyls, loss of free thiols and protein aggregation, in the outermost layer increased with longer aging time of beef muscles. Moreover, muscle fiber direction, feeding wet distillers grains or AGRAGO®PLUS antioxidant supplementation did not exert meaningful effects on protein oxidation in large beef clod heart muscle cuts packaged in high oxygen packages.
LITERATURE CITED


Table 1. Effects of aging time and layer location on carbonyls, free-thiols, and cross-linked proteins.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Aging, d</th>
<th>SEM(^{1})</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Carbonyls, nmole/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>2.38(^{a})(^{y})</td>
<td>3.28(^{a})(^{x})</td>
<td>0.10</td>
</tr>
<tr>
<td>Middle</td>
<td>1.82(^{b})</td>
<td>1.97(^{b})</td>
<td>0.10</td>
</tr>
<tr>
<td>Inner</td>
<td>1.56(^{c})(^{y})</td>
<td>2.14(^{b})(^{x})</td>
<td>0.10</td>
</tr>
<tr>
<td>(P) - value</td>
<td></td>
<td></td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Free-Thiols, nmoles/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>60.14(^{b})(^{x})</td>
<td>56.55(^{b})(^{y})</td>
<td>0.10</td>
</tr>
<tr>
<td>Middle</td>
<td>69.07(^{a})(^{y})</td>
<td>71.33(^{a})(^{x})</td>
<td>0.10</td>
</tr>
<tr>
<td>Inner</td>
<td>70.18(^{a})</td>
<td>70.79(^{a})</td>
<td>0.10</td>
</tr>
<tr>
<td>(P) - value</td>
<td></td>
<td></td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Protein Aggregation, Au</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>4.91(^{a})(^{y})</td>
<td>5.82(^{a})(^{x})</td>
<td>0.42</td>
</tr>
<tr>
<td>Middle</td>
<td>3.24(^{b})</td>
<td>2.85(^{b})</td>
<td>0.42</td>
</tr>
<tr>
<td>Inner</td>
<td>2.91(^{b})</td>
<td>2.55(^{b})</td>
<td>0.42</td>
</tr>
<tr>
<td>(P) - value</td>
<td></td>
<td></td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

\(^{1}\)SEM = pooled standard error of means.

\(^{a}\)\(^{y}\) Within a row, means without a common superscript differ at \(P \leq 0.05\).

\(^{a}\)\(^{c}\) Within a aging d, means without a common superscript differ at \(P \leq 0.05\).
Table 2. Effects of muscle fiber direction and layer location on \(^1\)Warner-Bratzler shear force and carbonyls.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Muscle fiber direction</th>
<th>SEM(^2)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parallel</td>
<td>Perpendicular</td>
<td>(P)-value</td>
</tr>
<tr>
<td>WBSF(^2), kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>5.39(^a)(^x)</td>
<td>5.04(^a)(^y)</td>
<td>0.15</td>
</tr>
<tr>
<td>Middle</td>
<td>4.20(^b)</td>
<td>4.47(^b)</td>
<td>0.15</td>
</tr>
<tr>
<td>Inner</td>
<td>4.25(^b)</td>
<td>4.25(^b)</td>
<td>0.15</td>
</tr>
<tr>
<td>(P)-value</td>
<td>&lt;.0001</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Carbonyls, moles/mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td>2.65(^a)(^y)</td>
<td>3.01(^a)(^x)</td>
<td>0.10</td>
</tr>
<tr>
<td>Middle</td>
<td>1.81(^b)</td>
<td>1.97(^b)</td>
<td>0.10</td>
</tr>
<tr>
<td>Inner</td>
<td>1.84(^b)</td>
<td>1.84(^b)</td>
<td>0.10</td>
</tr>
<tr>
<td>(P)-value</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)WBSF = Warner-Bratzler shear force.
\(^{2}\)SEM = pooled standard error of means.
\(^{x\,y}\) Within a row, means without a common superscript differ at \(P \leq 0.05\).
\(^{a\,b}\) Within a muscle fiber direction, means without a common superscript differ at \(P \leq 0.05\).
Table 3. Effects of diets on Warner-Bratzler shear force, free-thiols, and protein aggregates.

<table>
<thead>
<tr>
<th>Diet</th>
<th>No AG(^2) (0 mg/kg)</th>
<th>AG (150 mg/kg)</th>
<th>SEM(^4)</th>
<th>P-value</th>
<th>Contrasts, P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBSF(^1), kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 d aging</td>
<td>Corn</td>
<td>4.60(^x)</td>
<td>4.87(^x)</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>30% WDGS</td>
<td>4.65(^x)</td>
<td>4.21(^{b,y})</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>29 d aging</td>
<td>Corn</td>
<td>4.44</td>
<td>4.55</td>
<td>0.25</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>30% WDGS</td>
<td>4.74</td>
<td>4.73(^a)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.37</td>
<td>0.07</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.62</td>
<td>0.004</td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>Free-thiols, nmoles/mg proteins</td>
<td>65.70(^x)</td>
<td>67.68(^x)</td>
<td>65.96(^y)</td>
<td>1.58</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66.05(^y)</td>
<td>1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein aggregates, Au</td>
<td>4.37(^x)</td>
<td>3.40(^y)</td>
<td>3.59(^y)</td>
<td>0.49</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>3.74(^y)</td>
<td>3.71(^y)</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)WBSF = Warner-Bratzler shear force.
\(^2\)AG = AGRADO-PLUS.
\(^3\)WDGS = wet distillers grains plus solubles.
\(^4\)SEM = pooled standard error of means.
\(^x-y\) Within a row, means without a common superscript differ at P ≤ 0.05.
\(^a-b\) Within a diet, means without a common superscript differ at P ≤ 0.05.
Figure 1. Diagram showing the location of steaks and muscle strips removed from the clod heart (*m. triceps brachii*) roasts after retail display. A. clod heart roast; B. steaks cut perpendicular to the muscle fibers; and C. muscle strips removed both parallel and perpendicular to the muscle fibers from all three steaks.

1 perpendicular- oxygen exposure to the longitudinal surface of the muscle fibers

2 parallel- oxygen exposure to the cross sectional surface of the muscle fibers

Figure 2. SDS-PAGE separation of myofibrillar proteins (50 μg per lane) on 6% polyacrylamide gel under no-reducing and reducing conditions. Each gel was loaded with myofibrillar proteins isolated from muscle strips cut both parallel and perpendicular to the muscle fibers from each layer outside to the inside of the 8 and 29 d aged, frozen steaks from 13 cm-long beef clod heart (*m. triceps brachii*) cuts in high oxygen modified atmosphere packages.

1 CL-P: cross-linked protein aggregates.

2 MHC: myosin heavy chains.
Figure 1.
<table>
<thead>
<tr>
<th></th>
<th>8 d Aged</th>
<th>29 d Aged</th>
<th>8 d Aged</th>
<th>29 d Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>Middle</td>
<td>Outer</td>
<td>Middle</td>
</tr>
<tr>
<td>Perpendicular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>Middle</td>
<td>Outer</td>
<td>Middle</td>
</tr>
<tr>
<td></td>
<td>Outer</td>
<td>Middle</td>
<td>Outer</td>
<td>Middle</td>
</tr>
<tr>
<td></td>
<td>Inner</td>
<td></td>
<td>Inner</td>
<td></td>
</tr>
</tbody>
</table>

Non-reducing condition

Reducing condition

Figure 2.
Lipid oxidation rate affects postmortem free-calcium levels and tenderness of beef\textsuperscript{1, 2}

L. S. Senaratne-Lenagala,* C. R. Calkins,*\textsuperscript{3}

*Department of Animal Science, University of Nebraska, Lincoln, NE 68583

\textsuperscript{1}A contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE 68583

\textsuperscript{2}This project was funded, in part, by Novus International, Inc. (St. Charles, MO).

\textsuperscript{3}Corresponding author: ccalkins1@unl.edu
The effects of lipid oxidation on cytoplasmic free-calcium level and tenderness of beef were studied. Crossbred (British × Continental) steers (n = 467) were fed corn-based finishing diets containing 0 (corn) or 30% wet distillers grains plus solubles (WDGS) supplemented with (150 mg/kg) or without (0 mg/kg) antioxidants (AGRADO PLUS; AG) for the last 160 d. After 48 h postmortem, both short loins from a total of 80 (n = 20/diet) USDA Choice grade carcasses were vacuumed-packaged and transported under refrigeration to the University of Nebraska-Lincoln. Sub-primals were aged in vacuum packages for either 8 or 29 d at 2°C. After aging, *m. longissimus lumborum* were removed and cut into steaks. Two steaks (for 0 d lipid oxidation, free-calcium levels and shear force) were vacuumed packaged and frozen. Two steaks were cut into halves, packaged in oxygen permeable film (PVC-OW) or in a high oxygen atmosphere (HiOx-MAP; 80% O$_2$ and 20% CO$_2$) and displayed for 4 and 7 d for lipid oxidation and free-calcium level determination. The last two steaks were packaged in a PVC-OW and in a HiOx-MAP and displayed for 7 d for shear force analysis. Across all packaging treatments, feeding AG decreased lipid oxidation (*P* = 0.03), free-calcium levels (*P* = 0.007), and tenderness (*P* = 0.04) of steaks. Feeding WDGS generally tended to increase lipid oxidation, free-calcium levels (*P* = 0.07) and tenderness (*P* = 0.06) of steaks. Steak lipid oxidation (*P* < 0.05), free-calcium level (*P* = 0.0008) and tenderness (*P* < 0.0001) were higher when aged for a longer time. This study indicates lipid oxidation levels of beef can be altered by feeding WDGS, antioxidant supplementation, and time of aging. It also suggests that high lipid oxidation levels were associated with increased free-calcium levels in the sarcoplasm, which would account for increased tenderness of beef.
**Key Words:** free-calcium level, lipid oxidation, tenderness of beef
INTRODUCTION

The degree of proteolysis of cytoskeletal, costamere and myofibrillar proteins determines the ultimate tenderness of beef (Hopkins et al., 2002; Lametsch et al., 2003; Koohmaraei et al., 2006; Anderson et al., 2012). According to Koohmaraei et al. (2006), calpains, calcium dependent proteases, play the major role in postmortem tenderization of meat. In muscle cells, calcium is stored in a membranous organelle called sarcoplasmic reticulum. There is evidence that sarcoplasmic calcium level increasing at the onset of rigor mortis (Vignon et al., 1989; Jaecocke, 1993) and during aging (Parrish et al., 1981; Kuber et al., 2004) thereby activating calpains for meat tenderization. However, the mechanism of increasing calcium levels during extended aging (more than 7 d postmortem) has not been identified.

Phospholipids, which are mainly located in biomembranes, form a considerable proportion of lipids in muscle cells. According to Warren et al. (2008) and Woods et al. (2008), phospholipids in biomembranes contain more polyunsaturated fatty acids (PUFA) as their chain lengths and degree of unsaturation are important to maintain thickness and fluidity of membranes (Kummerow, 1983). Babka (1982) and Enser et al. (2000) reported that PUFA levels in biomembranes of pigs can be increased by feeding unsaturated fats. Feedlot studies also showed that feeding wet distillers grains (WDGS) increased PUFA levels in beef (Gill et al., 2008; Senaratne, 2009; de Mello Jr., 2010; Koger et al., 2010), likely at the biomembrane level. The PUFA in meat are more liable to oxidize during processing and retail display (Riley et al., 2000; Sheard et al., 2000; Kouba et al., 2003; Senaratne, 2009; de Mello Jr., 2010). Previous studies (Machlin and Bendich, 1987; Slater et al., 1987; Stanley, 1991) reported that lipid oxidation occurred at
an accelerated rate in biomembranes containing more PUFA, thereby collapsing the membrane structure. Perhaps lipid oxidation causes the release of calcium from the sarcoplasmic reticulum into the cytosol, thereby increasing calpain activity and tenderness.

None of studies have related lipid oxidation levels to postmortem tenderization of meat. Therefore, the objective of this study was to investigate the effects of lipid oxidation of beef *m. longissimus lumborum* from different diets on cytoplasmic free-calcium levels and tenderness. To get the different PUFA levels in beef, cattle were fed dry-rolled-corn based diets containing 0 or 30% WDGS (DM basis) with or without antioxidant supplementation. Antioxidants were included in some diets to reduce degree of PUFA oxidation.

**MATERIALS AND METHODS**

All procedures related to live animals for this study were approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln.

*Diets and cattle feeding*

Crossbred yearlings for this study were a subset of the feeding trial carried out by Moore et al. (2010). Crossbred (British × Continental) yearlings steers (n = 483; initial BW = 427 ± 37 kg) were randomly assigned to one of four dry-rolled-corn based finishing diets containing 0 (corn) or 30% WDGS (DM basis) with or without synthetic antioxidants (ethoxyquin and tertiary butyl hydroquinone in AGRADO PLUS; AG; 0 or 150 mg/kg/animal/d; Novus International, Inc., St. Charles, MO). The composition of diets are shown in Table1. Steers were fed a total of 160 d before slaughter.

*Slaughter and carcass fabrication*
When approximately 60% of steers within a block reached USDA Choice grade and 1.2 cm of back fat steers were slaughtered at a commercial packing plant (Tyson Fresh Meats, Inc., Lexington, NE). After 48 h of postmortem chilling, both beef loin, short loins (IMPS # 174; NAMP, 2007) from a total of 80 (n = 20 per each diet) USDA Choice grade carcasses were vacuumed-packaged and transported under refrigeration to Loeffel meat laboratory at the University of Nebraska-Lincoln. The left and the right sides of each beef sub-primal were aged for either 8 or 29 d at 2°C.

Muscle fabrication, steak allotment, and packaging

Strip loin, *m. longissimus lumborum* (IMPS # 180; NAMP, 2007) muscles were removed from 8 and 29 d aged beef loin, short loins. After trimming the surface exposed to the outside, each strip loin was cut into nine steaks (thickness of first and successive steaks were 1.27 and 2.54 cm, respectively) from the anterior to the posterior of the muscle. The first (0 d retail display sample for oxidation and free-calcium tests) and seventh (0 d retail display sample for tenderness test) steaks were immediately vacuum-packaged in vacuum pouches (3mil STD barrier, Prime Sources, St. Louis, OM) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas city, MO) and stored at -20ºC until they were tested. The second and third steaks were split into halves and assigned for 4 or 7 d retail display oxidation and free-calcium tests either under oxygen-permeable polyvinyl chloride film packages (PVC-OW) or high oxygen modified atmosphere packages (HiOx-MAP; 80% O₂ and 20% CO₂). The last two steaks were allotted for 7 d retail display tenderness testing and packaged either in PVC-OW or in HiOx-MAP. Steaks assigned for PVC-OW were packaged on Styrofoam trays (21.6 × 15.9 × 2.1 cm, Styro-Tech, Denver, CO). All steaks assigned for HiOx-MAP retail
display were packaged in high foam-barrier polypropylene trays (22.2 × 17.1 × 5.1 cm, 03200 series, Coexudred Plastic Technologies, Inc., Janesville, WI) with a gas mixture (80% O₂ and 20% CO₂) and mechanically sealed with oxygen impermeable film.

Retail display

All the steaks in PVC-OW and HiOx-MAP were displayed on a table in a cooler (at 0 ± 2°C) and exposed to continuous 1000-1800 lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions. Packages were randomly rotated daily to avoid location effects. Steaks assigned for 4 and 7 d retail display were removed from retail display, vacuum-packaged and stored at 20°C until they were used.

Lipid oxidation

The modified 2-thiobarbuteric acid reactive substances (TBARS) assay described by Ahn et al. (1998) was used to measure oxidation levels of 8 and 29 d aged steaks displayed for 0, 4 and 7 d. Briefly, steaks were diced, pulverized after dipping in liquid nitrogen using a Waring commercial blender (Model 51BL32, Warning Commercial, Torrington, CT) and stored at -80°C until they were tested. Fourteen mL of deionized-distilled water and 1 mL of butylated hydroxyanisole (10% BHA in 90% ethanol) were added to 5-g powdered sample. After homogenizing for 15 s using a polytron (POLYTRON Kinimatica CH-6010, Switzerland), the homogenate was centrifuged for 3000 × g for 5 min. One mL of homogenate was added to 2 mL of 2-thiobarbituric acid and trichloroacetic acid mixture (15% w/v TCA and 20 mM TBA in deionized ddH₂O) and vortexed for 5 s. The sample mixture was incubated at 70°C in a water bath for 30
min to develop color. After samples were cooled in a cold-water bath for 10 min, the sample mixture was centrifuged at 3000 × g for 15 min. Duplicate 200 µL aliquots of each sample were transferred into wells on a 96-well plate and the absorbance was read at 540 nm to calculate the mg of malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

Shear force evaluation

Shear force evaluation of steaks were performed using the Warner-Bratzler shear force test (WBSF). Steaks were thawed at 4°C for 24 h. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), turning over once at 35°C, until they reached an internal temperature of 71°C. All steaks from the same animal (including 8 and 29 d aged, 0 and 7 d retail displayed under PVC-OW and HiOx-MAP) were grilled in a single batch to avoid variation due to cooking sessions. Internal temperature during grilling was monitored by an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) with a ready-made insulated type T thermocouple (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) inserted into the geometric center of the steak. After grilling, steaks were cooled at 4°C for 24 h. Six cores with 1.27 cm diameter were removed parallel to the muscle fiber arrangement using a drill press. Cores were sheared on a tabletop WBSF analyzer (3000, WBS 25 kg scale, 115 motor, ½ coring cutter, G-R Manufacturing Co., Manhattan, KS) with a triangular Warner-Bratzler shear attachment. An average of the peak shear force (kg) of 6 cores was calculated for each steak. To determine the change in steak WBSF values during retail display time, delta (Δ) WBSF values of each steak were calculated by subtracting WBSF values of 0 d from 7 d retail display.
Free calcium level determination

The protocol described by Parrish et al. (1981) with some modifications was used to quantify free-calcium levels of each steak in duplicate. Briefly, 3 g of steaks, minced by using a stainless-steel knife were placed in polyallomer ultracentrifuge tubes (13 × 55 mm) and centrifuged at 196 000 × g at 4ºC for 30 min. Seven hundred μL of the supernatant were treated with 0.1 mL of 27.5% trichloroacetic acid and vortexed for 15 s. After standing for 10 min at room temperature, tubes were centrifuged for 10 min. Five hundred μL of supernatant were pipetted into plastic tubes and brought up to 5 mL with double distilled deionized water. Prepared samples were filtered through 13 mm diameter Millex-LG 0.20 μm syringe filters (Millipore, Bedford, MA). Calcium concentration of samples was quantified using the inductively-coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron Corporation, Cambridge, UK) with appropriate calcium concentration standards.

Statistical analysis

Data were analyzed by GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009) as a split-split-split-plot design with dietary treatments as the whole-plot, aging period and packaging system as sub-plots and retail display time as the repeated measures. Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P \leq 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, and No AG vs. AG.
RESULTS

Lipid oxidation

There were significant ($P = 0.05$; Table 2) three-way interaction effects of diet × aging × retail display d on steak lipid oxidation levels. Lipid oxidation levels in steaks (except 8 d aged from AG fed cattle) increased ($P < 0.05$) during retail display period. A significant ($P < 0.0001$) dietary effect on lipid oxidation was observed only on steaks aged 29 d and at the end of retail display period (d 7), when steaks from corn plus AG diets had the lowest oxidation level compared to steaks from non-AG fed cattle. However, feeding WDGS numerically increased steak lipid oxidation levels, irrespective to antioxidant supplementation. Overall contrast probabilities of no-AG vs. AG also showed that the antioxidant effect of AG was more ($P < 0.03$) prominent when steaks were aged and displayed for a longer time.

The HiOx-MAP packages did not significantly ($P > 0.05$) increase steak TBARS values over traditional overwrapped steaks (PVC-OW) as expected. Hayes et al. (2010) reported a similar observation in a study where raw pork patties were packaged in either HiOx-MAP or PVC-OW and displayed for 12 d under cold conditions. However, other studies (Jayasingh et al., 2002; de Mello Jr., 2010) reported that beef packaged in HiOx-MAP had higher TBARS values than beef in oxygen permeable film packages.

Shear force

The main effect of diets on steak shear force values was significant ($P = 0.02$; Table 3). Steaks from cattle fed AG supplemented corn diets were less ($P = 0.02$) tender than steaks from other diets. The contrast probability value of no-AG vs. AG showed that steaks from AG supplementations were considerably ($P = 0.04$) less tender than steaks
from non-AG supplemented diets. There was a trend (contrast $P$-value of Corn vs. WDGS; $P = 0.06$) that feeding WDGS increased steak tenderness compared to feeding corn.

Interaction effects of aging time $\times$ retail display $d$ were significant ($P < 0.0001$; Table 3) for WBSF. Steaks aged 29 d were more tender ($P < 0.0001$) than 8 d aged steaks. Interaction effects of packaging system $\times$ retail display $d$ were significant ($P < 0.0001$; Table 3). At the end of retail display time, steaks in HiOx-MAP were less ($P < 0.0001$) tender than the steaks in PVC-OW packages. The same trend could be seen in steak $\Delta$ WBSF values related to packaging systems ($P < 0.0001$).

**Free-calcium levels**

As shown in Table 3, only the main effects of diet ($P = 0.0032$), aging ($P = 0.0008$) and retail display time ($P < 0.0001$) for steak free-calcium levels were significant. The highest ($P = 0.0032$) free-calcium concentration was reported for steaks from WDGS without AG diets. The contrast $P$-value of no-AG vs. AG also showed that steaks from AG-supplemented diets had higher ($P = 0.007$) levels of free-calcium. In addition, the contrast $P$-value of Corn vs. WDGS showed that feeding WDGS tended ($P = 0.067$) to increase the free-calcium levels of steaks. Free-calcium levels increased ($P = 0.0008$) with longer aging time and decreased ($P < 0.0001$) with longer display time. There was no packaging effect on steak free-calcium levels ($P = 0.74$).

**DISCUSSION**

In the last few years, postmortem tenderization of meat has been extensively studied. However, relatively little attention has been given to the effect of lipid oxidation on meat tenderness. Jaecocke (1993) and Hawang et al. (2004) explained that pH drop and
depletion of ATP during onset of rigor slowed down ATP-driven sarcoplasmic reticulum calcium ion pumping capacity, thereby increasing cytoplasmic free-calcium accumulation which is needed for calpains to activate early postmortem. However, the process of increasing cytoplasmic free-calcium levels during extended aging (more than 7 d postmortem) is not yet fully explained. Therefore, this research was carried out as a preliminary step to study the effects of lipid oxidation rate on postmortem cytoplasmic free-calcium levels and tenderness of beef.

About 12 - 37% of total intramuscular fat in beef is represented by phospholipids (Lorenz et al., 2002; Warren et al., 2008; Varnold, in preparation). They are the major constituent in biological membranes. Phospholipids contain higher levels of PUFA (Warren et al., 2008; Woods et al., 2008). According to Babka (1982) and Enser et al. (2000), PUFA levels in phospholipid membranes (especially 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3 fatty acids) of pork can be increased by feeding animals with polyunsaturated fats. In feedlot studies, Senaratne (2009), de Mello Jr. (2010), and Segers et al. (2011) reported that feeding distillers grains, as a finishing diet, significantly increased PUFA levels in beef. Presumably, feeding WDGS to cattle would increase PUFA levels in membranal phospholipids, including membranes of the sarcoplasmic reticulum, where the calcium is stored. Steers were fed dry-rolled-corn based diets containing 0 (corn) or 30% WDGS. Polyunsaturated fatty acids are more prone to oxidize and this can be minimized by supplementing antioxidants. Therefore, half of the diets containing 0 or 30% WDGS were supplemented with antioxidants (AGRADO PLUS containing ethoxyquin and tertiary butyl hydroquinone).
Results showed a significant antioxidant effect of feeding AG when the beef was aged (29 d) and displayed 7 d. The lowest oxidation levels after 29 d aging were observed in steaks from cattle fed AG-supplemented corn diets. The contrast probability value also showed that steaks from antioxidant-supplemented diets had significantly lower lipid oxidation levels than steaks from non-antioxidant supplemented diets. A previous study (Krumseik and Owens, 1998) also showed that AG supplementation at the last month prior harvest was effective in increasing lipid stability of beef. Thus, feeding the antioxidant supplement AG helps to improve lipid stability of strip loins. However, when AG was fed with 30% WDGS diet it was not very effective in reducing lipid oxidation rate. The plausible reason would be the increased PUFA levels in beef due to WDGS feeding. The increase in PUFA level in beef due to WDGS feeding would occur at the membranal levels, thereby decreasing membranal lipid stability. This agrees with findings of previous studies by Hammer and Wills (1978) and Huang et al. (1998). They reported that feeding PUFA-rich diets increased PUFA levels in biomembranes (including sarcoplasmic reticulum as well) and their lipid oxidation levels. Ericson (2002) described that oxidation of PUFA changes fatty acid structure and produces hydroperoxides as intermediate products or decomposes further to non-radical volatile compounds (aldehydes, alcohols or ketones). Many other scientists (Eichenberger et al. 1982; Machlin and Bendich, 1987; Slater et al., 1987; Stanley, 1991; Ji and Takahashi, 2006) have revealed that higher levels of lipid oxidation in biomembranes disrupt membrane structure and function. Data from the current study suggests that biomembranes of steaks with lower lipid oxidation levels were less damaged (had greater functionality) compared to biomembranes of steaks with more lipid oxidation.
Apparently, biomembranes in 29 d aged steaks from corn plus AG diets were lesser
damaged compared to that of in steaks from other diets. Given the significant contrast $P$-
value of 29 d aged steaks from no-AG vs. AG diets, it appears that biomembranes of
steaks from AG-supplemented diets were less damaged than membranes in steaks from
non-AG diets during retail display. Such a situation could allow calcium to leach out of
the sarcoplasmic reticulum due to its damaged membranes. The current study supports
this assumption through increased free-calcium levels in steaks with greater lipid
oxidation levels (created by feeding WDGS, without-AG or by aging longer).

Shear force data also indicate that steaks from non-AG supplementation, WDGS
diets, or 29 d of aging were significantly more tender. A few other studies have reported
that feeding distillers grains diets (de Mello Jr. 2010; Segers et al., 2011; Larson et al.,
2012) decreased steak shear force values and antioxidant supplementation (Secrist et al.,
1995) increased shear force values. In addition, Parrish et al. (1981), Kuber et al. (2004),
and Segers et al. (2011) reported that longer aging time increased tenderness of beef. The
decrease in shear force values of beef during aging has been attributed mainly to
proteolysis by calcium-activated proteases ($\mu$-calpains and m-calpains). In general, $\mu$-
calpains need between 3-50 $\mu$M of calcium for its half-maximal activity while m-calpains
require 200-100 $\mu$M of calcium (Cong et al., 1989; Goll et al., 2003). Therefore, $\mu$-
calpain is known to be activated and responsible for tenderization of meat in early
postmortem period (Taylor et al., 1995). During extended aging, m-calpains are
recognized as the key enzyme responsible for meat tenderization (Sensky et al., 1996;
Boehm et al., 1998; Geesink et al., 2006). The current study also showed that steak
tenderness and free-calcium levels increased significantly with aging time. It may be that increased free-calcium levels increased m-calpain activity.

**CONCLUSION**

In conclusion, integrity of biomembranes of beef muscles can be altered by feeding wet distillers grains, antioxidant supplementation or time of aging. Loss of integrity in biological membranes influences escape of calcium from the sarcoplasmic reticulum into the cytosol, thereby improving beef tenderness during aging. To the best of the authors knowledge, this is the first manuscript reporting the effects of lipid oxidation levels on postmortem tenderization of beef by influencing cytosol calcium concentration. This provides conceptual foundation for a new research perspective on meat tenderization. Additional research should be conducted to further characterize biomembrane composition, particularly the sarcoplasmic reticulum, and relate changes to the release of calcium into the cytosol.

**LITERATURE CITED**


Table 1. Composition of finishing diets and formulated nutrient analysis

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<th>Diets</th>
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<tr>
<td>Dry-rolled corn</td>
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<tr>
<td>Calcium : Phosphorus</td>
<td>2.19</td>
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</table>

¹Values presented on a percentage DM per animal basis.
²WDGS = wet distillers grains plus solubles
³AG = AGRADO PLUS antioxidant supplementation
⁴Soybean:urea;1.86:1.
⁵Contained Rumensin (345 mg/animal/d; Elanco Animal Health, Greenfield, IN), Tylan (90 mg/hd/d; Elanco Animal Health, Greenfield, IN) with or without AGRADO-PLUS (150 ppm/animal/d; Novus International, Inc., St. Louis, MO).
Table 2. Least square means of thiobarbituric reactive substances (TBARS; mg malonaldehyde per kg of tissue) values of 8 and 29 d aged strip loin \((m.\ longissimus\ lumborum)\) steaks during retail display \((diet \times \ aging \times \ d, P = 0.05)\).

<table>
<thead>
<tr>
<th>Retail display, d</th>
<th>Diet</th>
<th>Pooled SEM</th>
<th>P - Value</th>
<th>Contrasts, P - Value</th>
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</thead>
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<tr>
<td></td>
<td>No AG(^1) (0 ppm)</td>
<td>AG (150 ppm)</td>
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</tr>
<tr>
<td></td>
<td>Corn</td>
<td>WDGS(^2)</td>
<td>Corn</td>
<td>WDGS</td>
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<tr>
<td>8 d aged</td>
<td></td>
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<td></td>
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<tr>
<td>0</td>
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<tr>
<td>7</td>
<td>0.44(^a)</td>
<td>0.67(^a)</td>
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<td>0.38</td>
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<td>0.0001</td>
<td>0.19</td>
<td>0.10</td>
</tr>
<tr>
<td>29 d aged</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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<td>0.66(^b)</td>
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<tr>
<td>7</td>
<td>2.07(^a),(^x)</td>
<td>2.17(^a),(^x)</td>
<td>1.11(^a),(^y)</td>
<td>1.62(^a),(^x),(^y)</td>
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<tr>
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<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

\(^1\)AG = AGRADO-PLUS antioxidant supplementation

\(^2\)WDGS = wet distillers grains plus solubles

\(^3\)Pooled SEM = pooled standard error of means

\(^a\)^\(^\text{x}\)Within a diet, means without a common superscript differ at \(P \leq 0.05\).

\(^a\)^\(^\text{x}\)Within a row, means without a common superscript differ at \(P \leq 0.05\).
Table 3. Least square means of $^1$Warner-Bratzler shear force (WBSF; kg) values, change in WBSF ($\Delta$ WBSF) values and free calcium concentrations ($\mu$M) of 8 and 29 d aged strip loin ($m.$ longissimus lumborum) steaks packaged in $^3$high oxygen (80% O$_2$: 20% CO$_2$) modified atmosphere packages (HiOx-MAP) and $^7$oxygen permeable polyvinyl chloride film packages (PVC-OW) during retail display.

<table>
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<tr>
<th>Dietary treatment</th>
<th>No AG$^2$ (0 ppm)</th>
<th>AG (150 ppm)</th>
<th>Pooled SEM$^4$</th>
<th>$P$ - Value</th>
<th>No AG vs. AG</th>
<th>Corn vs. WDGS</th>
<th>Contrasts, $P$ - Value</th>
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</thead>
<tbody>
<tr>
<td>WBSF$^5$, kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>2.77$^y$</td>
<td>2.75$^y$</td>
<td>2.98$^x$</td>
<td>2.79$^y$</td>
<td>0.11</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>2.98$^y$</td>
<td>3.02$^x$</td>
<td>3.02$^{ax}$</td>
<td>2.79$^y$</td>
<td>0.11</td>
<td>0.02</td>
<td>0.067</td>
</tr>
<tr>
<td>Free-calcium, $\mu$M</td>
<td>847.16$^y$</td>
<td>1015.78$^x$</td>
<td>806.80$^y$</td>
<td>806.12$^y$</td>
<td>90.89</td>
<td>0.0032</td>
<td>0.007</td>
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</table>

| Aging, d         | 8                | 29           |                |             |              |               |                        |
| WBSF, kg         | 3.03$^a$         | 2.69$^y$    | 0.07           | <.0001      |              |               |                        |
| 7                | 2.97$^x$         | 2.69$^y$    | 0.07           | <.0001      |              |               |                        |
| $P$ - Value      | 0.23             | 0.06         |                |             |              |               |                        |
| $\Delta$ WBSF$^5$, kg | -0.062$^y$ | 0.094$^a$ | 0.07           | 0.03        |              |               |                        |
| Free-calcium, $\mu$M | 791.18$^y$ | 946.76$^x$ | 45.45          | 0.0008      |              |               |                        |

| Packaging system |                  |              |                |             |              |               |                        |
| Retail display, d| PVC-OW$^7$       | HiOx-MAP$^6$ |                |             |              |               |                        |
| WBSF, kg         | 2.81$^a$         | 2.81$^b$    | 0.07           | 1.00        |              |               |                        |
| 7                | 2.64$^{ab}$      | 3.02$^{ax}$ | 0.07           | <.0001      |              |               |                        |
| $P$ - Value      | 0.001            | <.0001       |                |             |              |               |                        |
| $\Delta$ WBSF, kg| -0.174$^y$      | 0.207$^a$   | 0.07           | <.0001      |              |               |                        |
| Free-calcium, $\mu$M | 861.35     | 876.58      | 45.45          | 0.74        |              |               |                        |

| Retail display, d|                |              |                |             |              |               |                        |
| Free calcium, $\mu$M | 988.63$^x$ | 749.30$^y$  | 2.42           | < 0.0001    |              |               |                        |

---

$^2$AG = AGRADO-PLUS antioxidant supplementation
$^3$WDGS = wet distillers grains plus solubles
$^4$Pooled SEM = pooled standard error of means
$^5$WBSF = WBSF on 7 d – WBSF on 0 d
$^6$Within a packaging system, means without a common superscript differ at $P \leq 0.05$.
$^7$Within a row, means without a common superscript differ at $P \leq 0.05$. 

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RECOMMENDATIONS FOR FUTURE RESEARCH

1. It is a well-known fact that feeding wet distillers grains plus solubles (WDGS) increases polyunsaturated fatty acid (PUFA) levels in beef. According to previous literature, ethoxyquin (a lipid soluble antioxidant) can be retained in skeletal muscles better than tertiary-butyl-hydroquinone (TBHQ). Results of the current study showed that feeding WDGS as a finishing ration increased instrumental tenderness of beef and feeding AGRADO-PLUS (AG; ethoxyquin and tertiary-butyl-hydroquinone; TBHQ) as an antioxidant supplement decreased instrumental tenderness of beef. Also, the data showed that feeding AG decreased lipid oxidation, free-calcium and troponin-T degradation levels in beef. Also, feeding wet distillers grains showed a trend toward increasing free-calcium levels. Based on this, we hypothesized that feeding WDGS and AG influence lipid oxidation levels in biological membranes, including the membranes of the sarcoplasmic reticulum where intracellular calcium is stored, thereby affecting release of calcium for the activation of calpains in the postmortem tenderization process. To elucidate this hypothesis the following questioned should be answered;

• Does feeding WDGS increase PUFA levels in the membranes of sarcoplasmic reticulum in beef muscles?
• Do dietary lipid soluble antioxidant supplements protect sarcoplasmic reticulum membranes of beef muscles from lipid oxidation?
• Does lipid oxidation level affect release of free-calcium levels and calpain activity in beef muscles?
2. Results clearly showed that HiOx-MAP (80% O₂ and 20% CO₂) systems oxidized mainly myofibrillar (myosin) proteins and proteolytic enzymes responsible for residual postmortem tenderization process thereby toughening beef during retail display. The antioxidant blend we used contained ethoxyquin and TBHQ. Out of them, only ethoxyquin (lipid soluble antioxidant) can be retained in skeletal muscles (based on previous literature). Since ethoxyquin is a lipid soluble antioxidant, it would be present where lipids are present in a cell, such as adipose tissues and biological membranes. However, those myofibrillar proteins and proteases responsible for postmortem tenderization are present in the sarcoplasm. Therefore, those lipid soluble ethoxyquin antioxidants would not be able to provide much protection for myosin and proteolytic enzymes in beef muscles from oxidation under hypoxic conditions. Based on these observations and assumptions, it would be interesting to investigate how any dietary water-soluble antioxidant supplement or methionine hydroxy analogue supplementation, which would help to increase antioxidant levels in muscle (Xiao et al., 2011. Aquaculture Nutrition. 18:90-97), could protect myosin and proteolytic enzymes from oxidation under hypoxic condition (HiOx-MAP) during retail display.

3. Results of this study also showed that protein oxidation occurred at a descending gradient from the outside to the inside of beef roasts packaged in a HiOx-MAP system. Therefore, any water-soluble antioxidant coating (TBHQ, BHT, or BHA) on beef steaks before packaging in a HiOx-MAP system could help to minimize protein oxidation levels during retail display. This could be investigated by dipping beef steaks in any of above mentioned antioxidant solutions prior packaging in HiOx-
MAP and displaying under simulated retail display conditions. Then, instrumental
tenderness, and oxidation levels of protein, lipid, color of treated steaks can be
compared to the control steaks packaged in HiOx-MAP.
APPENDIX I

COMPOSITION OF FINISHING DIETS AND FORMULATED NUTRIENT ANALYSIS

<table>
<thead>
<tr>
<th>Item</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn</td>
</tr>
<tr>
<td>Ingredients, DM%(^1)</td>
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<tr>
<td>Dry-rolled corn</td>
<td>78.00</td>
</tr>
<tr>
<td>WDGS</td>
<td>0.00</td>
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<tr>
<td>Corn silage</td>
<td>12.00</td>
</tr>
<tr>
<td>Pellet(^2)</td>
<td>4.00</td>
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<td>Liquid supplementation(^3)</td>
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<td>Crude protein, %</td>
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<td>Fat (%)</td>
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<td>NE(_{\text{mn}}), Mcal/kg</td>
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<td>NE(_{\text{g}}), Mcal/kg</td>
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<td>Calcium, %</td>
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<td>Phosphorus, %</td>
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<td>Sulfur, %</td>
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<td>Vitamin A, IU/kg</td>
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<td>Vitamin D, IU/kg</td>
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<tr>
<td>Calcium : Phosphorus</td>
<td>2.19</td>
</tr>
</tbody>
</table>

\(^1\)Values presented on a percentage DM per animal basis. WDGS = wet distillers grains plus solubles; AG=AGRADO-PLUS.


\(^3\)Contained Rumensin (345 mg/animal/d; Elanco Animal Health, Greenfield, IN), Tylan (90 mg/hd/d; Elanco Animal Health, Greenfield, IN) with or without AGRADO\(^2\)PLUS (150 ppm/animal/d; Novus International, Inc., St. Louis, MO).
APPENDIX II
GUIDE FOR PERCENTAGE SURFACE DISCOLORATION

0%

5%

10%

20%

30%

40%
APPENDIX III

THIOBARBITURIC ACID REACTIVE SUBSTANCES ASSAY

(Buege and Aust, 1978, Modified by Ahn et al., 1998)

**TEP solution (1,1,3,3-Tetraethoxypropane)** (Make new weekly)
Stock Solution: Dilute 99 µl TEP (97%) bring volume to 100 mL ddH₂O.
Working Solution: Dilute stock solution to 1:3 (TEP Solution:ddH₂O) (1 × 10⁻³ M).

**TBA/TCA (2-Thiobarbituric Acid/Trichloroacetic Acid) Stock Solution: 1L**
15% TCA (w/v) and 20 mM TBA (MW 144.5) reagent in ddH₂O.
Dissolve 2.88 g TBA in warm ddH₂O first, then add TCA (150 g) and ddH₂O to 1L.

**BHA (Butylated Hydroxy Anisole) stock Solution:**
Make 10% stock solution by dissolving in 90% ethanol.
10 g BHA dissolved in 90 mL ethanol (90%) + 5 mL ddH₂O.

**Standards: In duplicate**

<table>
<thead>
<tr>
<th>Blank:</th>
<th>Moles of TEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mL ddH₂O</td>
<td></td>
</tr>
<tr>
<td>Standard 5:</td>
<td>100 µl working TEP + 1.90 mL ddH₂O</td>
</tr>
<tr>
<td>Standard 4:</td>
<td>1 mL Std. 1 + 1 mL ddH₂O</td>
</tr>
<tr>
<td>Standard 3:</td>
<td>1 mL Std. 2 + 1 mL ddH₂O</td>
</tr>
<tr>
<td>Standard 2:</td>
<td>1 mL Std. 3 + 1 mL ddH₂O</td>
</tr>
<tr>
<td>Standard 1:</td>
<td>1 mL Std. 4 + 1 mL ddH₂O</td>
</tr>
</tbody>
</table>

Remove 1 mL of Standard 1 and discard it, leaving 1 mL behind.

**Procedure:**
- Mix all reagents and standards before beginning.
- Transfer 5 g powdered sample into a 50 mL conical tube; add 14 mL of ddH₂O and 1.0 mL of BHA.
- Homogenize for 15 sec with a polytron.
- Centrifuge for 2000×g for 5 min.
- Transfer 1 mL of homogenate or standard to 15 mL conical tube.
- Add 2 mL of TBA/TCA solution, vortex.
- Incubate in a 70°C water bath for 30 min to develop color.
- Cool samples in a coldwater bath for 10 min.
- Centrifuge tubes at 2000×g for 15 min.
- Transfer duplicate aliquots of 200 µl from each tube into wells on a 96-well plate.
- Read absorbance at 540 nm.

**Calculations: mg of malonaldehyde/kg of tissue**

\[ K(\text{extraction}) = \left( \frac{S}{A} \right) \times \text{MW} \times (10^6/E) \times 100 \]

Where:
- \( S = \) Standard concentration \((1 \times 10^{-8} \text{ moles 1,1,3,3-Tetraethoxypropane})/5 \text{ mL}\)
- \( A = \) Absorbance of standard
- \( \text{MW} = \) MW of malonaldehyde (72.063 g/mole)
- \( E = \) Sample equivalent (1)
- \( P = \) percentage recovery

Final calculation: \( 0.012 \times \text{concentration} \times (72.063 \times 10^6) = \text{mg of Malonaldehyde/kg of tissue} \)

Reagents (Sigma): TBA- T5500; TCA – T9159; TEP – T9889; BHA – B1253
APPENDIX IV
DETERMINATION OF VITAMIN E LEVEL

2. Pulverize cubes by using Waring blender (Model 51BL32, Waring Commercial, Torrington, CT), after dipping in liquid nitrogen.
3. Measure 1 g of pulverized meat sample into a 50 mL centrifuged tube.
4. Add 3 mL of ethanol containing 6% pyrogallol and vortexed for 1 min.
5. Add 1 mL of saturated KOH and flue the mixture with nitrogen gas.
6. Digest the mixture for 30 min at 70ºC and cool it to the room temperature.
7. Add 5 mL of deionized ddH₂O and 3 mL of hexane containing 0.05% butylated hydroxytoluene (BHT).
8. Vortex the mixture for 1 min and centrifuge at 10 000 rpm for 2 min.
9. Collect the supernatant and kept it on ice in dark.
10. Repeat hexane extraction (step 7 – 9) for the pellet for 3 times.
11. Pool the supernatants collected and evaporate it to dry under nitrogen flush.
12. Re-suspend the residue in 200 μL of tetrahydrofuran and the total volume is brought up to 300 μL by adding the mobile phase (40:5:3:2, acetonitrile; tetrahydrofuran containing 0.1% BHT and triethylamine; methanol; 1% ammonium acetate in HPLC grade water.
13. Vortex the mixture for 1 min.
14. Inject 50 μL of the mixture is into the HPLC system* (Waters Associates, Milford, MA) attached with a reversed phase Microsorb-MV C<sub>18</sub> column (5 μm, 250 × 4.6 mm; Rainin, Woburn, MA) and a C<sub>18</sub> guard column (3 cm × 4.6 mm; packed with 5 μm sphere-5-C<sub>18</sub>).
15. Elute the column with the mobile phase under isocratic conditions and read absorbance at 292 nm for vitamin E.
16. Express vitamin E as mg/100 g of tissue.

*The HPLC system is equipped with Waters Associates instruments; 600E solvent delivery system, Pheodyne 484 UV detector, and 74SB integrator.
APPENDIX V

ISOLATION OF MYOFIBRILLAR PROTEINS
(Pietrzak et al., 1997, J. Anim. Sci. 75:2106-2116)

1. Knife-mince frozen steaks after trimming visible fats and connective tissues.
2. Weigh 3 g of minced meat into a 50 mL plastic conical tube.
3. Add 15 mL ice-cold rigor buffer (0.1 M KCl, 2 mM MgCl$_2$, 1 mM EGTA, and 10 mM K$_2$HPO$_4$; pH 7.4) and homogenize using the polytron (POLYTRON Kinematica CH-6010, Switzerland) at very low speed for 15 min.
4. Filter the homogenate through double-layered cheese cloth to remove connective tissue and fats into a new 50 mL plastic conical tube.
5. Pipette 1.4 mL homogenate into an eppendorf tube (2 mL safe-lock tubes; 02236352, Eppendorf AG, Hamburg, Germany).
6. Centrifuge eppendorf tubes at 4000 × g for 5 min.
7. Decant the supernatant and dismantle the pellet using a cleaned spatula after resuspending in 1 mL of ice-cold rigor buffer.
8. Vortex the mixture for 10 s and centrifuge for 5 min at 4000 × g.
9. Repeat step 6 and 7 three times to remove myoglobin as much as possible (until the supernatant is clear and free of myoglobin).
10. Decant the supernatant and remove the leftover supernatant using a pasture pipette.
11. Re-suspend the pellet in 250 μL of ice-cold rigor buffer.
12. Vortex thoroughly after dismantling the pellet.
13. Store eppendorfs on ice for immediate use or in -80°C for later use.

Reagent preparation:

a. Rigor buffer (0.1 M KCl, 2 mM MgCl$_2$, 1 mM EGTA, and 10 mM K$_2$HPO$_4$; pH 7.4)

Add 14.91 g of potassium chloride (KCl; MW 74.55), 380.84 mg of magnesium chloride (MgCl$_2$; MW 95.21), 760.7 mg of ethylene glycol tetraacetic acid (EGTA; MW 380.35, and 3.484 g of potassium phosphate dibasic (K$_2$HPO$_4$; MW 174.18) into 1900 mL of deionized ddH$_2$O and dissolved properly. Check the pH and volume up to 2000 mL. (If want to adjust pH to 7.4, adjust using conc. HCl).
APPENDIX VI

DETERMINATION OF CARBONYLS
( Oliver et al., 1987, J. Biol. Chem. 262:5488-5491)

1. Vortex myofibrillar proteins contained eppendorfs thoroughly.
2. Pipette two 50 μL aliquots of myofibrillar proteins (one for the carbonyl and other for the protein estimations) into eppendorf tubes (2 mL safe-lock tubes; 02236352, Eppendorf AG, Hamburg, Germany).
3. Add 1 mL of 10% trichloroacetic acid (TCA) and vortex for 5 s.
4. Centrifuge eppendorfs at 5000 × g for 5 min.
5. Remove the supernatant by using a pasture pipette.
6. a. Carbonyl estimation - derivatize with 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl.
   b. Protein estimation – mix with 500 μL of 2 N HCl, instead of DNPH.
      After adding reagents, protein pellets should be dismantled using a clean spatula.
      Derivatization is carried out for 40 min on a rocker and vortex every 10 min.
7. Add 500 μL of 20% TCA into both eppendorf tubes, vortex for 5 s, and centrifuge at 5000 × g for 5 min.
8. Decant the supernatant and the residual supernatant must be removed by using a pasture pipette.
9. Wash the protein pellets with 1 mL ethanol:ethyl acetate (1:1) solution followed by dismantling the pellet and vortexing for 20 s.
10. Centrifuge at 5000 × g for 5 min.
11. Repeat step 9 – 10 for 3 times.
12. Dry the pellet with a warm airflow by using a hair dryer (Revlon 1875 Watt Dryer, RVDR503, Revlon, El Pasco TX).
13. Dissolve the dried pellet in 1 mL of 6 M guanidine hydrochloride in 20 mM KH₂PO₄ (pH 2.3), vortex for 10 s.
14. Incubate the mixture at 4°C for overnight (12-14 h).
15. Centrifuge the final solution at 7000 × g for 5 min to remove any insoluble material.
16. Read absorbance of three 200 μL aliquots of carbonyl samples at 370 nm using a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California).
17. Read absorbance of three 200 μL aliquots of relevant protein samples at 280 nm to measure proteins contents. Actual protein concentrations are calculated using a concentration series of BSA in 6 M guanidine hydrochloride in 20 mM KH₂PO₄ as standard.
* Use UV/VIS microwell plates (07-200-623, Corning UV-Transparent Microplates)

To make BSA stock, 40 mg of BSA is dissolved in 5 mL of deionized ddH₂O. Then, concentration gradients are prepared as follows:

<table>
<thead>
<tr>
<th>Standard</th>
<th>BSA concentration (mg/mL)</th>
<th>BSA (mL)</th>
<th>6 M guanidine hydrochloride buffer (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St 1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>St 2</td>
<td>0.8</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>St 3</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>St 4</td>
<td>2.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>St 5</td>
<td>3.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Microplate is loaded as follows:

18. Carbonyl contents are calculated and expressed as nmoles of DNPH incorporated per mg of proteins on the basis of molar extinction coefficient of 22.0 nM⁻¹cm⁻¹ at 370 nm for protein hydrazines.
Reagent preparation:

a. 10% trichloroacetic acid
   Dissolve 20.20 g of trichloroacetic acid (TCA; MW 163.39, % assay ≥ 99.0) in 150 mL of deionized ddH₂O and volume up to 200 mL with deionized ddH₂O.

b. 20% trichloroacetic acid
   Dissolve 40.40 g of trichloroacetic acid (TCA; MW 163.39, % assay ≥ 99.0) in 150 mL of deionized ddH₂O and volume up to 200 mL with deionized ddH₂O.

c. 2 N HCl
   Dissolve 32.63 mL hydrochloric acid (HCl; MW -36.46, density -1.2g/mL, % assay -37.25) in 150 mL of deionized ddH₂O and volume up to 200 mL with deionized ddH₂O.

d. 6 M guanidine hydrochloride buffer (6 M guanidine hydrochloride in 20 mM KH₂PO₄ (pH 2.3)
   Dissolve 1.361 g of potassium phosphate monobasic (KH₂PO₄; MW 136.09) in 150 mL of deionized ddH₂O, add 286.59 g of guanidine hydrochloride (MW 95.53), and volume up to 500 mL with deionized ddH₂O. To adjust pH to 2.3, adjust using conc. HCl.

e. 0.2% 2,4- dinitrophenylhydrazine in 2 N HCL
   Dissolve 400 mg of 2,4- dinitrophenylhydrazine (DNPH; MW 198.14) in 200 mL of 2 N HCL at 50ºC.

f. Ethanol : ethyl acetate solution
   Mix 250 mL of ethanol with 250 mL of ethyl acetate.
APPENDIX VII

DETERMINATION OF SULFHYDRYLS (FREE THIOLS)
(Ellman, 1959, Arch. Biochem. Biophys. 82:70-77)

1. Vortex eppendorfs containing myofibrillar proteins thoroughly.
2. Pipette two 25 μL aliquots of myofibrillar protein (one for the free-thiols and other for the protein estimations) into eppendorf tubes (2 mL safe-lock tubes; 02236352, Eppendorf AG, Hamburg, Germany).
3. Add 1 mL of buffer (pH 8) containing 0.1 M tris-hydrochloride (Tris-HCl; MW 157.60), 1.25 mM ethylenediaminetetraacetic acid (EDTA; MW 292.24), and 5% sodium dodecyl sulfate (SDS; MW 288.38) to myofibrillar protein aliquots and vortex for 10 s.
4. a. Free-thiol estimation – Add 25 μL of 10 mM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB; MW 396.35) in 0.1 M tris-HCl at pH 8.
   b. Protein estimation – Add 25 μL of 0.1 M tris–HCl (pH 8).
      Vortex for 10 s and incubate at room temperature for 30 min.
5. Centrifuge the final solutions at 5000 g for 5 min to remove any insoluble matter.
6. Read absorbance of three 200 μL aliquots of each sample at 412 nm to measure free-thiols by using a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California).
7. Read absorbance of three 200 μL aliquots of each relevant protein sample at 280 nm to measure proteins contents and the actual protein concentrations are calculated using a concentration series of BSA in 0.1 M Tris-HCl, 1.25 mM EDTA, and 5% SDS buffer at pH 8.

* Use UV/VIS microwell plates (07-200-623, Corning UV-Transparent Microplates).

To make BSA stock, 40 mg of BSA is dissolved in 5 mL of deionized ddH₂O. Then, concentration gradients are prepared as follows:

<table>
<thead>
<tr>
<th>Standard</th>
<th>BSA concentration (mg/mL)</th>
<th>BSA (mL)</th>
<th>0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St 1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>St 2</td>
<td>0.8</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>St 3</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>St 4</td>
<td>2.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>St 5</td>
<td>3.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Microplate is loaded follows:

8. Free thiol levels are calculated and expressed as nmoles of sulfhydryls per mg of proteins on the basis molar extinction coefficient of 13.6 mM\(^{-1}\) cm\(^{-1}\) at 412 nm for protein sulfhydryls.

\[
\text{Free – thiols nmoles/mg of proteins} = \frac{(\text{Avg. Abs. free} \ - \ \text{thiols} \ - \ \text{Avg. Abs. free} \ - \ \text{thiol control})}{0.0068 \text{ nmoles/mL}} \times \frac{\text{protein concentration mg/mL}}{1000}
\]

Reagent preparation:

a. 0.1 M Tris-hydrochloride, 1.25 mM ethylenediaminetetraacetic acid, and 5% sodium dodecyl sulfate buffer (pH 8)

Dissolve 7.88 g of Tris-hydrochloride (Tris-HCl; MW 157.60), 0.1823 g of ethylenediaminetetraacetic acid (EDTA; MW 292.24), and 25 g of sodium dodecyl sulfate (SDS; MW 288.38) in 400 mL of deionized ddH\(_2\)O at 50°C and volume up to 500 mL with deionized ddH\(_2\)O.
b. 10 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 M Tris-hydrochloride (pH 8)

Dissolve 0.793 g of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; MW 396.35), and 3.152 g of Tris-hydrochloride (Tris-HCl; MW 157.60) in 150 mL of deionized ddH₂O and volume up to 200 mL with deionized ddH₂O.

c. 0.1 M Tris-hydrochloride (pH 8)

Dissolve 3.152 g Tris-hydrochloride (Tris-HCl; MW 157.60) in 150 mL of deionized ddH₂O, adjust pH with conc. HCl and volume up to 200 mL with deionized ddH₂O.
APPENDIX VIII

DETERMINATION OF PROTEIN LEVEL

(Pierce® bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL)

1. Dissolve the remaining amounts of myofibrillar protein stock samples in 1 mL of 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) and vortex properly.

2. Dilute 100 µL of diluted myofibrillar protein samples in a new eppendorf tube, with 100 µL of 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) and vortex properly.

3. Prepare a concentration series (20 - 2000 µg/mL) of bovine serum albumin (BSA) using 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) as the diluents as follows;

<table>
<thead>
<tr>
<th>Vial</th>
<th>Diluent (µL)</th>
<th>BSA (µL)</th>
<th>BSA concentration (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 of stock</td>
<td>2000</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 of stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 of stock</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 of vial B dilution</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 of vial C dilution</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 of vial E dilution</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 of vial F dilution</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 of vial G dilution</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0 = blank</td>
</tr>
</tbody>
</table>

4. Place 25 µL of BSA standards and diluted myofibrillar protein samples on a microwell plate.

5. Add 200 µL BCA working reagents (50:1, Reagent A:Reagent B) into respective wells in the microwell plate.

Microplate is loaded follows;
6. Mix protein samples and BCA working reagents thoroughly by rotating the well plate on a flat surface gently.
7. Incubate the microwell plate at 37°C for 30 min and cool to the room temperature.
8. Read absorbances at 562 nm on a microplate reader (SpectraMAX 250, Molecular devices, Sunnyvale, California) using 0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS buffer (pH 8) + BCA working reagent as the blank.
9. Protein concentrations are expressed as μg/mL.

Reagent preparation:

a. 0.1 M Tris-hydrochloride, 1.25 mM ethylenediaminetetraacetic acid, and 5% sodium dodecyl sulfate buffer (pH 8)

Dissolve 7.88 g of Tris-hydrochloride (Tris-HCl; MW 157.60), 0.1823 g of ethylenediaminetetraacetic acid (EDTA; MW 292.24), and 25 g of sodium dodecyl sulfate (SDS; MW 288.38) in 400 mL of deionized ddH₂O at 50°C and volume up to 500 mL with deionized ddH₂O.
APPENDIX IX

SODIUM DODECYL SULFIDE POLYACRYLAMIDE GEL ELECTROPHORESIS: SDS-PAGE
(BIO-RAD MINI-PROTEAN® II OR III ELECTROPHORESIS CELLS)

Assembling the Casting stand and Casting frame

1. Clean glass plates and casting frames with alcohol and dry.
2. Place the short plate on top of the spacer plate. (If you use long and short plates use two spacers of equal thickness along the short edges of the rectangular long plate and place the short glass plate on top of the spacers so that the bottom ends of the spacers and glass plates are aligned)
3. Slide the two plates into the clamp assembly keeping the short plate facing front and tighten four screws of the clamp assembly.
4. Place silicone gaskets on the casting slots in the casting stand and snap the clamp assembly in the casting stand.
5. Put some 2-propanol to make sure the gel sandwich assembly is not leaking.
6. Remove all the 2-propanol before pouring gels.

Running (or resolving) gel casting

1. Decide what percentage gel you want to use (Refer Table 1 below; for 2 gels).

<table>
<thead>
<tr>
<th>% Gel</th>
<th>DD H2O (mL)</th>
<th>30% Acrylamide/bis-acrylamide (mL)</th>
<th>1.5 M Tris-HCL gel buffer (mL)</th>
<th>10% w/v SDS (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.1</td>
<td>1.3</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>5.7</td>
<td>1.7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>6</strong></td>
<td><strong>5.4</strong></td>
<td><strong>2.0</strong></td>
<td><strong>2.5</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td>7</td>
<td>5.1</td>
<td>2.3</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>4.7</td>
<td>2.7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>4.4</td>
<td>3.0</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>4.1</td>
<td>3.3</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>11</td>
<td>3.7</td>
<td>3.7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>12</td>
<td>3.4</td>
<td>4.0</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>13</td>
<td>3.1</td>
<td>4.3</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>14</td>
<td>2.7</td>
<td>4.7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>15</strong></td>
<td><strong>2.4</strong></td>
<td><strong>5.0</strong></td>
<td><strong>2.5</strong></td>
<td><strong>0.1</strong></td>
</tr>
</tbody>
</table>

2. Make the monomer solutions in conical flasks.
3. Add 50 μL of 10% ammonium persulfate (freshly made) and 10 μL TEMED into the monomer mix.
4. Stirring slowly and pour the solution mixture gently between the glass plates to the mark using a plastic pipette. Immediately overlay the monomer solution with 2-propanol or t-amyl alcohol to get a smooth surface.
5. Allow the gel to polymerize for 1 hr and remove the alcohol overlay on the gel.
Stacking gel casting
1. Prepare the stacking gel monomer solution by combining chemical as below except 10% ammonium sulfate and TEMED.

<table>
<thead>
<tr>
<th>4% Stacking gel, pH 6.8</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DD H2O</td>
<td>3.075 mL</td>
<td></td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>1.25 mL</td>
<td></td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>50 μL</td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/bisacrylamide</td>
<td>0.67 mL</td>
<td></td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>50 μL</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.075 mL</td>
<td></td>
</tr>
</tbody>
</table>

2. Add ammonium persulfate and TEMED and mix gently.
3. Insert the desired comb (similar thickness of the spacers) between glass plates.
4. Pour the solution between the glass plates and the comb and remove air bubbles.
5. Allow the stacking gel to polymerize for 1 hr.

Electrophoresis cell assembly
1. After polymerization of stacking gel, remove the gel cassette sandwich from the casting stand and place it into the electrode assembly with short plate inward (if you use protein III cell, remove the gel sandwich from casting assembly, place gel sandwich into the electrode assembly, slide gel sandwich into the electrode assembly, press down on the electrode assembly while closing the two cam levers of the clamping frame).
2. Mark wells on the glass plates.
3. Lower the electrode assembly into the mini tank and add running buffer (1X) to the inner chamber up to the top level.
4. Add running buffer (1X) into the outer chamber up to the lower screw level.
5. Carefully remove combs pulling up straightly.

Sample preparation and loading
1. Get 333.33 μg proteins from the sample and dissolved in 20 μL sample dilution buffer.
2. Add 80 μL (4 times as sample volume) of sample buffer (composition is as follows), vortex and let it stand for 5 min.

<table>
<thead>
<tr>
<th></th>
<th>Non-Reducing (8 mL)</th>
<th>Reducing (8 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD H2O</td>
<td>4.40 mL</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>1.00 mL</td>
<td>1.00 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.80 mL</td>
<td>0.0 mL</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>1.60 mL</td>
<td>1.6 mL</td>
</tr>
<tr>
<td>Beta-mercaptoethanol</td>
<td>0.40 mL</td>
<td></td>
</tr>
<tr>
<td>0.05% w/v Bromophenol blue</td>
<td>0.20 mL</td>
<td>0.20 mL</td>
</tr>
</tbody>
</table>

3. Heat the mixture for 10 min at 95ºC and cool to the room temperature before loading.
4. Load 15 μL (50 μg of proteins) into each well gently. [If you use molecular marker (Bio-Rad Kaleidoscope Prestained standards) use 10 μL].

**Power setting and electrophoresis**

1. Place the lid on the mini tank aligning color coded banana plugs and jacks Close the electrode.
2. Insert the electrode leads into a suitable power supply.
3. Set voltage and current as follows;
   - 6% gel - voltage to 120 V and current to 0.08 A
   - 15% gel – voltage to 100 V and current to 50 mA
4. Run at the constant voltage as mentioned below (check the buffer level in the inner chamber during electrophoresis).
   - 6% gel – 90 min
   - 15% gel – 150 min

**Gel removal**

1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
2. Remove gel sandwich from the gel assembly.
3. Remove the gel by gently separating the two plates.

**Staining and de-staining** (For protein staining only; otherwise follow the protocol in Appendix X)

1. Place the gel in the 100 mL of staining solution for 1 hr on a rocker.
2. Transfer the gel into distilled deionized water to remove excess stain.
3. Place the gel in the 100 mL of de-staining solution and leave for overnight (12 h) on a rocker.

**Gel imaging and visualization**

1. Measure desired protein bands using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) at 700 nm channel as integrated intensity (K. pixels).

**Reagents preparation:**

- 10% Ammonium persulfate (fresh daily)
  Dissolve 100 mg Ammonium persulfate in 1 mL distilled deionized water.

- 10% w/v Sodium dodecyl sulfate (SDS)
Dissolve 10 g of SDS in 90 mL distilled deionized water and bring to 100 mL with distilled deionized water.

c. **1.5M Tris-HCL, pH 8.8**
   Dissolve 18.15 g of Tris-base in 80 mL distilled deionized water. Adjust to pH 8.8 with 6 N HCl and bring to 100 mL. Store at 4°C.

d. **0.5M Tris-HCL, pH 6.8**
   Dissolve 6 g Tris-base in 60 mL distilled deionized water. Adjust to pH 6.8 with 6 N HCl and bring to 100 mL. Store at 4°C.

e. **5X Running buffer, pH 8.3**
   Dissolve 15 g of Tris-base, 72 g of Glycine, and 5 g of SDS in 800 mL distilled deionized water. Bring to 1 L with distilled deionized water.
APPENDIX X

WESTERN BLOTTING
(BIO-RAD MINI-TRANS-BLOT® ELECTROPHORETIC TRANSFER CELL)

1. Cut a piece of PVDF (IPFL20200; 0.45 μm, Immobilon-FL transfer membrane, Millipore Corporation, Billerica MA) to the dimension of the gel.
2. Equilibrate the gel in transfer buffer for 20 min (change into fresh buffer after 10 min).
3. Soak the filter papers (170-3932, Bio-Rad Laboratories, Hercules CA) and fiber pads in transfer buffer for 5 min.
4. Soak PVDF in methanol for 3 min.
5. Prepare the gel sandwich as follow;
   Place the cassette with the gray side (anode) down on a clean surface
   Place one pre-wetted fiber pad on the gray side of the cassette
   Place a filter paper on the fiber pad
   Place the equilibrated gel on the filter paper (roll out all bubbles)
   Place the pre-wetted membrane on the gel (roll out all bubbles)
   Place the other filter paper (roll out all bubbles) and fiber pad respectively
   Close the cassette firmly without moving the gel and filter paper sandwich and lock the cassette with the white latch)
6. Place 2 prepared cassettes in module.
7. Place the module in the mini-tank and fill with the transfer buffer.
8. Transfer at a constant voltage
   6% gel - 100 V and 180 mA for 120 min
   15% gel – 100 V and 180 mA for 90 min
9. Block the membranes with 10 mL Odyssey Blocking Buffer (927-40100; LI-COR, Lincoln NE) for 120 min at room temperature.

Primary Antibody Preparation and incubation

1. Dilute primary antibodies as follow;
   a. Anti-Myosin Skeletal Muscle Monoclonal (MY-32; MA5-11748; Pierce Biotechnology, Rockford IL)
      1:2000 – 5 μL in 10 mL of Odyssey Blocking Buffer + 0.2% Tween®20 (20μL)
   b. Anti-Troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO)
      1:10000-10 μL in 10 mL of Odyssey Blocking Buffer + 0.2% Tween®20 (20μL)
2. Incubate blots in diluted primary antibody for 1 h at room temperature and for overnight at 4ºC while gently shaking.
3. Pour off primary antibody solution.
4. Wash membrane 3 times for 10 min each with 15 mL of 1X TBST (freshly made) while vigorous shaking.

Secondary Antibody Preparation and incubation

1. Reconstitute contents in the original IRDye® 680LT Conjugated Goat Anti-Mouse IgG1 vial with 0.5 mL of sterile distilled water. Mix gently by inverting and allow rehydrating for at least 30 min before use. (Centrifuge product if solution is not completely clear after standing at room temperature. After reconstitution, this is stable for up to 3 month at 4ºC without exposure to direct light.

2. Dilute the secondary antibody as follows;
   a. 1: 5000 – 2 μL in 10 mL of Odyssey Blocking Buffer + 0.2% Tween®20 (20 μL) + 0.02% SDS (10 μL from 20% SDS)

3. Incubate blots in diluted secondary antibody for 60 min at room temperature while gently shaking.

4. Pour off secondary antibody solution.

5. Wash membranes 3 times for 10 min each with 15 mL of 1X TBST (freshly made) while vigorous shaking.

6. Wash membrane with 15 mL of 1X TBS for 5 min to remove residual Tween 20.

6. Protect membrane from light during incubation (Use a microscope slide box for incubating with antibodies and washing steps until membrane is imaged.

Membrane Imaging

1. Membrane can be imaged wet or dry.

2. Measure desired bands using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) at 700 nm channel as integrated intensity (K. pixels).

3. Imaging conditions;
   - Resolution 169 nm
   - Quality medium
   - Focus offset 0 mm
   - Channels 700 nm
   - Intensity 3.0 or 3.5
   - Image size x-10 y-7
APPENDIX XI

DIAGRAM SHOWING THE LOCATION OF STEAKS AND MUSCLE STRIPS REMOVED FROM THE BEEF CLOD HEART (*M. TRICEPS BRACHII*) ROASTS AFTER RETAIL DISPLAY

c. Samples removed both parallel and perpendicular to the muscle fibers from all 3 steaks
APPENDIX XII

DETERMINATION OF FREE-CALCIUM LEVEL
(Parrish et al. 1981. J. Food Sci. 46:308-311 with modifications)

1. Analyze each sample in duplicates.
2. Mince frozen steak by using a stainless-steel knife (avoid fat and connective tissues).
3. Measure 3 g of minced meat into a thickwall polyallomer ultracentrifuge tube (13 × 55 mm) and centrifuge at 196 000 × g for 30 min at 4ºC.
4. Pipette 700 μL of the supernatant into an eppendorf tube.
5. Treat the supernatant with 0.1 mL of 27.5% trichloroacetic acid and vortex for 15 s.
6. After standing for 10 min at room temperature, centrifuge eppendorf tubes at 6000 rpm for 10 min.
7. Pipette 500 μL of the supernatant into a plastic tube and bring up the volume to 5 mL with deionized ddH₂O.
8. Filter prepared samples through a 13 mm diameter Millex-LG 0.20 μm syringe filter (Millipore, Bedford MA) in to a new tube.
9. Send prepared samples to the Ward Laboratories (Kearney, NE) for calcium level determination.
10. Calcium concentrations (ppm) of samples are quantified at the Ward Laboratories (Ward Laboratories, Kearney, NE) using the inductively-coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron Corporation, Cambridge, UK) with appropriate calcium concentration standards.
11. Calcium concentrations are calculated as follows;

\[
\text{Average ppm of Calcium in the sample} = \frac{X \text{ ppm}}{40.078 \text{ g}} = X \text{ mg/L}
\]
\[
\text{Molecular weight of Calcium} = 40.078 \text{ g}
\]
\[
\text{Micromolar (μM) concentration of calcium} = \frac{(X \times 1000)}{40.078} \mu\text{M}
\]