CALIFORNIA STATE UNIVERSITY NORTHRIDGE

LYOPHILIZED COFFEE'S OXIDATION INHIBITION IN TOP ROUND BEEF MUSCLE THROUGH THE INTERACTION WITH SARCOPLASMIC AND MYOFIBRILLAR PROTEINS

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Science in

Family and Consumer Sciences

by

Kityanun Angkinand

December 2014

The thesis of Kityanun Angkinand is approved:

Dr. Lilian Were, Ph.D.	Date
Dr. Yoko Mimura, Ph.D.	Date
Dr. Claudia Fajardo-Lira, Ph.D., Chair	Date

California State University, Northridge

DEDICATION

This thesis is dedicated to my family with a special feeling of gratitude to my loving parents and sister who have supported me each step of the way.

ACKNOWLEDGMENT

I would like to express my deepest gratitude to my advisor Dr. Lilian Were for her unrelenting support, expert guidance, stellar management, and glorious understanding throughout my research. Without her persistent help, incredible patience, meticulously inspection and timely wisdom, my thesis would not have been successfully completed. In addition, I also express my appreciation to the committee members, Dr. Claudia Fajardo-Lira and Dr. Yoko Mimura for their kindly support, thoughtful suggestions, and great patience at all times.

I would also like to thank Dr. Titiana Prytkova and Taylor Patti who put efforts to complete the docking experiment. I also wish to thank Dr. Ma Criselda Toto who expressed deep interests and provide substantial opportunities for practicing statistical analysis.

I would like to acknowledge California State University Northridge for providing necessary financial support, Michael Gaviña and F. Gaviña and Sons, Inc for carefully roasting and providing coffee beans, Kari Godbey (American Beef Packers, Inc.) for providing beef sample for this thesis.

This thesis would not have been possible without the help from Nahathai Stapornkul, my friend who has been working with me since starting until the very end. Thank you for your support and encouragement especially through the hard time.

Many thanks to my friends at Chapman University (Crystal Lin, Patrick Kim, Katrina Jully, and Hatouf Alnouman) for sharing good times in the lab and helping me upon request. Big thanks to Tiffany Hashimoto for allocating the room to stay over while running the full experiment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

LYOPHILIZED COFFEE'S OXIDATION INHIBITION IN TOP ROUND BEEF MUSCLE THROUGH THE INTERACTION WITH SARCOPLASMIC AND MYOFIBRILLAR PROTEINS

by

Kityanun Angkinand

Master of Science in

Family and Consumer Sciences

The antioxidant mechanism of roasted coffee to inhibit oxidation in beef is attributed to its metal chelating and free radical scavenging properties, but interaction with beef proteins may be another antioxidant mechanism. The purpose of this study was to investigate antioxidant mechanism of coffee on oxidation inhibition in beef muscle through the interaction with beef proteins. Sarcoplasmic (SP) and myofibrillar (MP) proteins extracted from top round beef muscle were treated with lyophilized coffee brew (0 - 160 μg/mL protein) and incubated with lipid oxidation (LOX) products (malonaldehyde/MDA and saturated aldehyde compounds/C_5-C_9) at 4˚C. The bound MDA and saturated aldehydes per g protein after incubation were determined over 9 days. Fluorescence, myoglobin (Mb) absorption spectra, and docking were performed to determine the interaction behavior of coffee on beef proteins. Moreover, metmyoglobin (MetMb) formation and thiol oxidation were also observed to determine the effect of coffee on protein oxidation (POX). The results showed that coffee increased ability to bind MDA and nonanal when interacting with SP. Specifically, coffee interacted at

surface of Mb in SP via hydrophilic interaction, and bound to aldehydes via hydrophobic interaction. Coffee did not increase thiol oxidation and MetMb formation. The finding help elucidate the antioxidant mechanism of coffee when added to beef and suggest that roasted coffee potentially inhibits LOX while does not cause POX.

Keywords: Antioxidant, Beef, Coffee, Docking, Lipid and Protein oxidation

CHAPTER I

INTRODUCTION

The largest global beef consumption is in the U.S., consuming approximately 27-28 billion pounds annually from 2002 - 2008 and 25 - 26 billion pounds from 2009-2013. Although beef consumption has slightly declined over the past decade due to the health concerns related to cholesterol effects, its consumption is still 31% and 56% higher than poultry and all red meats consumption (Davis & Lin, 2005; USDA, 2014). Main reasons for preferred consumption of beef over others meats are due to its protein density and flavor (Davis & Lin, 2005; USDA, 2014). However, these characteristics quickly deteriorate within 10-14 days; mainly caused by chemical oxidation of unsaturated lipid and protein in beef components (Delmore, 2009; Kim, Cadwallader, Kido, & Watanabe, 2013; Lin, Toto, & Were, 2015).

Beef muscle is highly susceptible to oxidation compared to pork, chicken, and fish due to the high concentration of iron content (~23 mg/kg in beef vs 15 mg/kg in pork and 6 mg/kg in chicken) (S. Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001). Free radicals, metals, and oxidation products are also involved in catalyzing oxidation in beef (Lynch, Faustman, Silbart, Rood, & Furr, 2001; Ramanathan, Konda, Mancini, & Faustman, 2009). Lipid oxidation mainly generates MDA and other saturated aldehydes (C_3-C_{10}) that are major causes of rancidity while protein oxidation (POX) mainly triggers protein conformational change causing texture and color alteration (Min & Ahn, 2005; Zhang, Xiao, & Ahn, 2013).

Roasted coffee, as a natural antioxidant source, has been studied to inhibit LOX in cookies, chocolate and meat (Budryn & Nebesny, 2013; Lin et al., 2015; Nissen, Byrne,

Bertelsen, & Skibsted, 2004). In beef, Lin et al. (2015) found that dark roasted coffee had the highest ability to inhibit MDA, pentanal, hexanal, and nonanal in ground beef muscle under 8 days refrigerated storage compared to light-, medium- roasted coffee and rosemary. The Maillard reaction products (MRPs), specifically melanoidins are the major antioxidants in roasted coffee in addition to chlorogenic acid/CGA and caffeic acid/CA (Yen, Wang, Chang, & Duh, 2005). The MRPs increase with roasting degree, while CGA and CA decrease with roasting. The dark roasted coffee thus contains the highest MRPs and lowest chlorogenic acid content compared to light and medium roast and often has the highest ability to inhibit LOX compared to light- and medium- roast as found in *vitro* studies and in beef (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Lin et al., 2015).

Statement of the Problem

The elucidated mechanism of coffee to inhibit LOX is attributed to free radical scavenging, metal chelating, and antiradical properties (Borrelli et al., 2002; Delgado-Andrade & Morales, 2005; Lin et al., 2015; Yen et al., 2005). However, these mechanisms may only partially explain its antioxidant effect when added to beef. More research needs to be done to increase the understanding of the multiple and complex reactions involved with beef oxidation and coffee.

Another possible mechanism that could explain coffee's antioxidant effect is attributed to the interaction of coffee with beef proteins. Pérez-Juan, Flores, & Toldrá (2006) found that SP and MP, comprising ~30% and 50-60% in beef muscle, could bind aldehyde compounds at 1-2 μmol per g protein. Specifically, histidine (His 81, 88, and 93) of Mb in SP and His, Try, Arg of myosin in MP are primary sites responsible for the

aldehydes binding (Buttkus, 1967; Pérez-Juan, Flores, & Toldrá, 2007; Suman, Faustman, Stamer, & Liebler, 2007; Tironi, Lopez, Pellegrino, Añtón, & Tomás, 2004). Coffee may interact with these amino acids side-chains and lower aldehydes to a higher extent. So far, this specific mechanism has yet to be studied and proposed work will investigate it.

Purpose

The aim of this study was to investigate the mechanism of coffee to inhibit oxidation in beef via the interaction with beef SP and MP. The effect of coffee and proteins on LOX was determined by monitoring (1) ability of protein-coffee to bind MDA and volatile aldehydes using thiobarbituric acid reactive substances (TBARS) and gas chromatography (GC) analysis (2) interaction of coffee with aromatic amino acids and Mb was determined via fluorescence, absorption spectra, and docking simulation. Additionally, the effect of coffee on POX was determined by measuring thiol and myoglobin oxidation during refrigerated storage.

Definitions

1. **Antioxidant** is any substances that can delay, inhibit, or retard the oxidation by donating electron to free radicals (Borrelli et al., 2002).
2. **Binding** is an ability of protein to bind LOX products and lower LOX products in the system (Pérez-Juan et al., 2006).
3. **Docking simulation** is a computational method used to depict the three-dimensional molecular structure of specific molecules (Lengauer & Rarey, 1996).
4. **Free radicals** are atoms or molecules that contain an unpaired valence electron which is unstable and highly reactive (Min & Ahn, 2005).

5. **Gas chromatography** is an analytical technique used to identify and quantify volatile compounds using gas as a carrier of separation (D'Arcy, 2007).
6. **High performance liquid chromatography** is an analytical technique used to identify and quantify the components in a mixture using polar and non-polar liquids as a mobile phase (D'Arcy, 2007).
7. **Interaction** refers to the properties of one compound to attach with another compound via chemical bond (Wang, Zhang, & Zhou, 2009).
8. **Lipid oxidation** is a free radical chain reaction, which occurs when reactive oxygen species interact with polyunsaturated fatty acids (Min & Ahn, 2005).
9. **Lipid oxidation products** refer to the primary and secondary products generated from the oxidation of unsaturated fatty acids (Min & Ahn, 2005).
10. **Malonaldehyde** is a major product of linolenic acid oxidation which is considered as a marker of lipid oxidation (Fernández, Pérez-Álvarez, & Fernández-López, 1997).
11. **Maillard reaction** is a non-enzymatic browning reaction between reducing sugar and free amino acids, catalyzed by heat (Borrelli et al., 2002).
12. **Maillard reaction products** refer to products developed from the Millard Reaction (Borrelli et al., 2002).
13. **Melanoidins** is one of the Maillard's reaction products, which is high molecular weight brown nitrogeneous-based compound (Borrelli et al., 2002).
14. **Myofibrillar protein** is a muscular fiber found in skeletal muscle that plays a key role in muscle contraction, consisting mainly of actin (23%) and myosin (50%) (Chiang, Byrem, & Strasburg, 2007)

15. **Myoglobin** is a protein containing iron (i.e., heme protein) found within sarcoplasmic protein (Chaijan, 2008).
16. **Prooxidants** are substances that catalyze oxidation (Jongberg, Tørngren, Gunvig, Skibsted, & Lund, 2013).
17. **Reactive oxygen species** are oxygen atoms or molecule that contains unpaired valence electron on oxygen atom which is unstable and highly reactive (Min & Ahn, 2005).
18. **Sarcoplasmic protein** is a muscular skeletal fiber, consisting mainly of glycolytic enzymes (56%), myoglobin (5%) (Chiang et al., 2007).
19. **Thiobarbituric acid reactive substances assay** is an analytical technique used to quantify lipid oxidation by measuring MDA (Fernández et al., 1997).
20. **Thiol** is a molecule containing a carbon-bonded sulfhydryl (R–SH) group found in the structure of cysteine (Romero, Ordonez, Arduini, & Cadenas, 1992).
21. **Volatile aldehydes** are secondary products of lipid oxidation which can cause odor alteration in meat (Min & Ahn, 2005).

Hypotheses

Null Hypotheses

The data analyses for this thesis were guided by the following null hypotheses:

1. Coffee's interaction with beef SP will not lower MDA.
2. Coffee's interaction with beef SP will not lower volatile aldehydes
3. Coffee's interaction with beef MP will not lower MDA.
4. Coffee's interaction with beef MP will not lower volatile aldehydes.
5. Coffee will not interact with aromatic amino acids in beef proteins.

6. Coffee will not interact with Mb.
7. Coffee will not prevent the loss of thiol during storage.
8. Coffee will not prevent the formation of MetMb during storage.

Research Hypotheses

Based on the literature review in Chapter II, the following research hypotheses were developed.

1. Coffee's interaction with beef SP will lower MDA (Buttkus, 1967; Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008).
2. Coffee's interaction with beef SP will lower volatile aldehydes (Pérez-Juan et al., 2006).
3. Coffee's interaction with beef MP will lower MDA (Buttkus, 1967; Estévez et al., 2008).
4. Coffee's interaction with beef MP will lower volatile aldehydes (Pérez-Juan et al., 2006).
5. Coffee will interact with aromatic amino acids in beef proteins via hydrophilic bond (Bekedam, 2008; Wang et al., 2009).
6. Coffee will interact with Mb (Wang et al., 2009).
7. Coffee will prevent the loss of thiol during storage (Borrelli et al., 2002).
8. Coffee will prevent the formation of MetMb during storage (Borrelli et al., 2002).

Objectives

This thesis was designed to address to following objectives

- To determine whether coffee increases ability to bind MDA and saturated aldehydes through the interaction with beef SP or MP

- To determine the location on beef proteins where coffee phenolics bind
- To determine the effect of coffee on thiol and Mb oxidation

Assumptions

- Dark roasted coffee contains antioxidant capacity, mainly from MRPs
- Equipment used in this study were properly calibrated
- All samples and reagents were prepared correctly.
- All samples and reagents were stored properly.
- All parameters e.g. pH, temperature, dilution, time were controlled correctly.
- No errors were made in the data entry
- No errors were made in the data analyses.

CHAPTER II

REVIEW OF LITERATURE

The focus of this literature review is divided into four parts, including (1) the chemical mechanism of LOX and POX, (2) the ability of beef proteins to bind to LOX products, (3) the ability of coffee to inhibit LOX and POX, and (4) the methods to monitor the interaction of coffee and beef proteins.

Lipid and Protein Oxidation in Beef

Beef is comprised of 67-73% water, 20-22% proteins, 1-13% lipids, and 1% ash (Roseland, Nguyen, Williams, & Patterson, 2013). The major proteins in beef include SP, MP, and connective tissue. The SP and MP are soluble proteins, with SP being solubilized in low (0.03-0.05 mmol/L) and MP in high (0.1- 0.15 mmol/L) concentrated salt solutions (Gianelli, Flores, & Toldrá, 2005; Morzel, Gatellier, Sayd, Renerre, & Laville, 2006; Pérez-Juan et al., 2006; A. Romero, Doval, Sturla, & Judis, 2005). The SP (30% of total muscle protein) is found in cytoplasm, and consists of glycolytic enzymes (54%) and Mb (5%) that is primarily responsible for beef color. The MP (50 – 60% of total protein) is comprised of myosin (45%) and actin (20%), and is responsible for the mechanical work of muscle contraction as well as contributes to the water holding capacity that provides tenderness and juiciness of beef (Chiang et al., 2007).

The lipid content in beef varies depending on cuts of beef. Beef contains 41-49% of saturated fatty acids in which triglycerides are the main component, found in cover fats and marbling. Another 34-37% is mono-unsaturated fatty acids (MUFA), including palmitoleic (C16:1, n-7) and oleic (C18:1, n-9) and 3-7% is polyunsaturated fatty acids (PUFA), including linoleic (C18:2, n-6), linolenic (C18:3, n-3), and arachidonic (C20:4,

n-6). Unsaturated fatty acids are found in phospholipids fraction functioning as a structural component and insulation of cell membranes (Pavan & Duckett, 2013).

Beef proteins and lipids are subject to oxidation. The mechanism of LOX and POX is fairly similar involving three steps: initiation, propagation, and termination (Estévez, 2011; Lund, Heinonen, Baron, & Estévez, 2011; Min & Ahn, 2005; Zhang et al., 2013).

(I) Initiation: The initiation step occurs when a hydrogen atom from a lipid or protein molecule is abstracted by initiators e.g., reactive oxygen species (ROS), transition metals, resulting in the formation of lipid or protein radical (R·). The methylene groups of unsaturated fatty acids and amino acids containing sulfhydryl groups (e.g., cysteine, methionine) along with tryptophan residues are the primary target for initiators (Estévez, 2011; Lund et al., 2011; Min & Ahn, 2005; Zhang et al., 2013).

(II) Propagation: This step is the reaction of R· with oxygen molecule to form peroxyl radical (ROO·) which is highly reactive and can react with nearby lipid and protein molecules. In lipid, this reaction leads to formation of aldehydes and ketones e.g., MDA, hexanal, 1-octen-3-one which are major causes of rancidity due to their low odor threshold (Hun Kim et al., 2013; Min & Ahn, 2005) In protein, ROO· can catalyze the formation of carbonyl derivative compounds. The carbonyl formation leads to protein denaturation, protein cross-linking, and MetMb formation, altering texture, flavor, color, and water holding capacity of beef (Chaijan, Benjakul, Visessanguan, Lee, & Faustman, 2007; Mestdagh, Kerkaert, Cucu, & De Meulenaer, 2011; Utrera & Estévez, 2012, 2013). Moreover, POX causes the loss of hydrophobic conformation; consequently lowering

protein's digestibility by proteolytic enzymes; thereby lessening the nutrient absorption (Morzel et al., 2006).

(III) Termination: This step occurs when free radicals react with each other to form non-radical products e.g., ketone, and alcohols, stopping the oxidation process (Frankel, 1980). Antioxidants (A•) including coffee can also bind to radicals, terminating the propagation stage (Delgado-Andrade & Morales, 2005).

Reciprocal Transfer between Lipid and Protein Oxidation

Lipid and protein oxidation typically occur concurrently. The oxidation of both reactions involves the reciprocal transfer of the radicals and oxidation products between each other (Lund et al., 2011). The rate of LOX compared to POX in beef is difficult to predict. One may go faster than the other, depending on the storage conditions, preservative treatments and cuts of beef (Balentine, Crandall, O'Bryan, Duong, & Pohlman, 2006; Jung, Nam, Ahn, Kim, & Jo, 2013; Utrera & Estévez, 2013). However, the onset of LOX is likely faster than POX, therefore LOX possibly facilitates POX more than POX facilitating LOX (Estévez et al., 2008; Lund et al., 2011; Utrera & Estévez, 2013). Studies related to the relationship between LOX and POX are summarized in Table 1.

Mestdagh et al. (2011) indicated that the rate of POX was dependent on the degree of unsaturated fatty acids and aldehyde formation. The aldehydes can interact with amino acids residues via Michael additions or Schiff base reaction at which cysteine, lysine, histidine, and tryptophan are primary targets (Figure 1; Elias, Kellerby, & Decker, 2008; Utrera, Morcuende, & Estevez, 2014). The reaction causes the formation of aldehyde-protein adducts (Elias et al., 2008; Lynch et al., 2001; Mestdagh et al., 2011;

Utrera et al., 2014). The rearrangement of these adducts (amadori rearrangement) subsequently forms α-dicarbonyl compounds e.g., MDA and methylglyoxal that continuously interacts with α-amino acid via Strecker degradation (Figure 2), resulting in the formation of Strecker aldehyde such as 4,5(E)-epoxy-2(E)-heptenal and 4,5(*E*)-epoxy-2(*E*)-decenal derived from n-3 and n-6 fatty acids, respectively (Hidalgo & Zamora, 2004). The reaction between LOX products and proteins finally results in the modification of protein, along with promoting rancidity in meat from new aldehydes produced (Frankel, 1980; Lynch et al., 2001; Pavan & Duckett, 2013; Suman et al., 2007).

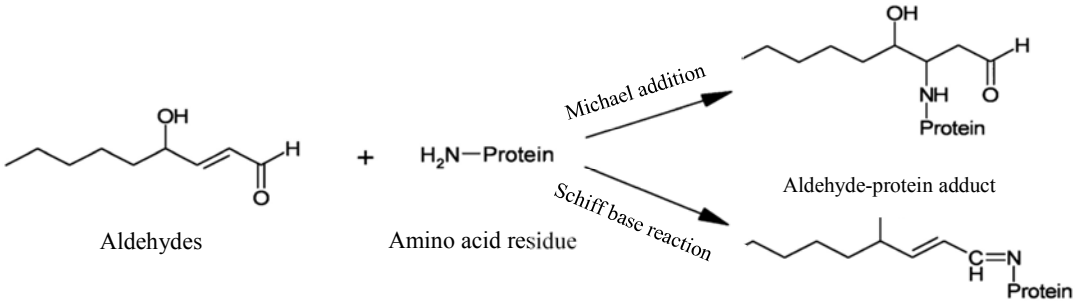


Figure 1 Modification of protein by aldehydes via Schiff based reaction and Michael addition (adapted from Elias et al., 2008)

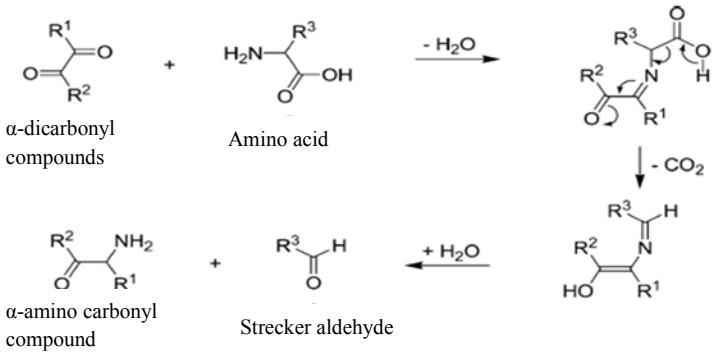


Figure 2 Modification of protein by dicarbonyl compound via Strecker degradation (adapted from Hidalgo & Zamora, 2004)

Protein, likewise, can also catalyze LOX. The reaction is induced by endogenous

iron present within Mb, the main fraction of meat SP. The Mb contains heme protein that can exist in the ferrous state/Fe(II) - deoxymyoglobin (DeoxyMb) and oxymyglobin (OxyMb) - exhibiting the bright cherry-red color of beef (Chaijan, 2008). The oxidation of OxyMb causes transition of ferrous to ferric state/Fe(III), resulting in metmyoglobin (MetMb) formation (brown color) measured at 500-600 nm by spectroscopic method (J. Tang, Faustman, & Hoagland, 2004).

The transition of ferrous to ferric state results in heme denaturation, releasing iron from the structure and initiating LOX. In addition, the rate of LOX in cooked meat is greater than raw meat due to heat catalyzing the release of heme iron to interact with lipids (Grunwald & Richards, 2006). The OxyMb can react with oxygen and generate ROS, initiating free radical chain reactions. The interaction of MetMb and H_2O_2 produces ferrylmyoglobin (FerrylMb) which is a stronger prooxidant than other free metals e.g., Cu(II), Cu(III), Fe(II), Fe(III), catalyzing oxidation of lipids especially under physiological condition (pH 7.4) and high iron condition found in beef muscle (Chaijan, 2008; Ramanathan et al., 2009; Rao, Wilks, Hamberg, & Montellano, 1994). The formation of FerrylMb is also accelerated when catalase (enzyme catalyzing H_2O_2 breakdown) is impaired due to the storage time or in the presence of catalase inhibitor such as sodium azide (Pradhan, Rhee, & Hernández, 2000). The concentration of heme iron is thus a major indicator of LOX (Grunwald & Richards, 2006; S. Tang et al., 2001; Tokur & Polat, 2010). The rate of LOX in raw beef is ~7.0-fold higher than white meats e.g., fish and poultry and 2.6-fold higher than pork because of the higher in heme protein content (1-2.5 fold higher than white meats and 0.65 fold higher than pork) (S. Tang et al., 2001).

In summary, free radicals and heme protein can promote the continuous radical chain reaction, causing oxidation of beef lipids and proteins. Roasted coffee, the antioxidant source in the current study can stop this chain reaction via free radical and metal scavenging properties (Borrelli et al., 2002; Delgado-Andrade & Morales, 2005; Lin et al., 2015; Nissen et al., 2004). The current study will investigate if coffee inhibits beef oxidation via the interaction with beef proteins.

Ability of Proteins to Bind to Lipid Oxidation Products

Despite the fact that proteins and lipids can facilitate oxidation of each other, some proteins, peptides, or amino acids (e.g., cysteine, carnosine, whey, bovine serum albumin/BSA, SP, and MP) have anti-oxidative properties that lower the susceptibility of oxidation (Estévez et al., 2008; Gianelli et al., 2005; Lynch et al., 2001; Pérez-Juan et al., 2006; A. Romero et al., 2005; S. Zhou & Decker, 1999). There are a number of assumptions on how proteins decrease oxidation in meat systems. Firstly, under acidic condition, proteins carry a positive net charge, repelling metals (Hu, McClements, & Decker, 2003). Secondly, dipeptide carnosine in protein molecules can act as metal chelators (Gianelli et al., 2005). Thirdly, amino acids containing sulfhydryl groups (cysteine, methionine) can act as radical scavengers, terminating oxidation (Tong, Sasaki, McClements, & Decker, 2000). Fourthly, muscle proteins contain catalase enzyme, inhibiting H_2O_2 that is major cause of FerryMb formation, thus inhibiting oxidation in beef (Pradhan et al., 2000). Furthermore, when protein interacts with LOX products under heat, it induces the formation of antioxidant compounds such as oxidized lipid/amino acid reaction products or MRPs (A. Romero et al., 2005).

Particularly to beef SP and MP, they can function as reservoir and bind to aldehydes and other LOX products, remarked as contributing factor to limit rancidity in beef (Buttkus, 1967; Pérez-Juan et al., 2006). Selected studies related to effect of proteins on LOX products binding are summarized in Table 1. Histidine in Mb component is the main binding site in SP, whereas His, Tyr, and Arg within myosin component are binding sites in MP (Buttkus, 1967; Suman et al., 2007; Tironi et al., 2004; Yin et al., 2011). Although Mb in SP acts as prooxidant (Grunwald & Richards, 2006; Ramanathan et al., 2009; S. Tang et al., 2001), SP has properties to bind LOX products. In fact, the binding ability of SP is greater than MP (SP bound hexanal at ~2 μmol/g while MP bound at ~1 μmol/g in MP) due to the configuration favored for Michael additions (Pérez-Juan and others 2006, 2007, 2008; Suman and others 2007). Specifically in beef, there are 13 His in beef in which His 81, 88, and 93 could potentially adduct with aldehydes due to b- and y- ion series configuration existed for a Michael adduct (Suman et al., 2007; Yin et al., 2011). Although myosin contains more His residues than Mb (36 His sites of 1,940 amino acids in total) (Chikuni, Muroya, & Nakajima, 2004), no studies investigated the relationship between its configuration and its affinity for Michael addition or Schiff based reaction. More research needs to be done on this particular relationship in order to elucidate why Mb has more LOX binding ability than myosin.

The current study determined if the interaction of coffee with beef proteins could be antioxidant mechanism of coffee when added to beef that could lower LOX products to a greater extent than without added coffee.

Table 1 Interaction effect of meat proteins and lipids on oxidation in meats

Protein type (meat source)	Lipid or LOX[a] products	Additional compounds	Condition (time, temp, pH)	Analytical methods			Key findings	References
				Binding ability	POX[a]	LOX[a]		
Beef								
Beef Patties	Low-, medium-, high- fat content	-	15 days, 5˚C	-	Tryptophan and Schiff base structure, formation of AAS[d]	TBARS[c]	Higher fat content resulted in higher POX[a] due to the higher oxidation products produced from LOX[a].	Utrera et al. (2014)
OxyMb[b] (bovine, porcine, ovine, cervine, equine, and turkey)	4-HNE[d]	-	6 hr, 25˚C and 96 hr, 4˚C, pH 5.6	-	MetMb[b] formation	TBARS[c]	4-HNE[d] increased MetMb[b] formation and TBARS[c] in all species (~5% of MetMb[b] and ~30% of TBARS[c] higher than no 4-HNE[d] in bovine sample).	Yin et al. (2011)
Ground beef	Propional, Pentanal, Hexanal, 4-HNE, MDA	-	9 days, 4˚C, pH 7.2	ELISA[c]	MetMb[b] formation	Aldehydes, TBARS[c]	The interaction of beef protein with 4-HNE[d], MDA[d], and C6-C9enal-adducts detected by ELISA[c]. TBARS[c] and MetMb[b] formation concurrently increased with degree of interaction.	Lynch et al. (2001)

Pork

MP[b] (pork muscle), BSA	purified rapeseed oil	Gallic acid, cyanidin-3-glucoside, chlorogenic acid, epicatechin, rutin, genistein, and tocoperol	10 days, 37 ˚C, pH 7.0	-	Loss of tryptophan, formation of carbonyls	Conjugated diene, hexanal content	MP[b] inhibited hexanal formation and carbonyl to a greater extent than BSA by 80% and 30%, respectively. Cyanidin-3-glucoside, genistein, and gallic acid enhanced inhibiting ability of MP[b] and BSA[b] >20-70%.	Estévez et al. (2008)
SP[b], MP[b], actomyosin, G-actin, (pork muscle)	3-methyl-butanal, 2-methyl-butanal, 2-pentanone, hexanal, methional Octanal	Salts (NaCl, KCl, $MgCl_2$, $CaCl_2$)	16 hr, 30˚C, pH 7.4	SPME[c] and GC[c]	-	-	SP[b] bound to all volatile compounds except 2-pentanone. MP[b] bound only octanal. Actomyosin bound to hexanal and octanal, but actin didn't show binding effect. Salts acted as prooxidant. NaCl decreased 50% of SP[b] binding ability. $MgCl_2$ and $CaCl_2$ increased aldehydes production in MP[b].	Pérez-Juan, Flores, & Toldrá (2008)
Actomyosin and G-actin (pork	3-methyl-butanal, 2-methyl-	-	16 hr, 30˚C, pH 7.4	SPME[c] and GC[c]	-	-	Actomyosin decreased hexanal and octanal by 40% and 60%,	Pérez-Juan et al. (2007)

muscle)	butanal, 2-pentanone, hexanal, methional, Octanal						respectively. No significant change of volatile compounds was found from the effect of G-actin.	
SP[b], MP[b] (pork muscle and 7-, 12-month dry cured ham)	3-methyl-butanal, 2-methyl-butanal, 2-pentanone, hexanal, methional, Octanal	-	16 hr, 30˚C, pH 7.4	SPME[c] and GC[c]	-	-	SP bound to all volatile compounds except 2-pentanone (methional>octanal> hexanal>2- and 3- methyl-butanal). MP bound only 3-methyl-butanal, hexanal, and octanal. 7-, 12- month ham had lower ability to bind than raw pork due to the loss of carnosine content during processing.	Pérez-Juan et al. (2006)
OxyMb[b] (pork heart)	4-HNE[d]	-	4hr at 25˚C and 2 hr at 37˚C, pH 5.6,7.2	SDS-Page, LC-MS[c]	MetMb[b] formation	-	4-HNE[d] bound to pork Mb at His residues. The binding increased MetMb[b] formation by 10% at pH 7.2.	Lee, Phillips, Liebler, & Faustman (2003)
Horse								
Mb[b], arnisine,	3-methyl-butanal, 2-	-	15 hr, 30˚C, pH	SPME[c] and	-	-	Carnosine bound to 20-80% of all volatile	Gianelli et al. (2005)

and carnosine (horse muscle)	methyl-butanal, 2-pentanone, hexanal, methional, Octanal		6.0	GC[c]			compounds except 2-pentanone. Arnisine bound to 10-20% of 2-methylbutanal, 3-methylbutanal, hexanal, and methional. Mb[b] bound to 0-15% 2-methylbutanal and hexanal.	
OxyMb[b] (horse heart)	4-HNE[d]	-	120 min, 37 ˚C , pH 7.2	-	OxyMb[b] oxidation	TBARS[c]	40% of 4-HNE[d] was bound to OxyMb[b]. The binding decreased OxyMb[b] oxidation and TBARS by ~25% and 50%, respectively.	Lynch & Faustman (2000)
Fish								
Myosin (cod fish)	hexanal, 2-hexenal, 2,4-hexadienal, 2,6-nonadienal	-	72 hr, 25˚C, pH 7.0	SPME[c] and GC[c]	Thiol, free amino acids, carbonyl content	-	The aldehyde-myosin binding increased with the hydrophobic affinity of aldehydes. At 24 hr, 75%, 40%, 20% of 2 ,6-nonadienal, 2,4-hexadienal, 2-hexenal were bound to fish myosin, but no significant change in hexanal. All aldehydes catalyzed POX[a] by increasing carbonyl content while decreasing thiol and	Chopin, Kone, & Serot (2007)

							amino acids content.	
OxyMb[b] (yellowfin tuna)	hexanal, hexenal, 4-HNE[d]	-	2 hr, 37˚C, pH 7.2	-	OxyMb[b] oxidation, MetMb[b] formation	-	MetMb[b] formation increased in the presence of aldehydes (4-HNE[d]>hexenal>hexanal).	Lee, Joo, Alderton, Hill, & Faustman (2003)
MP[b] (sea salmon)	MDA[d]	-	8 hr, 27 ˚C, pH 7.0	-	The loss of lysine	-	Interaction of MP[b] and MDA[d] caused microstructural changes, protein aggregation, and loss of lysine due to the covalent linkage and conjugated Schiff base formation.	Tironi et al. (2004)
Myosin (trout)	MDA[d]	-	6 days, 0-20 ˚C, pH 6.8	Interaction of amino acids and MDA[d]	-	-	40% of amino groups reacted with MDA[d] within 8 hours at room temp. The major site of reaction was histidine, followed by tyrosine, arginine, and methionine.	Buttkus (1967)

a LOX – lipid oxidation; POX – protein oxidation

b Mb – myoglobin; OxyMb – oxymyoglobin; MetMb – metmyoglobin; MP – myofibrillar protein; SP – sarcoplasmic protein

c ELISA - enzyme-linked immunosorbent assay; GC – gas chromatography; SPME – solid phase microextraction; TBARS – thiobarbituric acids reactive substances

d 4-HNE – 4 hydroxynonenal; AAS - α-aminoadipic semialdehyde; MDA - malonaldehyde

Antioxidant Capacity of Coffee to Inhibit Oxidation in Foods

Coffee is the second ranked most consumed beverage in the world; consumed for its stimulant caffeine content as well as its flavor. However, the value of coffee is not only limited to the brew, but it is also an effective antioxidant source (Budryn & Nebesny, 2013; Lin et al., 2015; Nissen et al., 2004).

Coffee Antioxidant Composition and Mechanism

Antioxidant capacity of coffee exists in both green and roasted coffee beans; however, the chemical composition of these two beans is different. In green coffee beans, the antioxidant properties are dominantly from CGA and CA (Sacchetti, Di Mattia, Pittia, & Mastrocola, 2009). During roasting, CGA content decreases 56 - 99% with roasting time and temperature of 170 - 220˚C for 6 - 15 min (Perrone, Farah, & Donangelo, 2012; Sacchetti et al., 2009). Roasting, however, induces the formation of newly MRPs antioxidant compounds, specifically melanoidins, which are high molecular weight nitrogenous compounds, comprise approximately 25 g/100g of dry coffee matter (Borrelli et al., 2002).

While CGA decreases with roasting, MRPs increase with roasting (Perrone et al., 2012; Vignoli, Bassoli, & Benassi, 2011). Dark roasted coffee thus contains the highest amount of MRPs which is often found to have the highest antioxidant capacity, compared to light- and medium- roast (Borrelli et al., 2002; Lin et al., 2015; Nicoli, Anese, Manzocco, & Lerici, 1997; Vignoli et al., 2011). However, Sacchetti et al. (2009) demonstrated that medium roasted coffee had greater antioxidant capacity than dark roast. The discrepancy is due to the differences of the types of coffee used, roasting time

and temperature, and the assays used to determine antioxidant capacity (Perrone et al., 2012; Sacchetti et al., 2009; Vignoli et al., 2011).

So far, the investigated antioxidant mechanisms of coffee include peroxyl radical scavenging and antiradical and metal chelating (Borrelli et al., 2002; Delgado-Andrade & Morales, 2005; Lin et al., 2015; Vignoli et al., 2011). Delgado-Andrade and Morales (2005) explained that, possibly, the enediol structure reductones produced throughout the Maillard reaction donate electrons to free radicals and terminate the radical chain reactions.

Another possible mechanism of coffee to work as antioxidant in meat is via the interaction with meat proteins. Low molecular weight compounds in MRPs such as coffee phenolics (CGA and CA) can interact with proteins via covalent and hydrophobic bond (Kroll, Rawel, & Seidelmann, 2000), whereas hydrophilic with proteinous moieties and negatively charged groups of high molecular weight compound, melanoidins, can bind to positive charged groups on protein (Bekedam, 2008). Coffee, therefore, is hypothesized to exhibit antioxidant capacity via the interaction with beef proteins, and this particular mechanism was investigated in the current study.

Capacity of Coffee to Inhibit Oxidation in Food Matrices

Antioxidant effect of coffee has been mainly investigated *in vitro.* Studies that specifically tested coffee in food matrices are still lacking. Other studies testing antioxidant effect of MRPs from other sources (e.g., synthesized MRPs) beside roasted coffee can explain antioxidant effect of coffee in foods (summarized in Table 2).

Roasted coffee and MRPs can lower LOX in foods, yet it is still less effective than BHT/BHA (Fernandez, Sturla, Doval, Romero, & Judis, 2012; Miranda, Rakovski,

& Were, 2012). Nevertheless, consumers, nowadays, prefer to consume food products without addition of chemicals (Shah, Bosco, & Mir, 2014). Thus, roasted coffee may be more preferable for consumers and can be a potential source of natural preservative used to slow down the oxidation and extend shelf-life of food products (Shah et al., 2014). The recommended coffee concentration to inhibit LOX in beef is 0.1g coffee/100g beef as it was found to be more effective than rosemary – a commercial natural preservative (Lin et al., 2015). On the other hand, when coffee was used at 0.05 g/100 g, it will be less effective than rosemary (Nissen et al., 2004). However, besides different coffee concentrations, Lin et al. (2015) and Nissen et al. (2004) used different types of coffee and rosemary which should be taken into account in terms of comparing their antioxidant effectiveness.

Although coffee effectively lowers LOX, we cannot assume that it can lower POX to the same extent as LOX. Some well-known plant-based antioxidants e.g., green tea and rosemary that effectively reduce LOX, can function as prooxidant on protein, resulting in the loss of thiol and myosin content (Jongberg et al., 2013). To date, no studies have determined the effect of coffee on POX. Thus, the current study also investigated this particular effect.

Table 2 Effect of coffee or Maillard reaction products in food systems

Antioxidant sources	Food system	Key findings	References
		Coffee as an antioxidant source	
Blend			
Coffee extracts (0.05 g/100g) and rosemary, green tea, and grape skin extracts (0.2 g/100g)	Cooked pork patties	The antioxidant efficiency in inhibiting TBARS and aldehydes production in declining order was as follow: 0.2 g/100g rosemary>grape skin>tea> 0.05 g/100g coffee>control.	Nissen et al. (2004)
Lyophilized coffee, spices, tea, grape skin, and tomato peel slurry (0.4 mg/g)	Pork lard	Coffee and rosemary were the most effective extracts in inhibiting the formation of conjugated diene.	Schwarz et al. (2001)
Brazil			
Light-, medium-,dark-ground roasted coffee (0.1 g/100g), rosemary (0.1 ml/100g)	Raw ground beef	Dark roasted coffee was the most effective antioxidant that lowered TBARS[b] and hexanal by 60% and 90% respectively in 8 days ground beef stored at refrigerated storage.	Lin et al. (2015)
Robusta			
Green and roasted lyophilized coffee (0.1, 0.5, 1.0 g/100g)	Cookies and chocolates	Green and roasted lyophilized coffee decreased peroxide values by 35-48%, conjugated diene by 15% and conjugated triene by 15-33%. No significant differences were found between doses of coffee.	Budryn & Nebesny (2013)
		Millard reaction products as an antioxidant source	
Soy sauce (0.2 L/kg beef)	Raw beef	Soy sauce limited TBARS[b] and MetMb[a] formation by ~40% and	Hyun Kim et al. (2013)

in the presence of 100 g/L of NaCl	patties	~10%, respectively due to the presence of melanoidins and phenolic compounds.	
Beef SP[a]/MDA[a] reaction (0, 0.5, 1, 2, 3 g/100g) and BHA (0.01 g/100g)	Cooked beef patties enriched with PUFA	The 3g/100g of MRPs effectively inhibited peroxide and TBARS[b] value by 83% and 85%, respectively. However, the effectiveness was still less than BHA[c] by ~10%.	Fernandez et al. (2012)
Amino acids/glucose reaction (0.01, 0.02 g/100g) and BHT[c] (0.01g/100g)	Ground chicken breast	MRPs[a] (0.2 mg/g) from amino acids/glucose reaction had higher ability than BHT[a] by ~30% to inhibit TBARS[b] formation. However, BHT[a] decreased aldehyde compounds ($p<0.05$) more significant than MRPs[a].	Miranda et al. (2012)
Autoclaving egg albumin hydrolysate/glucose (0.5 and 1.0 g/100g)	Cooked ground beef	Adding 0.5 and 1.0 g/100g of MRPs[a] to cooked ground beef inhibited the formation of TBARS[b] by 17 and 39% on day 8, respectively. MRPS[a] prevented the change of beef aroma and flavor, determined by sensory evaluation.	Smith & Alfawaz (1995)

a MDA – malonaldehyde, MetMb – metmyoglobin MRPs – Millard Reaction products, SP – sarcoplasmic protein

b TBARS – thiobarbituric acid reactive substances

c BHA - Butylated hydroxyanisole, BHT - Butylated hydroxytoluene

d 4-HNE – 4 hydroxynonenal; AAS - α-aminoadipic semialdehyde; MDA - malonaldehyde

Monitoring Effect of Coffee and Beef Proteins on Oxidation Inhibition

Lipid Oxidation Products Binding

In order to investigate the binding ability of proteins with LOX products, the LOX products are usually incubated with protein over time. The incubation allows protein to interact and bind to LOX products, consequently lowering the free LOX products in an aqueous phase (Ganhão, Estévez, & Morcuende, 2011; Gianelli et al., 2005; Pérez-Juan et al., 2006). The ability of protein to lower LOX products is usually expressed as bound LOX products/g protein or % free LOX products (i.e., % recovery). The bound LOX products/protein represents the amount of bound LOX products per gram of protein, while % recovery represents the amount of free LOX products in aqueous phase that are unbound by protein after incubation (Ganhão et al., 2011; Gianelli et al., 2005; Pérez-Juan et al., 2006). The higher bound LOX products or less % recovery indicates the higher ability of protein to bind LOX products. With addition of antioxidants such as coffee into protein; researchers expect that coffee could inhibit LOX products to a higher extent as it might interact with protein and aldehydes (Bekedam, 2008; Borrelli et al., 2002; Budryn & Nebesny, 2013; Lin et al., 2015).

The LOX products of interest in the current study include common lipid oxidative markers, often used in assessment of LOX in beef: MDA and saturated aldehyde compounds (C_5-C_9) (Lin et al., 2015; Pavan & Duckett, 2013). The higher level of MDA and aldehydes represents higher degree of LOX, which increase with days of storage (Jung et al., 2013; Lin et al., 2015; Lynch et al., 2001). Generally, MDA is determined colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) or using

HPLC, whereas aldehyde compounds are usually determined using gas chromatography /GC (Ganhão et al., 2011; Gianelli et al., 2005; Pérez-Juan et al., 2006).

Determination of malonaldehyde. Malonaldehyde (MDA) is a three-carbon dialdehyde with carbonyl groups at the C-1 and C-3 positions, formed through the decomposition of linolenic acids hydroperoxides in beef (Fernández et al., 1997). In the TBARS assay, MDA is reacted with thiobarbituric acids (TBA), resulting in the formation of a pink color of MDA-TBA adduct (Figure 3) that can be measured at absorbance 532 nm. The amount of MDA in the sample is usually calculated against the standard curve of MDA made from acid hydrolysis of 1,1,3,3 tetramethoxypropane (TMP) or 1,1,3,3 tetraethoxypropane (TEP). Malonaldehyde is not a stable compound in nature; however, it can be prepared by acid hydrolysis of TMP or TEP (Fernández et al., 1997).

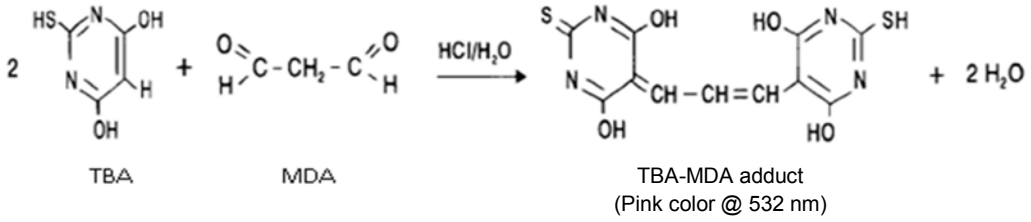


Figure 3 Reaction of thiobarbituric acid with malonaldehyde (adapted from Fernández et al., 1997)

Although TBARS have been widely used to determine LOX in various meat studies, it lacks specificity and is less accurate than chromatography assays (Tug, Karatas, Terzi, & Ozdemir, 2004). However, performing chromatography techniques are more expensive and require specific equipment. Thus, TBARS is still preferable in an assessment of MDA. The lack of specificity of TBARS occurs because other carbonyl containing compounds such as methylglyoxal in coffee may react with TBA and form the

pink color similar to MDA-TBA adduct and interfere TBARS measurement (Ganhão et al., 2011; IARC, 1991). Moreover, amino acids or proteins, if present in the sample, can also bind to MDA and decrease TBARS value (Kwon, Menzel, & Olcott, 1965). The additional steps such as filtration and centrifugation could eliminate the interference from protein to obtain more accurate TBARS value (Siu & Draper, 1978).

Determination of saturated aldehydes. Saturated aldehydes C_3-C_{10} are a result of the oxidation of unsaturated fatty acids in beef. Hexanal (C_6) is an abundant saturated aldehyde produced from the oxidation of linoleic and arachidonic acids which is used as a common marker of LOX in beef (Lin et al., 2015; Pavan & Duckett, 2013). Other aldehydes produced from beef oxidation include propanal, pentanal, octanal, nonanal, MDA, and (E)-2,4-epoxy-(E)-2-decenal, derived from the oxidation of linolenic and oleic acids (Kim et al., 2013; Lin et al., 2015; Lynch et al., 2001; Pavan & Duckett, 2013).

Gas chromatography is used to detect the volatile compounds in the sample (Lin et al., 2015; Pérez-Juan et al., 2006; Teets & Were, 2008). The sample is heated to release the volatile compounds and interact with the stationary phase when injecting to GC machine. Each compound has different partition coefficient that is separated depending on its volatility and solubility. The result is shown as chromatogram peak of each compound. The peak area of each compound is calculated against internal response factor (IRF) and internal standard (IS) (Equation 1 and 2).

$$\text{Response factor} = \frac{\text{conc. of volatile standard}}{\text{peak area of volatile standard}} \times \frac{\text{peak area of IS}}{\text{conc. of IS}} \quad (1)$$

$$\text{Conc. of interested volatile} = \frac{\text{conc. of IS x peak area of volatile x IRF}}{\text{peak area of IS}} \quad (2)$$

Determination of the Interaction of Coffee on Specific Protein Components in Beef

The current study determined the binding behavior between coffee and aromatic amino acids in Mb beef protein components to elucidate the antioxidant mechanism of coffee when added to beef. The methods used included fluorescence, absorption spectra, and docking simulation.

Fluorescence quenching. Tryptophan and tyrosine are aromatic amino acids that absorb high energy at excitation wavelength ~280 nm and emit the stored energy at ~308-355 nm. The efficiency of molecule to emit the energy is also defined by the quantum yield (Möller & Denicola, 2002; Vivian & Callis, 2001). Fluorescence quenching is a process which decreases the intensity of the fluorescence emission by activity of the quencher to lower quantum yield of protein. Coffee compounds such as caffeine and chlorogenic acid can act as quenchers perturbing aromatic nature and decrease in fluorophore, indicating the interaction of compounds with protein aromatic groups (Galinato, Fogle, & Galan, 2013; Wang et al., 2009; J. Zhou et al., 2007).

Myoglobin absorption spectra. The interaction between coffee and Mb can also be determined using spectroscopic technique. The change in absorption intensity at specific wavelength by coffee effect indicated the interaction between coffee and specific protein components. Different protein components exhibit different peak at specific UV/vis wavelengths. Prominent absorption peaks of protein include the peak at 280 nm that indicates the presence of aromatic amino acids (mainly Trp and Tyr). The peak at around 409 nm (soret peak) indicates the absorption of heme iron, specifically FerrylMb (Rao et al., 1994). The peak of Qv and Q band at 450-600 nm region indicate the presence of oxygenated myoglobin which are also be used to calculate formation of

MetMb (Galinato et al., 2013; J. Tang et al., 2004).

Docking Simulation. The docking simulation is a computational method that predicts the molecular orientation of specific complexes. The docking can reveal the specific binding sites and the binding affinity between protein and ligands (Lengauer & Rarey, 1996). The current study uses docking to explain the binding orientation and affinity between beef proteins, aldehydes and antioxidant components in coffee.

Beef Mb contains 153 amino acids of which 13 are His, 2 are Trp and 2 are Tyr. The His 81, 88, and 93 are the main binding sites of interest, as these sites contain b- and y- ion series configuration playing a key role in the quaternary state change upon ligand binding via Michael additions (Suman et al., 2007; Wang et al., 2009). Other His binding sites of interest include His 64 and 97. The His 64 is the distal His, the one which hydrogen bonds to oxygen while His 97 is located at the entrance of the hole were the heme group sits (Alderton, Faustman, Liebler, & Hill, 2003; Suman et al., 2007). Antioxidant compounds of interest in the docking included coffee CGA and CA, as docking could not be done with coffee MRPs since its molecular structure remains unknown at this point.

Thiol Oxidation as an Indicator of Protein Oxidation

Analysis of thiol content determines the degree of POX. The thiol group (-SH) in meat is mainly present within the structure of cysteine. Thiol oxidation leads to the formation of disulfide (RSSR) that consequently forms the amide linkage with protein, promoting protein cross-linking. Thiol oxidation in beef mainly occurs via the interaction with oxygen or redox transition of Mb. Romero et al. (1992) found that the cysteine oxidation is coupled with the Mb oxidation with higher rate constant ($2.19 \pm 0.28 \times 10^5$)

than other thiol forms e.g., glutathione, ergothioneine ($1.09 \pm 0.17 \times 10^4$ and 1.64 ± 0.33 10^5).

Many studies determine protein thiol to assess the effect of antioxidant compound in preventing thiol oxidation (Eymard, Baron, & Jacobsen, 2009; Jongberg et al., 2013). Coffee, likewise, is expected to prevent the loss of thiol and delay the deterioration of protein in beef. The assessment of protein thiol often depends on the reaction of Ellman's reagent (5,5'-Dithiobis-(2-Nitrobenzoic Acid)/DTNB) with thiol groups to yield a yellow-colored adduct of 5-thio-2-nitrobenzoate (TNB) that can be measured absorbance at 412 nm (Figure 4; Peng et al., 2012).

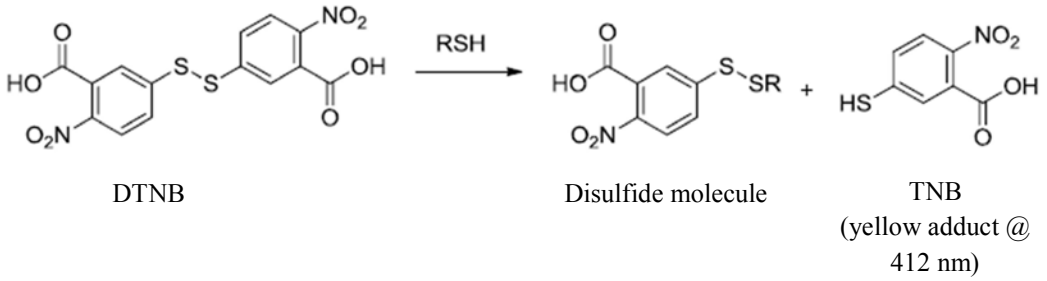


Figure 4 The reaction of Ellman's reagent and thiol group (adapted from Peng et al., 2012)

Rationale and Significance

Antioxidant mechanism of roasted coffee when added to beef is attributed to metal and radical scavenging (Borrelli et al., 2002; Delgado-Andrade & Morales, 2005; Lin et al., 2015; Vignoli et al., 2011). Beside these mechanisms, coffee is hypothesized to interact with beef proteins and inhibit oxidation (Lin et al., 2015; Pérez-Juan et al., 2006; Suman et al., 2007; Wang et al., 2009). Therefore, the objective of the current study was to investigate the interaction effect of coffee and proteins on oxidation inhibition in beef (Figure 5).

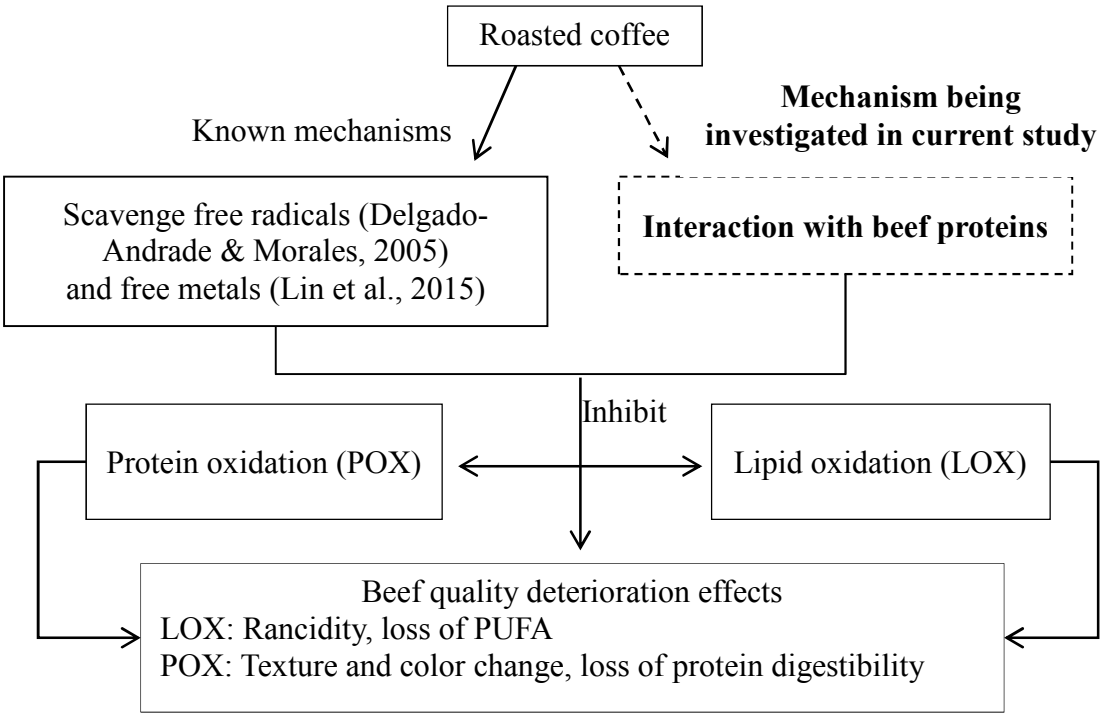


Figure 5 Known and investigating mechanism of roasted coffee to inhibit oxidation in beef

CHAPTER III

METHODOLOGY

Chemical Reagents

Sodium phosphate monobasic was purchased from Spectrum Chemical (Gardena, CA). Sodium phosphate dibasic anhydrous, HPLC water, methanol HPLC grade, bovine albumin serum (BSA), biuret reagent and trichloroacetic acid (TCA) were purchased from Fisher Scientific (Tustin, CA). Potassium iodide was purchased from Cynmar Corporation (Carlinville, IL). Sodium azide, thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP) and GC standards (4-heptanone, pentanal, hexanal, heptanal, octanal, and nonanal) were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction of Sarcoplasmic and Myofibrillar Proteins

Skinned top round beef muscle from both sides of three carcasses was obtained from American beef packers (Chino, CA, USA) 24 h post-slaughter. Animal weights were 496.7 – 594.2 kg at slaughter. After slaughter, carcasses were kept frozen for 1 day. Beef were cut into one-inch cubes and ground through a 3 mm fine grinding plate attached to a Kitchen Aid Professional 5-quart food processor (St Joseph, MI, USA). Ground beef from three carcasses were hand-mixed for 5 min.

Sarcoplasmic and myofibrillar proteins were extracted according to Pérez-Juan et al. (2006) with slight modifications. Ground top round beef was vortexed (1:5 w:v) for 1 min with 30 mM sodium phosphate pH 7.4 buffer, containing 0.02% NaN_3. The mixture was centrifuged at 1,882 g^{-1} for 15 min at 4˚C using accuSpin^{TM} 1R (Fisher Scientific, Pittsburg, PA, USA). The supernatant containing SP was filtered through eight layers of cheesecloth. The remaining precipitate was vortexed (1:6 w:v) with 100 mM sodium

phosphate pH 7.4 buffer, containing 0.02% NaN_3 and 0.7 M KI for 1 min. The mixture was centrifuged at 1,882 g^{-1} for 15 min at 4˚C using accuSpin™ 1R. The supernatant containing MP was filtered through eight layers of cheesecloth. The protein concentration in solution was 0.013 g/ml for SP and 0.015 g/ml for MP determined using the Biuret method. Sarcoplasmic and myofibrillar protein homogenates were divided into 3 replicates for coffee treatments preparation.

Preparation of Protein-Coffee Treatments

Dark roasted Brazil 2/3 coffee beans produced by roasting green coffee beans at 242°C for 9.52 min were obtained from Gaviña Gourmet Coffee Company (Vernon, CA, USA). The whole coffee beans were ground for 1 min using a Waring Commercial WSG30 Spice Grinder (Stamford, CT, USA) and passed through a 0.841 mm sieve. Coffee was brewed in HPLC water (1:5.75 w:v) at 90˚C for 5 min according to Budryn and Nebesny (2013). Then, coffee was filtered and lyophilized under room temperature using Dura-Dry mP manifold lyophilizer (FTS Systems, model #FD2085C0000, Stone Ridge, NY). Lyophilized coffee was dissolved in DDW (0.02 g/mL) and added to SP and MP homogenates in an amount of 0, 20, 40, and 80 µL per mL protein. Each coffee concentration was prepared in triplicates for SP and MP. The final concentration of four protein-coffee treatments were 0 (control/no coffee), 40, 80, and 160 µg coffee/mL protein (or approximately 0 – 0.12 g/100g protein). The concentration used was based on preliminary data (shown in Chapter IV) and Lin et al. (2015) that used 0.1g coffee/100 g beef. Each protein-coffee treatment was examined for LOX and POX inhibition on day 1, 3, 5, 7, and 9. The experimental design is outlined in Figure 6.

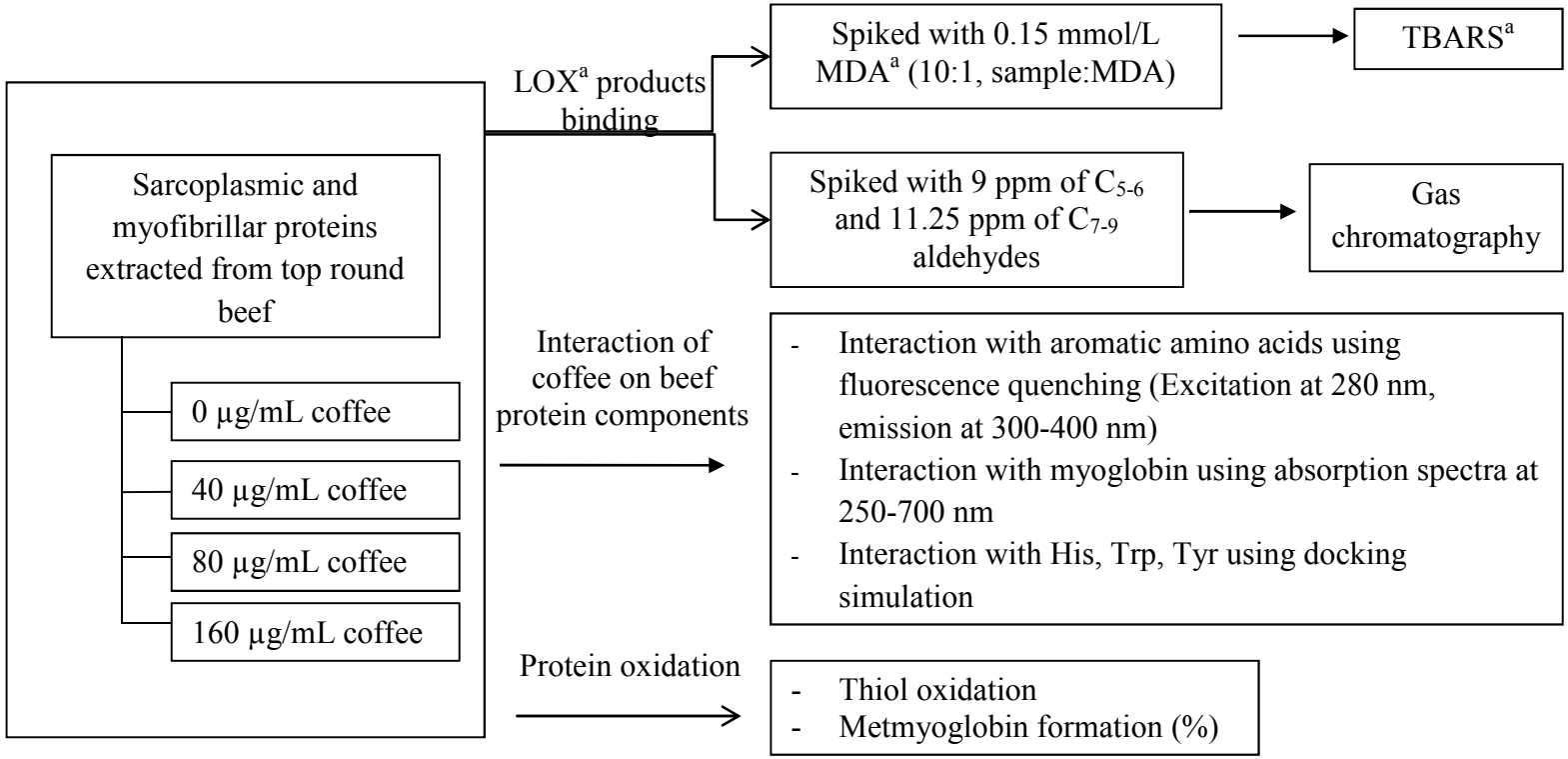


Figure 6 Proteins-coffee treatments for lipid and protein oxidation analyses
[a] LOX – lipid oxidation, MDA – malonaldehyde, TBARS – thiobarbituric acid reactive substances

Effect of Bovine Proteins and Coffee on Malonaldehyde Binding

Thiobarbituric acid reactive substances assay was performed according to Miller (1998) with modifications. Malonaldehyde was prepared by acid hydrolyzing 164 µl of TMP in 10 g TCA/100 ml DDW at 70˚C for 15 min. Serial dilution was performed to obtain a final concentration of 0.15 mmol/L of MDA. The MDA was spiked into protein-coffee samples (1:10 v:v) and incubated at 4˚C. TBARS was performed to determine free MDA in each treatment. Samples (0.6 mL) were mixed with 0.75 mL of 10g TCA/100 mL in microcentrifuge tubes to precipitate the protein and centrifuged using accuSpinTM micro???? at 8,000 g^{-1} for 5 min. The supernatants were obtained and mixed with 0.02 mol/L of TBA (1:1 v:v). All samples were incubated at 60˚C for 90 min. Sample absorbances were measured at 532 nm using a FLUOstar Omega Microplate Reader (Cary, NC, USA). Bound MDA (mg/g) protein was calculated using Equation 3.

$$\text{Bound MDA (mg/g protein)} = \frac{\frac{[\text{MDA}]_B - [\text{MDA}]_{SP} + [\text{MDA}]_{UN} + [\text{MDA}]_{CF}}{[\text{MDA}]_B} \text{ x I}}{\text{Protein conc. (g/mL)} \; x \; 1000 \text{ (mL)}} \quad (3)$$

; where $[\text{MDA}]_B$ refers to concentration of MDA in spiked buffer sample (no protein) (mg/L), $[\text{MDA}]_{SP}$ was the MDA concentration in spiked protein sample (mg/L), $[\text{MDA}]_C$ (mg/L) refers to MDA concentration in unspiked protein sample (mg/L), $[\text{MDA}]_{CF}$ refers to MDA concentration unspiked buffer sample with corresponding concentration of coffee (mg/L) (to eliminate interference at 532 nm from coffee pigment), I refers to initial concentration of MDA added.

Effect of Bovine Proteins and Coffee on Aldehyde Binding

Standard aldehydes (pentanal/C_5, hexanal/C_6, heptanal/C_7, octanal/C_8, nonanal/C_9,

and 4-heptanone) were prepared by diluting each aldehyde (0.1 mL) in 10 mL methanol. Then, serial dilution was performed in DDW to obtain stock solution of 250 mg/L of C_5 and C_6, 200 mg/L of C_7-C_9, and 2 mg/L of 4-heptanone (internal standard). A standard aldehyde cocktail mix was prepared by mixing 20 mL of C_5-C_9. Final concentration of each aldehyde was 40 mg/L of C_5 and C_6 and 50 mg/L of C_7-C_9.

Only proteins treated with 0, 40, and 160 µg/mL coffee were tested for aldehyde binding. The SP-coffee were mixed with 4 mg/L of C_5 and C_6, 6 mg/L of C_7-C_9, while samples of MP-coffee were mixed with 2 mg/L of all aldehyde compounds prior to incubation at 4˚C. The concentration of aldehydes in SP samples was increased to 9 mg/L for C_5 and C_6 and 11.25 mg/L for C_7-C_9 on day 3 since all aldehydes were bound by SP and were bdl (discussed in Chapter IV). Aldehyde concentration after incubation was quantified using gas chromatography (GC). The condition used for GC was as outlined in Teets & Were (2008) with slight modifications.

Protein-coffee samples (2.375 mL) were mixed with 0.125 mL of 4-heptanone which served as an internal standard. Samples were purged at 70˚C for 15 min, trapped at 75 ˚C using a Tenax trap (Trap #7, 0.125" O.D. x 0.105" I.D.) and desorbed for 1 min at 220 ˚C using a purge and trap (OI Analytical 4560, College Station, TX). An 8:1 split inlet was used to inject volatiles into a HP-1 fused silica capillary column, 30 m x 0.32 mm x 0.25 l m film thickness (Agilent Technologies, Inc. Santa Clara, CA) equipped with a flame ionizing detector (FID) using ramped oven temperature conditions (30˚C for 2 min, increased to 40 ˚C at 2˚C/min, increased to 50 ˚C at 5 ˚C/min, increased to 100 ˚C at 10 ˚C/min, increased to 140 ˚C at 20˚C/min, increased to 200˚C at 30 ˚C/min, and held for 4.5 min). Helium was used as the carrier gas and column flow was 2.2 mL/min. The

area of each peak was integrated using ChemStation software (Agilent Technologies, Inc. Santa Clara, CA). Each sample was analyzed in duplicate. Aldehyde concentration was expressed in mg/L after determining the internal response factor based on the ratio between internal standard and each respective standard aldehyde. Bound aldehyde per g protein was calculated using Equation 2.

$$\text{Bound aldehyde (mg/g protein)} = \frac{\frac{[A]_B - [A]_{SP} + [A]_{UN} + [A]_{CF}}{[A]_B} \text{ x I}}{\text{Protein conc. (g/mL) x 1000 (mL)}} \quad (4)$$

; where $[A]_B$ refers to aldehyde concentration in spiked buffer sample (no protein) (mg/L), $[A]_{SP}$ refers to aldehyde concentration in spiked protein sample (mg/L), $[A]_C$ (mg/L) refers to aldehyde concentration in protein sample unspiked (mg/L), $[A]_{CF}$ refers to aldehyde concentration in coffee (mg/L), I refers to initial concentration of aldehyde added.

Determination of Interaction of Proteins and Coffee

Fluorescence Quenching

Tryptophan fluorescence of each protein-coffee treatment was measured on days 1, 3, 5, 7, and 9 using a Fluoromax-4 spectrofluorometer (Horiba Scientific, Edison, NJ, USA). Samples were diluted with DDW (1:9, v:v). The emission spectra of tryptophan were recorded from 300 to 400 nm with the excitation wavelength set at 280 nm.

Metmyoglobin Absorption Spectra and Formation

The interaction of coffee on Mb was tested by scanning SP-coffee samples through 250 - 700 nm using a FLUOstar Omega Microplate Reader (Cary, NC, USA). The absorption at wavelength 503, 525, 557, and 582 was also used to determine percent metmyoglobin (MetMb) formation calculated using following equation according to J.

Tang et al. (2004).

$$\text{MetMb (\%)} = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$$

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$ and $R_3 = A_{503}/A_{525}$

Docking Simulation Studies

Beef myoglobin structure was developed using HARLEM (Molecular Modeling Package). A PDB file (1Z2H) of apoprotein of beef myoglobin was attained (http://www.ebi.ac.uk/pdbsum/1Z2H) and saved as an HLM file. The heme group from beef hemoglobin (1G09) (http://www.rcsb.org/pdb/explore.do?structureId=1g09) was isolated using HARLEM and attached to the proximal His of Mb structure. A solvated and an unsolvated 100,000 step minimization calculation were conducted on the molecule in order to attain an energetically stable and accurate structure. The minimized beef Mb was then desolvated and stripped of its non-polar hydrogen.

Docking simulation was conducted to test binding affinity of coffee phenolics (CA and CGA) on entire exterior surface of Mb, aromatic amino acids (Trp-7, 14 and Tyr-103, 146), and potential binding His sites (His-81, 88, 93) according to Suman et al. (2007). In addition, docking with His 64 and 97 was also determined as His 64 is the distal His, the one which hydrogen bonds to oxygen while His 97 is located at the entrance of the hole were the heme group sits (Alderton et al., 2003; Suman et al., 2007). Additionally, the binding affinity of MDA and C_5-C_9 aldehydes at the same binding locations on Mb was also determined.

The coordinates of the search space were visualized using the Python Molecular Viewer from Autodock Tools (version 1.5.6). The binding affinity was determined using Autodock Vina (version 1.1.2). The entirety of the molecular surface was considered by

constructing a parallelepiped search space of dimensions 42, 45, and 40 angstroms and docking with an exhaustiveness of 200. Particular amino acids of Trp and His were docked by limiting the search space to a cube with vertices of 15 angstroms and docking with an exhaustiveness of 40.

Protein Thiol Oxidation

Thiol oxidation was determined according to Eymard et al. (2009) with slight modifications. Proteins-coffee samples (1 mL) were mixed with 14.3 µl of 0.01 mol/l of 5, 5'-Dithiobis-(2-Nitrobenzoic Acid)/DTNB in 0.05 mol/l of sodium acetate and incubated at 40˚C for 15 min. The sample absorbance was measured at 412 nm using FLUOstar Omega Microplate Reader (Carry, CA, USA). Thiol content was expressed in µmol/g protein, calculated using a molar extinction coefficient of 13,600 $(mol/l)^{-1}$ cm^{-1}.

Statistical analysis

The current study determined the effect of four concentrations of dark lyophilized coffee (0, 40, 80, and 160 µg/ml protein) and two beef proteins (SP and MP) on beef oxidation's inhibition on day 1, 3, 5, 7, and 9. Analysis of Variance (ANOVA) and Tukey's Multiple Comparison Test were conducted to identify the difference in means between coffee concentrations and proteins in inhibiting MDA, saturated aldehydes (C_5-C_9), MetMb formation, and thiol oxidation at significant level 0.05 using R Studio (version 0.98.1074). Linear mixed-effects models with interactions between coffee concentration and day were developed for MDA and saturated aldehydes.

CHAPTER IV

RESULTS

Chapter IV presents the results and statistical analyses of the data collected in this study. The present study was designed to determine whether coffee could inhibit LOX and POX in beef when combining with beef proteins. There were two experiments conducted, including preliminary and actual study. The preliminary study was conducted to determine experimental conditions to be used, and the results were used to adjust conditions in actual study. The actual study determined the interaction effect of protein-coffee on MDA and aldehydes binding, tryptophan fluorescence quenching, Mb absorption spectra, MetMb formation, and thiol oxidation. Additionally, molecular docking explained binding behavior between protein and coffee.

Preliminary Study: Effect of Proteins-Coffee on Lipid Oxidation

The preliminary study was conducted starting on April 5th, 2104 to determine experimental conditions of TBARS, GC, and Trp florescence assays on day 1, 4, and 7. The preliminary study used chuck beef steak purchased from Ralph's supermarket (Orange, CA). The SP and MP were extracted and treated with coffee as outlined in Chapter III.

Figure 7 presents TBARS results of each protein-coffee treatment on day 1, 4, 7. The lower absorbance values indicated the higher extent of MDA bound by protein-coffee effect. Sarcoplasmic protein treated with coffee (all concentrations) significantly lowered TBARS value compared to SP control (p<0.05), in which the higher coffee concentrations resulted in lower TBARS value. On the other hand, coffee did not affect

TBARS value of MP solutions. Since the coffee concentrations used in this study exhibited significant effect on MDA binding in SP, these concentrations thus were used for all experiments in this study.

(a) Sarcoplasmic protein (b) Myofibrillar Protein

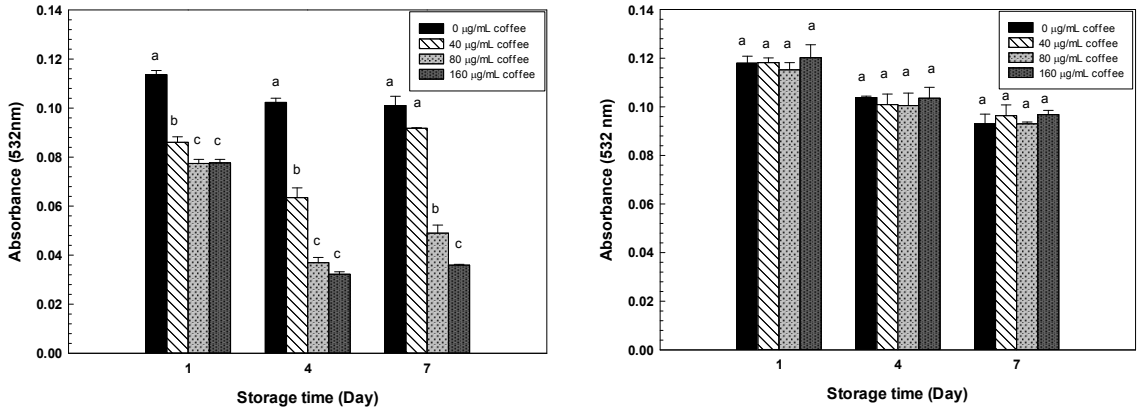


Figure 7 Absorbance of (a) sarcoplasmic and (b) myofibrillar protein treated with 0 - 160 µg/mL of dark lyophilized coffee at 4˚C over 7 days storage determined using thiobarbituric acids reactive substance assay. Means on each day with the same letter above the bar are not significantly different ($p < 0.05$).

Figure 8 shows chromatogram of SP and MP spiked with 1 mg/L of C_5-C_9 aldehydes, treated with and without coffee on day 4. The lower the chromatogram peak area, the higher the aldehyde bound by proteins-coffee effect. The results showed that coffee lowered chromatogram peaks of all aldehydes in SP, while coffee had no effect when interacting with MP. On day 7, all aldehydes were below detection limit (all were bound by proteins), thus the concentration of spiked aldehydes was increased in actual study from 1 to 4 mg/L for C_5 and C_6 and 6 mg/L for C_7-C_9 in SP samples and to 2 mg/L of all aldehydes in MP samples.

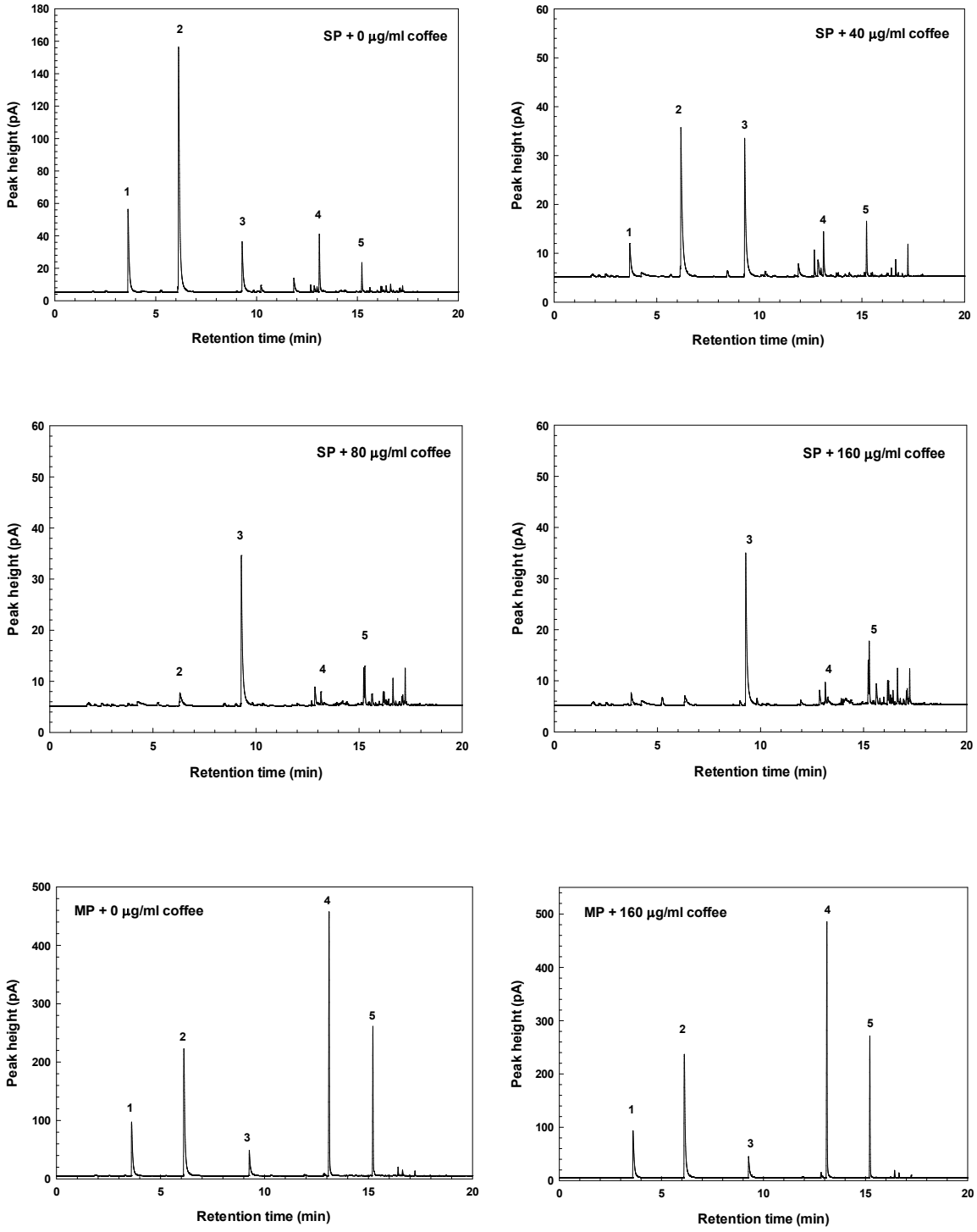


Figure 8 Chromatogram of sarcoplasmic (SP) and myofibrillar (MP) spiked with 1 mg/L of aldehydes treated with different coffee concentrations. Quantified peaks include (1) pentanal, (2) hexanal, (3) 4-heptanone [internal standard], (4) octanal, (5) nonanal.

Preliminary results suggested that coffee inhibited MDA and aldehydes by interacting with SP not MP. Fluorescence quenching study was conducted further to confirm this interaction by testing the effect of coffee as a quencher of SP and MP aromatic amino acids residue (Trp and Tyr) (Figure 9). The fluorophore in both SP and MP decreased with coffee concentrations, and coffee quenched fluorescence to a higher extent when interacting with SP compared to MP. The result indicated that coffee mainly interacted with aromatic groups in beef SP rather than MP.

In this study, fluorescence was also used to quantify the formation of protein carbonyls (indicator of POX) or Schiff base product induced by the reaction between radicals or LOX products and amino acids. The fluorescence of protein-lipid carbonyl adducts was measured at excitation and emission wavelength 350 and 400-500 nm, respectively (Estévez et al., 2008; Viljanen, Kylli, Kivikari, & Heinonen, 2004). Unfortunately, this technique was not successfully used since coffee contains carbonyl compounds (3-ethylcyclopentane-1,2 dione, furaneol, 2-acetylfuran and methygloxal) that largely interfered with fluorescence measurements (Figure 10; IARC, 1991). The analysis of thiol thus was used in the actual experiment as an alternative method to determine the effect of coffee on POX.

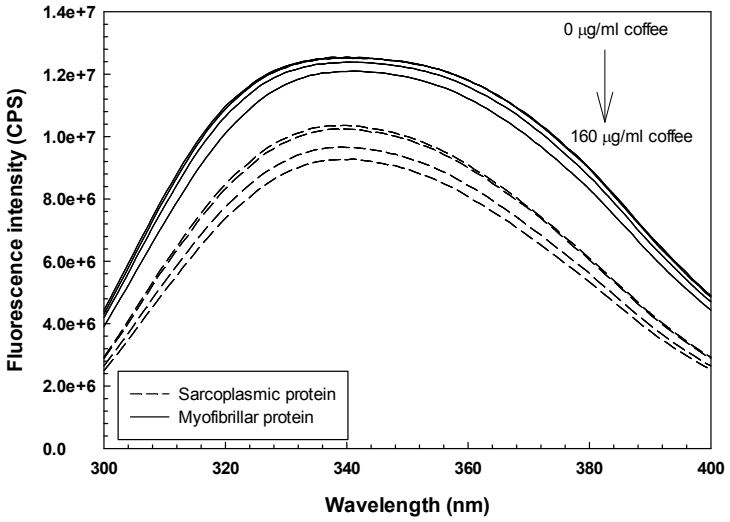


Figure 9 Fluorescence intensity of sarcoplasmic and myofibrillar protein treated with 0, 40, 80, and 160 μg/ml of dark lyophilized coffee on day 4 of refrigerated storage. ↓ indicates decrease of fluorophore with coffee concentration.

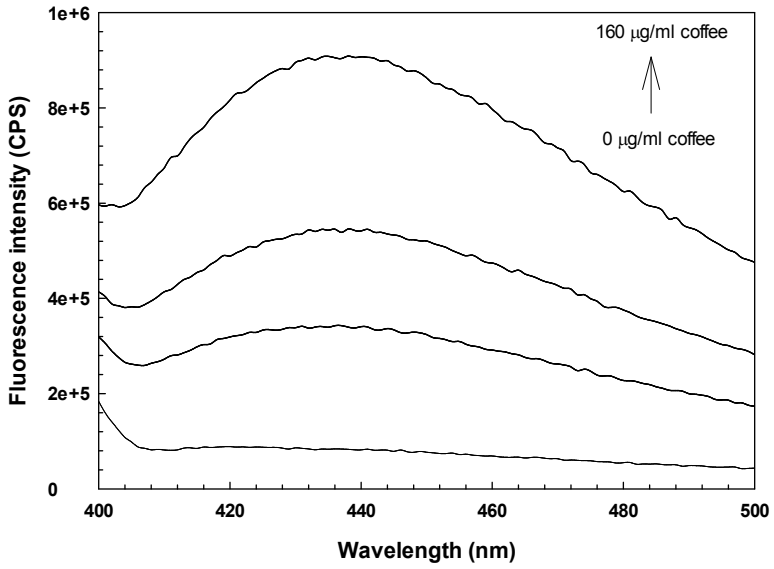


Figure 10 Fluorescence intensity of 0, 40, 80, and 160 μg/ml of dark lyophilized coffee in deionized water. ↑ indicates increase of fluorophore with coffee concentration.

The results from the preliminary study implied that coffee inhibited LOX products via the interaction with aromatic groups in beef SP. The actual study was conducted to determine the specific location in SP that coffee interacted with. The interaction of coffee with Mb was determined by scanning absorption spectra. Additionally, docking simulation was implemented to test binding affinity between coffee phenolics and Mb.

Another adjustment that was done in the actual experiment was the change in beef supply. In the preliminary study, chuck beef purchased from a local supermarket was used; however, its origin and storage conditions after slaughtered could not be tracked. Actual experiment thus obtained beef from a local abattoir as indicated in Chapter III. Moreover, since the current study focused on unsaturated lipids and protein components of beef, top round beef muscle was used in place of chuck muscle as top round muscle is high in unsaturated lipids that is highly susceptible to oxidation compared to other cuts (Pavan & Duckett, 2013; Roseland et al., 2013).

Effect of Beef Proteins and Coffee on Malonaldehyde

The ability of proteins-coffee to bind MDA expressed as bound MDA per g of protein is presented in Figure 11. In control samples (no coffee added), both SP and MP showed the same extent in MDA binding ability. In protein-coffee treatments, the interaction of coffee with SP significantly increased MDA binding compared to control, while addition of coffee with MP did not affect the MDA binding, similar to preliminary results. However, in this actual experiment, no significant binding effect was found between coffee concentrations in SP ($p>0.05$). In addition, significant two-way interactions between coffee concentration and day were also observed ($p<0.05$). Storage

day also affected the ability of coffee to bind MDA. When comparing day 1 and 9, bound MDA increased ~1.85 - 2.3 fold in SP control and in all MP samples, whereas increased ~3.0 fold in SP treated with coffee in all concentrations.

(a) Sarcoplasmic protein (b) Myofibrillar protein

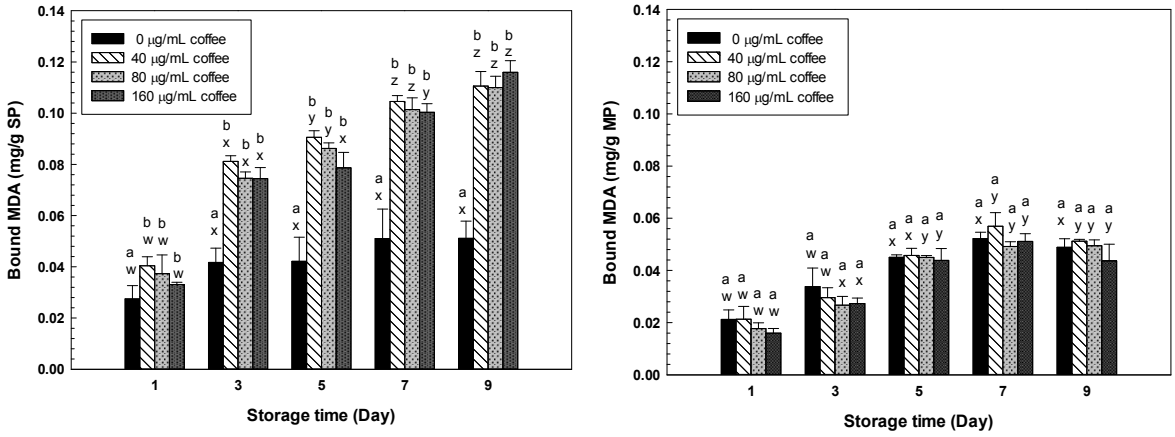


Figure 11 Bound MDA (mg/g protein) by (a) sarcoplasmic (SP) and (b) myofibrillar (MP) protein treated with different concentrations of dark lyophilized coffee at 4˚C over 9 days of storage. a,b denotes the significant difference between coffee concentrations within day (p<0.05). x-z denotes the significant difference between day with the same coffee concentration (p<0.05).

Effect of Beef Proteins and Coffee on Aldehydes Binding

The results of bound aldehydes by proteins-coffee are presented in Figure 12. The binding increased with aldehyde carbon-chain length (C9>C8>C7>C6>C5) in both SP and MP. Sarcoplasmic protein bound to aldehydes at >10-fold higher than MP. In fact, all aldehydes were bound by SP on day 3. As a result, higher aldehyde concentrations were added into SP samples in order to continue the observation beyond day 3. The final concentration of aldehyde added was 9 mg/L for C_5 and C_6 and 11.25 mg/L for C_7-C_9.

Surprisingly, coffee had little effect on aldehyde binding either combining with SP or MP, which contradicted the preliminary results, where coffee significantly

increased ability to bind aldehydes in SP. The significant increase in binding was only found with medium chain-length aldehyde (octanal and nonanal) on day 9 when SP was treated with coffee, whereas no obvious effect of coffee was found in MP. Storage time and protein types had greater effect on binding than added coffee. Figure 13 shows the main effect that impacted binding of nonanal. The same effect was also found for other aldehydes (data not shown). The binding significantly increased with storage time for all aldehydes except pentanal and hexanal in MP samples that remained constant since day 1. However, storage time still had larger effects than coffee in these samples.

The ability of coffee without proteins to bind aldehydes was also observed. Table 3 shows the ability of coffee in 30 mmol/L sodium phosphate buffer on free aldehyde inhibition calculated against 1 mg/L of C_5-C_9 of standard aldehydes. The results indicated that the ability of coffee to lower free aldehyde increased with aldehyde chain length. In other word, coffee showed a tendency to bind longer chain-length of aldehyde to a greater extent than shorter chain-length aldehydes.

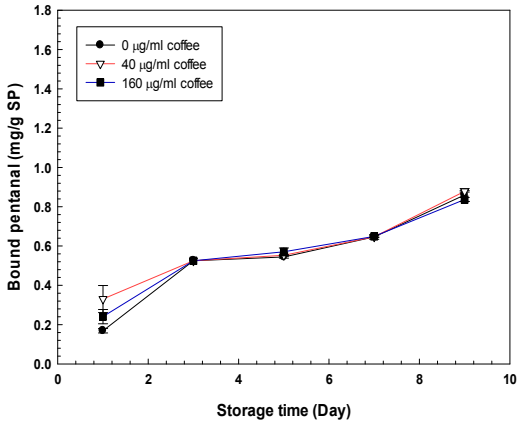
In summary, coffee and proteins preferably bound to longer chain-length rather than shorter chain-length aldehydes. Coffee increased the binding of octanal and nonanal when interacting with SP. However, storage time and protein types still had the larger effect than coffee treatments on the binding ability.

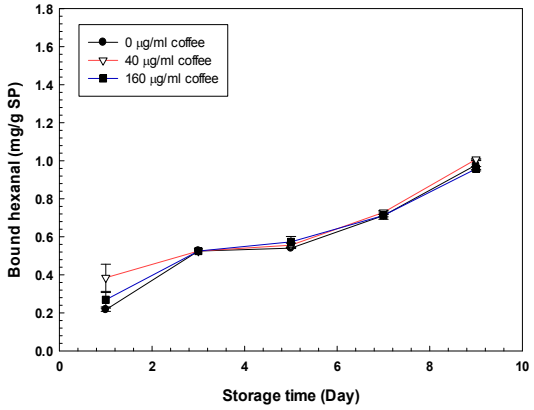
Table 3 Free aldehyde (%) in 30 mmol/L of sodium phosphate buffer pH 7.4 treated with 40 and 160 µg/ml coffee after incubation with 1 mg/L of C_5-C_9 aldehydes on day 1, 5, and 9.

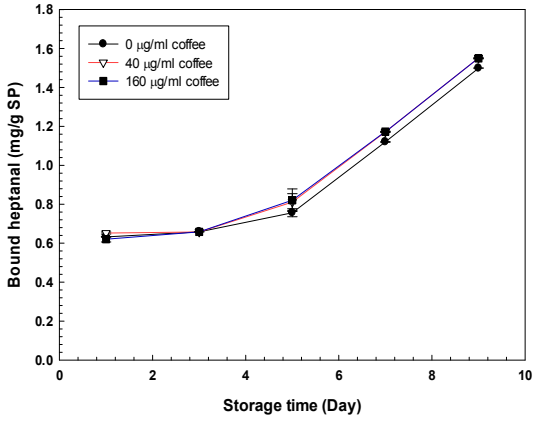
	Day 1		Day 5		Day 9	
Aldehyde	40 µg/ml	160 µg/ml	40 µg/ml	160 µg/ml	40 µg/ml	160 µg/ml
Pentanal	107.7 ± 5.9^{a}	100.7 ± 0.8^{a}	108.9 ± 3.2^{a}	105.9 ± 2.4^{a}	107.4 ± 5.1^{a}	97.2 ± 1.9^{a}
Hexanal	109.2 ± 5.0^{a}	103.3 ± 0.7^{a}	104.9 ± 1.8^{ab}	101.9 ± 2.0^{ab}	97.8 ± 2.3^{ab}	89.0 ± 1.5^{b}
Heptanal	108.1 ± 0.1^{a}	107.4 ± 0.8^{b}	85.4 ± 4.8^{b}	97.8 ± 1.8^{b}	82.3 ± 6.5^{b}	80.4 ± 0.2^{c}
Octanal	100.6 ± 9.1^{a}	109.0 ± 0.8^{b}	61.1 ± 1.4^{c}	84.6 ± 1.1^{c}	63.2 ± 4.3^{c}	60.9 ± 1.0^{d}
Nonanal	97.4 ± 5.0^{a}	106.9 ± 1.2^{b}	31.6 ± 4.5^{d}	63.0 ± 0.6^{d}	37.8 ± 6.1^{d}	37.2 ± 0.7^{e}

Data are represented as mean ± SD (each sample was analyzed in duplicate). Values in the same column with the same letter are not significantly different ($p < 0.05$).

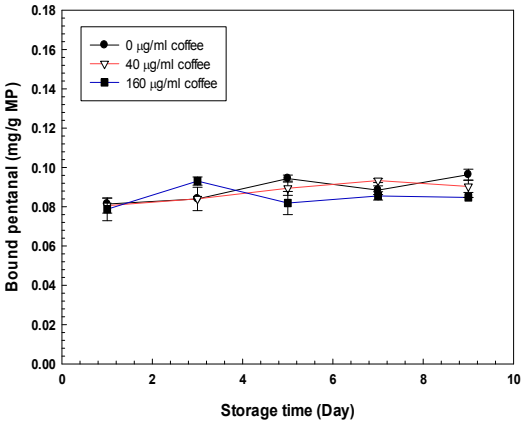
Sarcoplasmic protein

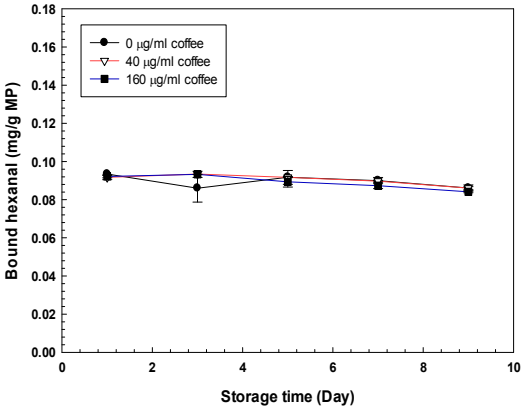


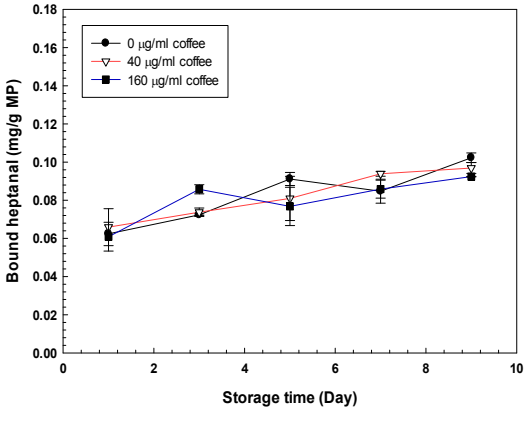




Myofibrillar protein







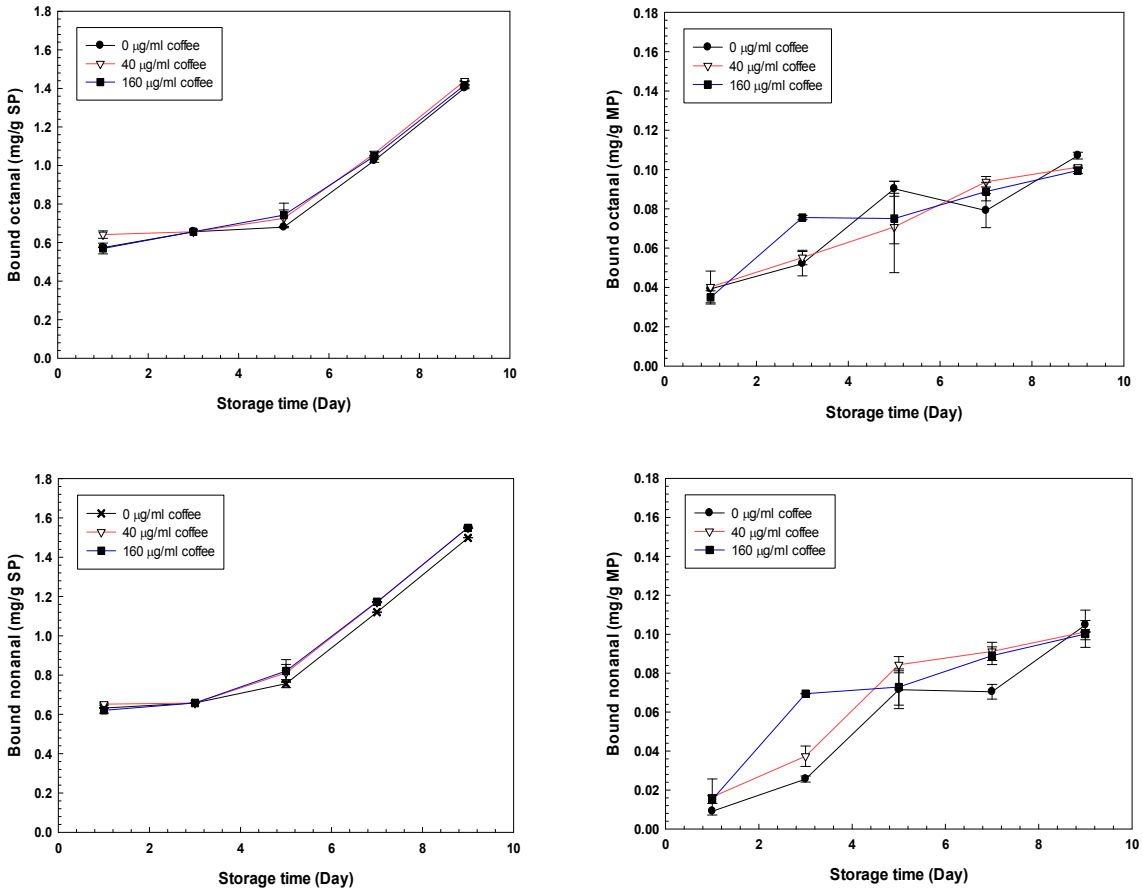


Figure 12 Bound aldehyde compounds (mg/g protein) by sarcoplasmic protein/SP (left column) and myofibrillar protein/MP (right column) treated with 0, 40, and 160 μg/mL of dark lyophilized coffee at 4˚C over 9 days storage.

(a) Sarcoplasmic protein (b) Myofibrillar protein

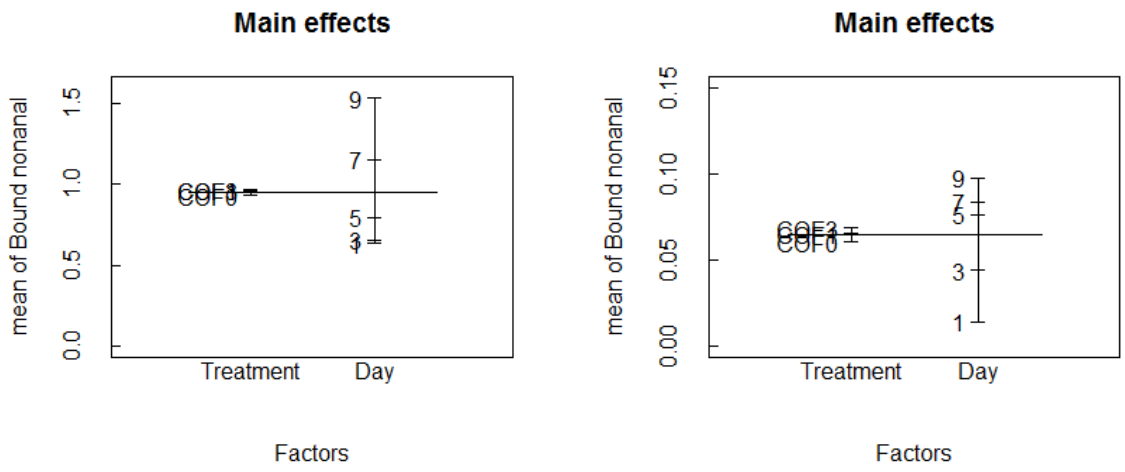


Figure 13 Factors affecting bound nonanal (mg/g protein) in (a) sarcoplasmic protein (b) myofibrillar protein. Treatment included COF0, COF1, COF3 (0, 40, 160 µg/ml protein, respectively).

Metmyoglobin Absorption Spectra and Formation

The UV/vis absorption scans of SP treated with coffee is presented in Figure 14. The difference in absorption intensity compared to control indicated the interaction of coffee with SP. The peak between 250-300 nm could not be detected since the peak of 30 mM sodium phosphate buffer overshadowed the protein peak in this region, resulting in no difference between buffer and protein peak. Thus, the interaction of coffee on aromatic amino acids was only quantified using fluorescence. The prominent peak change was found between 300-380 nm in which the protein band intensity was hypochromic shifted with coffee concentration. However, the difference on absorption in this region between coffee treatments and control was lesser with storage time. The interaction of coffee with Mb pocket was determined at soret peak (418 nm). Coffee caused significant hypochromic shift of soret peak on day 3, but no significant effect was

observed afterwards.

The hyperchromic shift of soret peak in all samples was observed on later day of storage on all treatments. The increase in soret peak denoted FerrylMb formation, influenced by the promotion of H_2O_2 formed due to the decrease of catalase enzyme during storage time and the presence of sodium azide in buffer (Pradhan et al., 2000; Rao et al. 1994). The increase in peak at 500-600 nm region denoted MetMb formation (Rao et al. 1994; Tang et al., 2004) which was also used to calculate MetMb formation as indicated in Chapter III. The %MetMb formation of SP-coffee treatments on day 1-9 is shown in Figure 15. The MetMb was lower in coffee treated samples compared to control on day 1-3. Nonetheless, %MetMb between control and treatments was not different after day 5.

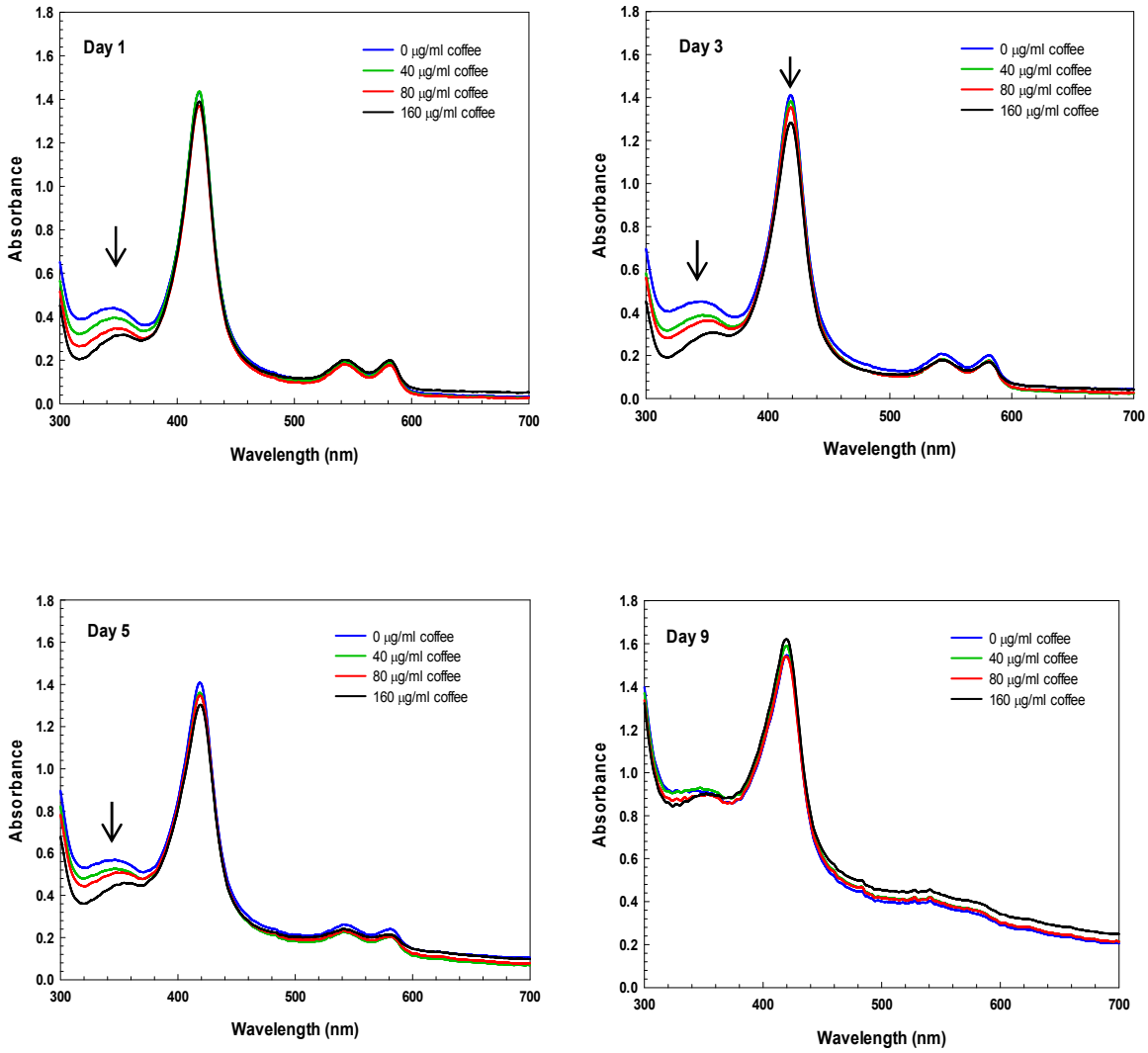


Figure 14 Absorption spectra of sarcoplasmic protein treated with 0- 160 µg/mL of dark lyophilized coffee at 4˚C over 9 days storage. ↓ indicated decreased absorption spectra with coffee concentration.

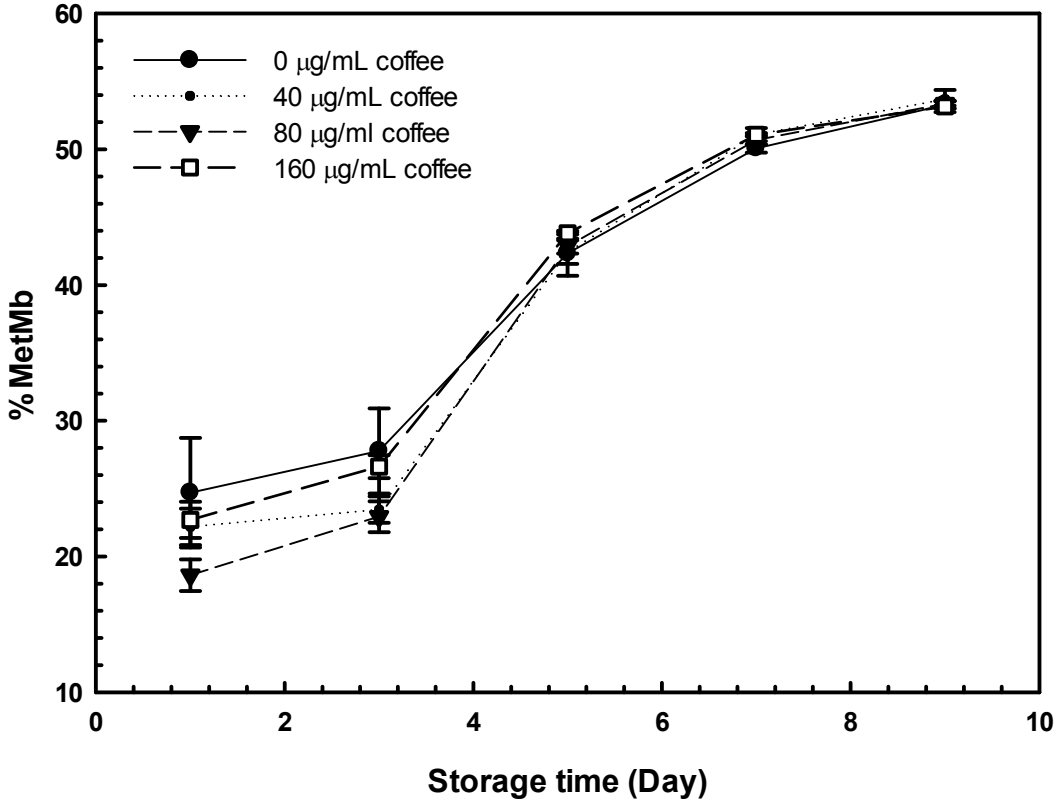


Figure 15 Metmyoglobin formation (%) of sarcoplasmic protein treated with 0-160 μg/mL of dark lyophilized coffee over 9 days at 4˚C storage

Tryptophan Fluorescence Quenching

The effect of coffee fluorescence quencher of SP and MP aromatic amino acids residue (Trp and Tyr) on day 9 is shown in Figure 16. Coffee quenched Trp fluorophore to a higher extent when interacting with SP compared to MP. The SP fluorophore decreased with coffee concentration was observed from day 3 through 9, while MP fluorophore remained constant. This result confirmed that coffee mainly interacted with SP in beef muscle.

(a) Sarcoplasmic protein (b) Myofibrillar protein

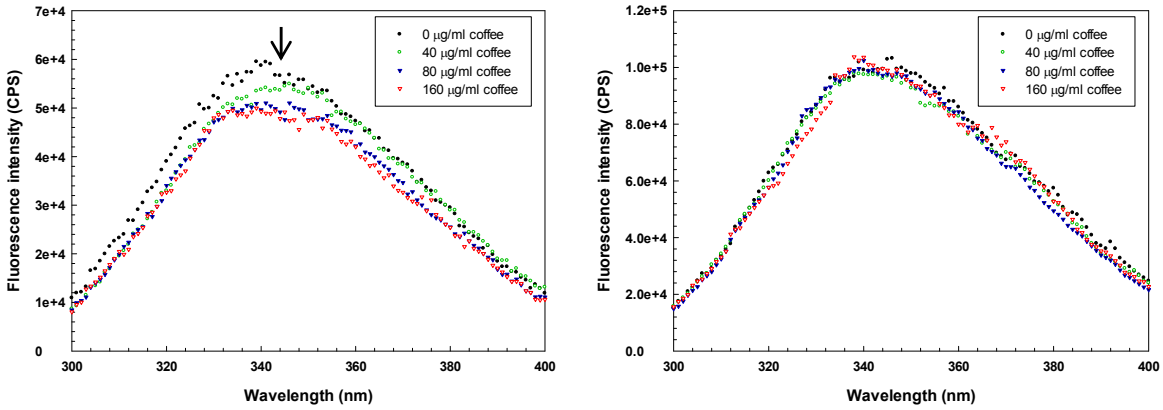


Figure 16 Tryptophan fluorescence of (a) sarcoplasmic and (b) myofibrillar proteins treated with 0 - 160 μg/mL of dark lyophilized coffee after 9 days of refrigerated (4˚C) storage. ↓ indicates decrease of fluorophore with coffee concentration.

Docking Simulation Studies

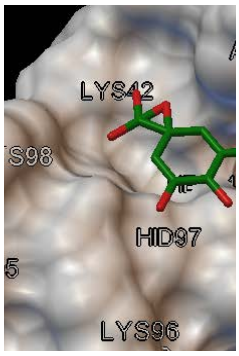
Coffee inhibited aldehydes (MDA and saturated aldehydes) through the interaction with beef SP, mainly on the surface of Mb. Docking was conducted to determine binding affinity between coffee phenolics (CA and CGA) and specific binding sites on Mb as indicated in Chapter II.

The docking simulation of CA and CGA on the surface of Mb is shown in Figure 17. The lowest binding affinity of CA and CGA was found at Leu 76 and His 97, respectively. The binding affinity of coffee phenolics on His and aromatic amino acids is also shown in Table 4. The lowest binding affinity was found at His 64, His 97 and Tyr 103 (< -5.0 kcal/mol) while the highest binding affinity was found at His 93 (> -4.0 kcal/mol). In addition, the binding of CGA with aromatic amino acids and His mostly resulted in lower binding energy than CA except on His 93 and Tyr 146 at which binding energy of CGA was higher than - 1.2 kcal/mol.

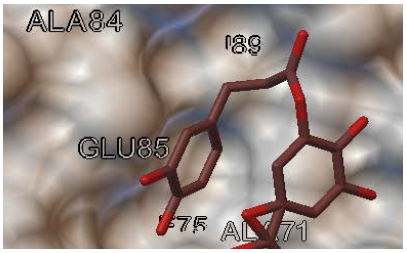
CA (binding affinity = - 6.7 kcal/mol)

CA (binding affinity = - 5.6 kcal/mol)

CGA (binding affinity = - 6.5 kcal/mol)



CGA (binding affinity = - 6.4 kcal/mol)



CGA (binding affinity = - 6.2 kcal/mol)

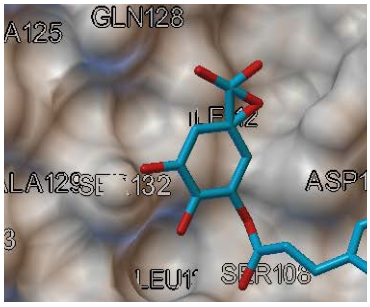


Figure 17 Docking simulation and binding affinity (kcal/mol) of caffeic acid (CA) or chlorogenic acid (CGA) on surface of beef myoglobin.

Table 4 Binding affinity (kcal/mol) of caffeic acid and chlorogenic acid on histidine and aromatic amino acids of beef myoglobin

	Binding affinity (kcal/mol)	
Binding site	Caffeic acid	Chlorogenic acid
Histidine		
His 64	- 5.4	- 5.9
His 81	- 4.2	- 5.3
His 88	- 4.1	- 4.8
His 93	- 3.9	- 0.2
His 97	- 5.3	- 5.0
Aromatic amino acids		
Trp 7	- 4.2	- 4.7
Trp 14	- 3.9	- 4.3
Tyr 103	- 4.8	- 5.8
Tyr 146	- 4.6	- 1.2

Table 5 Binding affinity (kcal/mol) of aldehydes on histidine and aromatic amino acids of beef myoglobin

	Binding affinity (kcal/mol)					
Binding site	Malonaldehyde	Pentanal	Hexanal	Heptanal	Octanal	Nonanal
Histidine						
His 64	- 2.5	- 3.2	- 3.4	- 3.7	- 4.1	- 4.3
His 81	- 2.1	- 2.3	- 2.5	- 2.6	- 2.6	- 2.7
His 88	- 2.1	- 2.3	- 2.5	- 2.7	- 2.9	- 2.9
His 93	- 2.3	- 2.4	- 2.6	- 2.5	- 2.5	- 2.2
His 97	- 2.4	- 2.6	- 2.9	- 3.1	- 3.2	- 3.2
Aromatic amino acids						
Trp 7	- 2.5	- 2.4	- 2.4	- 2.7	- 2.6	- 2.7
Trp 14	- 1.9	- 2.2	- 2.4	- 2.5	- 2.6	- 2.6
Tyr 103	- 2.3	- 2.6	- 2.8	- 2.9	- 3.1	- 2.8
Tyr 146	- 2.5	- 3.0	- 3.1	- 3.2	- 3.4	- 3.5

The docking was also conducted to test binding affinity between aldehydes and Mb on the same locations done with coffee phenolics. Aldehydes could adduct to all determined locations on His and aromatic amino acids. The lowest binding affinity was found at His 64. The binding affinity was in increasing order: nonanal<octanal<heptanal< hexanal<pentanal<MDA for all binding sites except His 93 and Tyr 103 that nonanal had higher binding affinity than heptanal and octanal (Table 5).

Protein Thiol Oxidation

The effect of coffee on thiol oxidation is shown in Figure 18. The lower thiol content indicated higher thiol oxidation. Coffee caused thiol oxidation in both SP and MP samples but the effect was not significant (p>0.05). During 9 days of storage, thiol content significantly decreased in SP (p<0.05) while remained stable in MP. In addition, the gradual decrease of SP thiol was negatively correlated (-0.87) with MetMb formation across all days of analysis, indicating that as thiol content decreased, MetMb formation increased.

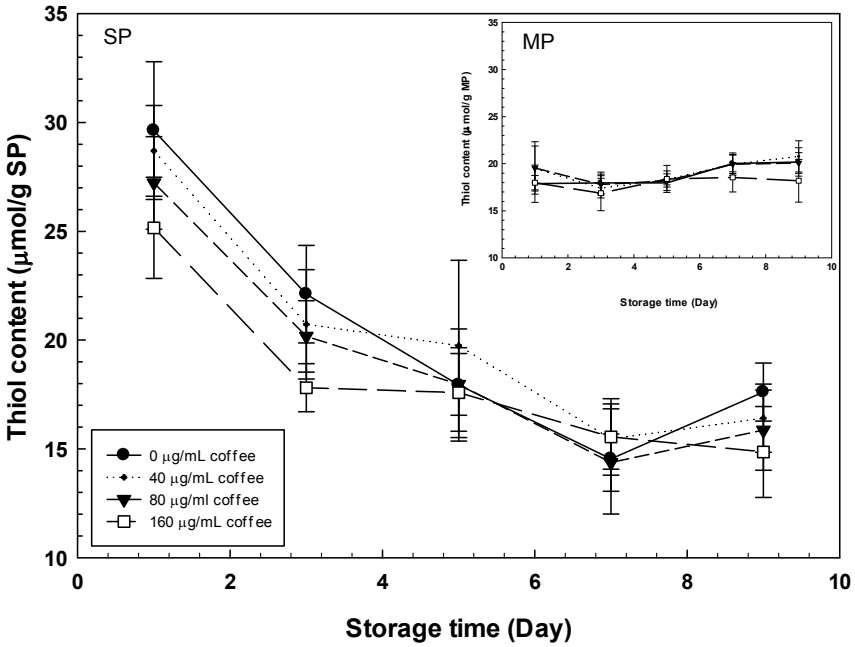


Figure 18 Thiol content (µmol/g protein) of sarcoplasmic protein (SP) treated with 0 - 160 µg/mL of dark lyophilized coffee at 4˚C over 9 days storage. Thiol content in myofibrillar protein (MP) treated with same coffee concentrations is shown in the inset.

CHAPTER V

DISCUSSION

The present study was designed to examine the interaction between coffee and proteins on oxidation inhibition in top round beef muscle. The interaction effect of coffee and proteins on LOX was determined by monitoring ability of protein-coffee to bind MDA and saturated aldehydes using TBARS and GC analysis. The binding locations between protein and coffee were determined via fluorescence, absorption spectra, and docking simulation. Lastly, the loss of thiol and MetMb formation was assessed to reveal the effect of coffee on POX.

Binding Effect of Beef Proteins and Coffee on Malonaldehyde and Aldehydes

Both SP and MP beef proteins bound MDA and saturated aldehydes (C_5-C_9) in which the binding increased with likelihood of hydrophobic conformation of aldehydes. The result is also supported by Chopin et al. (2007) who found that the increase in the hydrophobic chain-length of aldehydes increased the affinity for myosin to bind, mainly due to the globular protein existed in the core structure is hydrophobic nature.

. Beef SP showed higher ability to bind aldehydes than MP similar to the findings in pork muscle (Pérez-Juan et al., 2006). The higher ability of SP to bind aldehydes than MP are possibly due to b- and y- ion series configuration favored for Michael additions (Alderton et al., 2003; Suman et al., 2007). Lipid oxidation products are bound at His, Tyr, and Arg in myosin MP, while bound at His (especially His 81, 88, and 93) in sarcoplasmic Mb, the fifth coordination site occupying in the imidazole ring (Buttkus, 1967; Suman et al., 2007; Tironi et al., 2004; Yin et al., 2011).

Coffee significantly increased binding ability of SP to inhibit MDA and medium-

chain length of aldehydes (octanal and nonanal), but had no effect on MP. This result implied that coffee mainly interacted with SP and increased ability of SP on LOX products binding. The higher interaction of coffee with SP than MP could be due to higher of hydrophilic affinity of SP than MP that possibly interacted with hydrophilic patches of coffee melanoidins (Bekedam, 2008; Gianelli et al., 2005; Morzel, et al., 2006; Pérez-Juan et al., 2006; Phillips & Pettitt, 1995). Furthermore, the ability of coffee to bind aldehydes also increased with the likelihood of aldehydes hydrophobic conformation. This result indicated the possibility of hydrophobic interaction between coffee and aldehydes.

However, effect of coffee on aldehydes binding in the actual study was lower than the preliminary results as protein and storage time had greater effect than coffee. The discrepancy could be due to the difference of time when coffee was added to beef. The current study added coffee to fresh beef (within 3 days after slaughter) while in preliminary study, coffee was added to beef purchased from local supermarket that might be significantly oxidized (10-14 days after slaughter as the time for inspection is required) (USDA, 2014). The fresh proteins may contain high auto-antioxidative properties due to the catalase enzyme, dipeptide carnosine, sulfhydryl groups presented in non-oxidized proteins, inhibiting oxidation in beef and limiting the effectiveness of coffee (Gianelli et al., 2005; Pradhan et al., 2000; Tong et al., 2000). This result suggested that coffee might not necessary be effective in beef immediately after slaughter since proteins still have their own defense mechanism to limit oxidation. After beef started to lose the defense properties, coffee may inhibit oxidation in beef (Lin et al., 2015).

Interaction of Coffee with Beef Proteins

According to aldehydes binding results, coffee increased ability of SP to bind MDA and medium chain-length aldehydes. Fluorescence, spectroscopic technique, and docking simulation were conducted to investigate the specific locations that coffee interacted on SP.

Interaction of Coffee with Aromatic Amino Acids using Fluorescence

The decrease in fluorophore when coffee was added to beef proteins indicated the interaction between coffee with aromatic protein groups (mainly Trp and Tyr) (Möller & Denicola, 2002). The lower fluorophore in SP-coffee samples than MP-coffee samples denoted a higher interaction of coffee with beef SP than MP. This result corresponded to LOX products binding results in which coffee mainly interacted with SP not MP.

Interaction of Coffee with Myoglobin using Absorption Spectra

Since Mb is a major component in SP, Mb absorption spectra was measured to determine the location on Mb that coffee interacted with. The decreased absorption in 300-380 nm region with coffee concentrations indicated the interaction of coffee and Mb at ground-state or exterior surface of Mb, forming prosthetic groups (complex of heme with non-polypeptide via weak interaction, perturbing the absorption spectra (Antoine & Dugourd, 2013; Galinato et al., 2013). In soret region, the significant hypochromic shift by coffee was observed on day 3; however, the significant shift was not observed afterwards. The decrease in soret peak indicated some interaction between coffee and interior Mb structure without deforming the structure Mb structure since the peak was not bathochromic shifted (Antoine & Dugourd 2013).

The interior and exterior of Mb are well-distinguished by hydrophobic and hydrophilic nature, respectively (Phillips & Pettitt, 1995). The change of absorption at 300-380 nm thus indicated the interaction of coffee with Mb via hydrophilic bond. Compounds in coffee that could interact with Mb via hydrophilic bond could possibly be Maillard reaction products since it might contain partly of hydrophilic sections in its structure (Bekedam, 2008). However, the structure of melanoidins is not yet elucidated and since higher melanoidins concentration compared to phenolics are contained in dark coffee, more research needed to be done.

The compounds in coffee that interact with interior structure of Mb (hydrophobic bond) could be CGA, CA, and caffeine (Kroll et al., 2000; Wang et al., 2009). Kroll et al. (2000) found that CGA and CA were the main cause of the loss in hydrophilic environment of Mb (less solubility). Therefore, the less interaction between coffee and Mb in 300-380 nm region observed on later days of storage could be due to the coffee phenolics weakening the hydrophilic interaction (Estévez et al., 2008; Grunwald & Richards, 2006; Kroll et al., 2000; Phillips & Pettitt, 1995).

The results implied that coffee mainly interacted with Mb at exterior surface rather than interior surface, specifically via hydrophilic bond. Docking simulation was conducted further to determine the binding affinity between coffee and Mb.

Docking Simulation

Low binding affinity indicated less intermolecular force required for ligand-receptor binding while high binding affinity refers to the opposite. The binding affinity between Mb and coffee phenolics was negative, indicating the possibility of these molecules to interact with each other. Tthe binding affinity of Mb and coffee phenolics is

relatively intermediate (-0.2 to -6.7 kcal/mol) compared to the binding affinity of enzyme-ligand system (-2.6 to -13.4 kcal/mol) (Böhm & Klebe.1996; Galinato et al., 2013*).* The lowest binding affinity was found when coffee phenolics bound to the surface of Mb, suggesting that coffee phenolics mainly interacted on Mb surface as expected according to absorption results.

Chlorogenic acid required lower binding energy than CA when attached to His and aromatic amino acids located on the surface of Mb, while CA required lower energy when attached to amino acids at interior structure such as His 93 which is a proximal His attached to the heme group that is located in the interior structure (Suman et al., 2007). The higher ability of CA to bind at interior structure of Mb than CGA could be due to the lower molecular weight of CA (M.W. of CA = 180.16 g/mol, CGA = 354.31 g/mol). The smaller molecule could move into the interior structure of Mb easier than the larger CGA molecule. However, no studies directly determined the relationship between the size of ligand and binding position on Mb. More research needs to be done on this topic. Since the size of ligand could impact the binding energy, melanoidins, the high molecular weight compounds, thus was also hypothesized to bind to the surface of Mb rather than the interior structure (Perrone et al., 2012). Possibly, hydrophilic with negative charged groups in melanoidins can form hydrophilic bond with Mb surface (Bekedam, 2008; Phillips & Pettitt, 1995). Nevertheless, docking between melanoidins and Mb needs to be done to confirm this hypothesis once the structure of coffee melanoidins is elucidated.

Docking results of Mb-aldehydes also agreed with GC results in which the binding energy between protein and aldehydes decreased with hydrophobic nature of aldehyde compounds, demonstrating the hydrophobic interaction between Mb and

aldehydes. However, at His 93 and Tyr 103, nonanal needed higher energy to bind with these amino acids than other aldehydes. This result denoted that the hydrophobic interaction between aldehydes and protein might possibly be also dependent on the binding location and structure of protein favored for Michael addition (Elias et al., 2008; Suman et al., 2007). More research is needed to conclude the hydrophobic interaction of aldehydes on each specific location in protein.

Coffee, likewise, showed the hydrophobic interaction with aldehydes as binding ability increased with aldehyde chain-length as well as interacted with exterior surface of Mb which is hydrophilic nature (Phillips & Pettitt, 1995). The results implied that coffee interacted to SP at Mb surface via hydrophilic interaction and increased ability of SP to inhibit aldehydes by binding with aldehydes via hydrophobic bond.

Effect of Coffee on Thiol Oxidation and Metmyoglobin Formation

The coffee concentration used in the current study (0 – 160 μg/mL protein or ~0 – 0.12 g/g protein) was based on Lin et al. (2015) who found that added coffee 0.1 g/ 100 g beef could effectively inhibit LOX. Within this concentration, coffee neither prevented nor triggered thiol and Mb oxidation. Hence, coffee is amongst the antioxidants that could inhibit LOX while not increasing POX, unlike what has been found in green tea and rosemary that increased thiol oxidation in pork (Jongberg et al., 2013). Thiol oxidation in meat caused by green tea and rosemary was mainly induced by quinone in their phenolic compounds. However, phenolic compounds (found in CGA and CA) are not predominant antioxidant in dark roasted coffee since they are degraded with roasting degree (Perrone et al., 2012). Nonetheless, POX may be greater if light- or medium-roasted coffee or higher concentration of coffee was used since they contain higher

phenolic acids content than dark roast (Jongberg et al., 2013; Perrone et al., 2012).

Thiol content decreased gradually in SP samples while remained constant in MP during 9 days of refrigerated storage. The result suggested that thiol oxidation in beef muscle is mainly in the SP fraction. The loss of protein thiol in SP was primarily induced by the redox transition of Mb as negatively correlated (-0.87) between thiol content and MetMb formation was observed. This result was also supported by Romero et al. (1992) who indicated that free radical products produced from thiol and Mb oxidation pathways could reciprocally transfer and trigger the oxidation between each other.

Limitations

This thesis addressed antioxidant mechanism of coffee on oxidation inhibition through interaction with beef SP and MP; however, certain limitations to the study exist.

- The antioxidant compositions of coffee were not directly determined in this study but drawn from conclusion of existing studies. The compositions may vary depending on types of coffee and roasting degree.
- The structure of MRPs in coffee is not yet elucidated, thus the binding behavior between Mb and MRPs could not be determined in the current study. The focus in this study was on the low molecular phenolic compounds which are minor constituents of dark compared to light and medium roasted coffee.
- Gas chromatography required approximately 45 min analyzing time per sample. Thus, only coffee concentrations 0, 40, and 160 μg/ml could be analyzed in duplicate.
- Protein carbonyl formation could not be determined by fluorescence technique since coffee contains high level of carbonyl compounds from 3-

ethylcyclopentane-1,2 dione, furaneol, 2-acetylfuran and methygloxal (IARC, 1991).

Implications

The study findings could elucidate how coffee interacted with proteins in beef and if interaction could be an antioxidant mechanism in limiting oxidation in beef. The understanding of the mechanism adds to the knowledge for future research that attempts to apply coffee as a natural antioxidant to inhibit oxidation in beef and possibly other meats.

Conclusions

Antioxidant mechanism of roasted coffee when added to top round beef muscle could also be attributed to the interaction with beef SP that binds to free MDA and saturated aldehydes. Coffee mainly interacts with exterior structure of Mb in SP via hydrophilic interaction and increases ability of SP to bind aldehydes via hydrophobic interaction. The main binding locations of coffee on Mb based on docking simulation are at Leu 76, His 64, His 97, and Tyr 103.

Roasted coffee did not cause the loss of thiol content or MetMb formation in beef when used within the concentration 0 – 160 μg coffee/ml protein. Coffee, as a natural antioxidant source, is thus recommended to use to inhibit oxidation in beef as it lowers LOX while does not trigger POX.

Recommendations for Future Research

Recommended future work includes examining the interaction of proteins with other roasting degree of coffee or other physical forms of coffee (e.g., ground, spent) as they contain different antioxidant compositions. The findings will increase the

understanding on how different antioxidant compositions affect the interaction of how it influences LOX and POX.

The docking should be done between Mb and melanoidins once the coffee melanoidins' structure is elucidated. This is because melanoidins are predominant antioxidant compounds found in dark roasted coffee (Perrone et al., 2012). The docking between Mb and melanoidins may explain the binding behavior between protein and coffee in more detail than what was determined in present study.

The antioxidant mechanism of coffee should be examined in other meats that are susceptible to oxidation e.g., fish, pork, poultry. The antioxidant mechanism of coffee in different meats could vary due to the differences in lipid and protein composition and structure.

Protein was found to have a greater effect than coffee on saturated aldehydes binding in the current study. Future research should store beef several days under refrigerated storage after slaughtered prior to use in the experiment in order to avoid the effect from auto-antioxidative properties from meat proteins and to be better mimic supermarket conditions (Estévez et al., 2008; Gianelli et al., 2005; Lynch et al., 2001; Pérez-Juan et al., 2006; A. Romero et al., 2005; S. Zhou & Decker, 1999). This way, significant effect of coffee to lower aldehydes when interacting with beef proteins, could be observed (Lin et al., 2015).

REFERENCES

Alderton, A. L., Faustman, C., Liebler, D. C., & Hill, D. W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry, 42*(15), 4398-4405. doi: 10.1021/bi0271695

Antoine, R., & Dugourd, P. (2013). UV-visible absorption spectroscopy of protein ions. In S. Nielsen & J. A. Wyer (Eds.), *Photophysics of ionic biochromophores* (pp. pp. 141-153). New York, NY: Springer.

Balentine, C. W., Crandall, P. G., O'Bryan, C. A., Duong, D. Q., & Pohlman, F. W. (2006). The Pre- and post-grinding application of rosemary and its effects on lipid oxidation and color during storage of ground beef. *Meat Science, 73*(3), 413-421. doi: 10.1016/j.meatsci.2005.12.003

Bekedam, E., Koen. (2008). *Coffee brew melanoidins. structural and functional properties of brown-colored coffee compounds* (Doctoral dissertation). Wageningen University, Netherlands.

Böhm, H., & Klebe, G. (1996). What can we learn from molecular recognition in protein–ligand complexes for the design of new drugs?. *Angewandte Chemie International Edition in English*, *35*(22), 2588-2614.

Borrelli, R. C., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002). Chemical characterization and antioxidant properties of coffee melanoidins. *Journal of Agricultural and Food Chemistry, 50*(22), 6527-6533. doi: 10.1021/jf025686o

Budryn, G., & Nebesny, E. (2013). Effect of green and roasted coffee antioxidants on quality and shelf life of cookies and chocolates. *Journal of Food Processing and Preservation, 37*(5), 835-845. doi: 10.1111/j.1745-4549.2012.00710.x

Buttkus, H. (1967). The reaction of myosin with malonaldehyde. *Journal of Food Science, 32*(4), 432-434. doi: 10.1111/j.1365-2621.1967.tb09703.x

Chaijan, M. (2008). Review: lipid and myoglobin oxidations in muscle moods. *Songklanakarin Journal of Science and Technology, 30*, 47-53.

Chaijan, M., Benjakul, S., Visessanguan, W., Lee, S., & Faustman, C. (2007). The effect of freezing and aldehydes on the interaction between fish myoglobin and myofibrillar Proteins. *Journal of Agricultural and Food Chemistry, 55*(11), 4562-4568. doi: 10.1021/jf070065m

Chiang, W., Byrem, T., M, & Strasburg, G., M. (2007). Red meats. In Y. Hui, H (Ed.), *Food Chemistry: Principles and Applications* (2 ed.). CA: Science Technology System.

Chikuni, K., Muroya, S., & Nakajima, I. (2004). Myosin heavy chain isoforms expressed in bovine skeletal muscles. *Meat Science*, *67*(1), 87-94.

Chopin, C., Kone, M., & Serot, T. (2007). Study of the interaction of fish myosin with the products of lipid oxidation: the case of aldehydes. *Food Chemistry, 105*(1), 126-132. doi: 10.1016/j.foodchem.2007.03.058

D'Arcy, B., R. (2007). Analytical food chemistry. In Y. Hui, H (Ed.), *Food Chemistry: Principles and Applications* (2 ed.). CA: Science Technology System.

Davis, C., G, & Lin, B.-H. (2005). Factors affecting U.S. beef consumption. *Electronic Outlook Report from the Economic Research Service*: U. S. Department of Agriculture.

Delgado-Andrade, C., & Morales, F. J. (2005). Unraveling the contribution of melanoidins to the antioxidant activity of coffee brews. *Journal of Agricultural and Food Chemistry, 53*(5), 1403-1407. doi: 10.1021/jf048500p

Delmore, R., J. (2009). Beef shelf-life *Beef facts: Product enhancement research*. Centennial, CO: National Cattlemen's Beef Association.

Elias, R., J, Kellerby, S., S, & Decker, E., A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition, 48*(5), 430-441. doi: 10.1080/10408390701425615

Estévez, M. (2011). Protein carbonyls in meat systems: A Review. *Meat Science, 89*(3), 259-279. doi: 10.1016/j.meatsci.2011.04.025

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Oxidation of skeletal muscle myofibrillar proteins in oil-in-water emulsions: interaction with lipids and effect of selected phenolic compounds. *Journal of Agricultural and Food Chemistry, 56*(22), 10933-10940. doi: 10.1021/jf801784h

Eymard, S., Baron, C. P., & Jacobsen, C. (2009). Oxidation of lipid and protein in horse mackerel (*Trachurus Trachurus*) mince and washed minces during processing and storage. *Food Chemistry, 114*(1), 57-65. doi: 10.1016/j.foodchem.2008.09.030

Fernandez, C. L., Sturla, M. A., Doval, M. M., Romero, A. M., & Judis, M. A. (2012). Inhibition of lipid oxidation by MRP antioxidant in enriched cooked beef patties with polyunsaturated fatty acids. *Journal of Food Research, 1*(3), 128. doi: 10.5539/jfr.v1n3p128

Fernández, J., Pérez-Álvarez, J. A., & Fernández-López, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry, 59*(3), 345-353. doi: 10.1016/S0308-8146(96)00114-8

Frankel, E., N. (1980). Lipid oxidation. *Progress in Lipid Research, 19*, 1-22.

Galinato, M. G. I., Fogle, R. S., & Galan, J. F. (2013). Binding Interaction of Hypocrellin B to Myoglobin: A Spectroscopic and Computational Study. *Spectrochimica Acta Part A-molecular and Biomolecular Spectroscopy, 115*, 337-344. doi: 10.1016/j.saa.2013.06.052

Ganhão, R., Estévez, M., & Morcuende, D. (2011). Suitability of the TBA method for assessing lipid oxidation in a meat system with added phenolic-rich materials. *Food Chemistry, 126*(2), 772-778. doi: 10.1016/j.foodchem.2010.11.064

Gianelli, M. P., Flores, M., & Toldrá, F. (2005). Interaction of soluble peptides and proteins from skeletal muscle with volatile compounds in model systems as affected by curing agents. *Journal of Agricultural and Food Chemistry, 53*(5), 1670-1677. doi: 10.1021/jf040357c

Grunwald, E. W., & Richards, M. P. (2006). Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. *Journal of Agricultural and Food Chemistry, 54*(21), 8271-8280. doi: 10.1021/jf061231d

Hidalgo, F. J., & Zamora, R. (2004). Strecker-type degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. *Journal of Agricultural and Food Chemistry, 52*(23), 7126-7131. doi: 10.1021/jf048883r

Hu, M., McClements, D. J., & Decker, E. A. (2003). Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 51*(5), 1435-1439. doi: 10.1021/jf0203794

IARC. (1991). *IARC Monographs on the Evaluation of Carcinogenic Risks to Human* (Vol. 51). UK: International Agency for Research on Cancer.

Jongberg, S., Tørngren, M., Ann, Gunvig, A., Skibsted, L., H, & Lund, M., N. (2013). Effect of green tea or rosemary extract on protein oxidation in bologna type sausages prepared from oxidatively stressed pork. *Meat Science, 93*, 538-546.

Jung, S., Nam, K. C., Ahn, D. U., Kim, H. J., & Jo, C. (2013). Effect of phosvitin on lipid and protein oxidation in ground beef treated with high hydrostatic pressure. *Meat Science, 95*(1), 8-13. doi: 10.1016/j.meatsci.2013.04.005

Kim, H., Cadwallader, K. R., Kido, H., & Watanabe, Y. (2013). Effect of addition of commercial rosemary extracts on potent odorants in cooked Beef. *Meat Science, 94*(2), 170-176. doi: 10.1016/j.meatsci.2013.01.005

Kim, H., Choi, Y.-S., Choi, J.-H., Kim, H.-Y., Hwang, K.-E., Song, D.-H., . . . Kim, C.-J. (2013). Antioxidant effects of soy sauce on color stability and lipid oxidation of raw beef patties during cold storage. *Meat Science, 95*(3), 641-646. doi: 10.1016/j.meatsci.2013.06.006

Kroll, J., Rawel, H. M., & Seidelmann, N. (2000). Physicochemical properties and susceptibility to proteolytic digestion of myoglobin-phenol derivatives. *Journal of Agricultural and Food Chemistry, 48*(5), 1580-1587. doi: 10.1021/jf991172m

Kwon, T. W., Menzel, D. B., & Olcott, H. S. (1965). Reactivity of malonaldehyde with food constituents. *Journal of Food Science, 30*(5), 808-813. doi: 10.1111/j.1365-2621.1965.tb01845.x

Lee, S., Joo, S. T., Alderton, A. L., Hill, D. W., & Faustman, C. (2003). Oxymyoglobin and lipid oxidation in yellowfin tuna (*Thunnus Albacares*) Loins. *Journal of Food Science, 68*(5), 1664-1668. doi: 10.1111/j.1365-2621.2003.tb12310.x

Lee, S., Phillips, A. L., Liebler, D. C., & Faustman, C. (2003). Porcine oxymyoglobin and lipid oxidation in vitro. *Meat Science, 63*(2), 241-247. doi: 10.1016/S0309-1740(02)00076-1

Lengauer, T., & Rarey, M. (1996). Computational methods for biomolecular docking. *Current Opinion in Structural Biology, 6*(3), 402-406.

Lin, C., Toto, C., & Were, L. (2015). Antioxidant effectiveness of ground roasted coffee in raw ground top round beef with added sodium chloride. *LWT - Food Science and Technology, 60*(1), 29-35. doi: 10.1016/j.lwt.2014.08.010

Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: a review. *Molecular Nutrition & Food Research, 55*(1), 83-95. doi: 10.1002/mnfr.201000453

Lynch, M. P., & Faustman, C. (2000). Effect of aldehyde lipid oxidation products on myoglobin. *Journal of Agricultural and Food Chemistry, 48*(3), 600-604. doi: 10.1021/jf990732e

Lynch, M. P., Faustman, C., Silbart, L. K., Rood, D., & Furr, H. C. (2001). Detection of lipid-derived aldehydes and aldehyde: protein adducts *in vitro* and in beef.

Journal of Food Science, 66(8), 1093-1099. doi: 10.1111/j.1365-2621.2001.tb16087.x

Mestdagh, F., Kerkaert, B., Cucu, T., & De Meulenaer, B. (2011). Interaction between whey proteins and lipids during light-induced oxidation. *Food Chemistry, 126*(3), 1190-1197. doi: 10.1016/goodchem.2010.11.170

Miller, D., D. (1998). *Food Chemistry: A Laboratory Manual*. New York: John Wiley & Sons.

Min, B., & Ahn, D. (2005). Mechanism of lipid peroxidation in meat and meat products. *Food Science and Biotechnology, 14*(1), 152-163.

Miranda, L. T., Rakovski, C., & Were, L. M. (2012). Effect of maillard reaction products on oxidation products in ground chicken breast. *Meat Science, 90*(2), 352-360. doi: 10.1016/j.meatsci.2011.07.022

Morzel, M., Gatellier, P., Sayd, T., Renerre, M., & Laville, E. (2006). Chemical oxidation decreases proteolytic susceptibility of skeletal muscle myofibrillar proteins. *Meat Science, 73*(3), 536-543. doi: 10.1016/j.meatsci.2006.02.005

Möller, M., & Denicola, A. (2002). Protein tryptophan accessibility studied by fluorescence quenching. *Biochemistry and Molecular Biology Education, 30*(3), 175-178. doi: 10.1002/bmb.2002.494030030035

Nicoli, M. C., Anese, M., Manzocco, L., & Lerici, C. R. (1997). Antioxidant properties of coffee brews in relation to the roasting degree. *LWT - Food Science and Technology, 30*(3), 292-297. doi: 10.1006/fstl.1996.0181

Nissen, L. R., Byrne, D. V., Bertelsen, G., & Skibsted, L. H. (2004). The antioxidative activity of plant extracts in cooked pork patties as evaluated by descriptive

sensory profiling and chemical analysis. *Meat Science, 68*(3), 485-495. doi: 10.1016/j.meatsci.2004.05.004

Pavan, E., & Duckett, S. K. (2013). Fatty acid composition and interrelationships among eight retail cuts of grass-feed beef. *Meat Science, 93*(3), 371-377. doi: 10.1016/j.meatsci.2012.09.021

Peng, H., Chen, W., Cheng, Y., Hakuna, L., Strongin, R., & Wang, B. (2012). Thiol reactive probes and chemosensors. *Sensors (Basel, Switzerland), 12*(11), 15907-15946. doi: 10.3390/s121115907

Perrone, D., Farah, A., & Donangelo, C. M. (2012). Influence of coffee roasting on the incorporation of phenolic compounds into melanoidins and their relationship with antioxidant activity of the brew. *Journal of Agricultural and Food Chemistry, 60*(17), 4265-4275. doi: 10.1021/jf205388x

Phillips, J. G. N., & Pettitt, B. M. (1995). Structure and dynamics of the water around myoglobin. *Protein Science : A Publication of the Protein Society, 4*(2), 149-158. doi: 10.1002/pro.5560040202

Pérez-Juan, M., Flores, M., & Toldrá, F. (2006). Model studies on the efficacy of protein homogenates from raw pork muscle and dry-cured ham in binding selected flavor compounds. *Journal of Agricultural and Food Chemistry, 54*(13), 4802-4808. doi: 10.1021/jf060374x

Pérez-Juan, M., Flores, M., & Toldrá, F. (2007). Binding of aroma compounds by isolated myofibrillar proteins: effect of protein concentration and conformation. *Food Chemistry, 105*(3), 932-939. doi: 10.1016/j.foodchem.2007.04.051

Pérez-Juan, M., Flores, M., & Toldrá, F. (2008). Effect of pork meat proteins on the binding of volatile compounds. *Food Chemistry, 108*(4), 1226-1233. doi: 10.1016/j.foodchem.2007.04.037

Pradhan, A. , Rhee, K. , & Hernández, P. (2000). Stability of catalase and its potential role in lipid oxidation in meat. *Meat Science*, *54*(4), 385-390.

Ramanathan, R., Konda, M. K. R., Mancini, R. A., & Faustman, C. (2009). Species-specific effects of sarcoplasmic extracts on lipid oxidation in vitro. *Journal of Food Science, 74*(1), C73-C77. doi: 10.1111/j.1750-3841.2008.01021.x

Rao, S. I., Wilks, A., Hamberg, M., & Montellano, P. R. (1994). The lipoxygenase activity of myoglobin. Oxidation of linoleic acid by the ferryl oxygen rather than protein radical. *Journal of Biological Chemistry, 269*(10), 7210-7216.

Romero, A. M., Doval, M. M., Sturla, M. A., & Judis, M. A. (2005). Antioxidant behaviour of products resulting from beef sarcoplasmic proteins-malondialdehyde reaction. *European Journal of Lipid Science and Technology, 107*(12), 903-911. doi: 10.1002/ejlt.200501196

Romero, F. J., Ordonez, I., Arduini, A., & Cadenas, E. (1992). The reactivity of thiols and disulfides with different redox states of myoglobin. Redox and addition reactions and formation of thiyl radical intermediates. *Journal of Biological Chemistry, 267*(3), 1680-1688.

Roseland, J., M, Nguyen, Q., V, Williams, J., R, & Patterson, K., Y. (2013). *USDA nutrient data set for retail beef cuts from SR, release 3.0*. West Beltsville, MD.

Sacchetti, G., Di Mattia, C., Pittia, P., & Mastrocola, D. (2009). Effect of roasting degree, equivalent thermal effect and coffee type on the radical scavenging activity of

coffee brews and their phenolic fraction. *Journal of Food Engineering, 90*(1), 74-80. doi: 10.1016/j.jfoodeng.2008.06.005

Schwarz, K., Bertelsen, G., Nissen, L. R., Gardner, P. T., Heinonen, M. I., Hopia, A., . . . Tijburg, L. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research and Technology, 212*(3), 319-328. doi: 10.1007/s002170000256

Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science, 98*(1), 21-33. doi: 10.1016/j.meatsci.2014.03.020

Siu, G. M., & Draper, H. H. (1978). A survey of the malonaldehyde content of retail meats and fish. *Journal of Food Science, 43*(4), 1147-1149. doi: 10.1111/j.1365-2621.1978.tb15256.x

Smith, J. S., & Alfawaz, M. (1995). Antioxidative activity of maillard reaction-products in cooked ground-beef, sensory and TBA values. *Journal of Food Science, 60*(2), 234-234. doi: 10.1111/j.1365-2621.1995.tb05644.x

Suman, S. P., Faustman, C., Stamer, S. L., & Liebler, D. C. (2007). Proteomics of Lipid Oxidation-induced Oxidation of Porcine and Bovine Oxymyoglobins. *Proteomics, 7*(4), 628-640. doi: 10.1002/pmic.200600313

Tang, J., Faustman, C., & Hoagland, T. A. (2004). Krzywicki revisited: Equations for spectrophotometric determination of myoglobin redox forms in aqueous meat

extracts. *Journal of Food Science, 69*(9), C717-C720. doi: 10.1111/j.1365-2621.2004.tb09922.x

Tang, S., Kerry, J. P., Sheehan, D., Buckley, D. J., & Morrissey, P. A. (2001). Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. *Food Research International, 34*(8), 651-657. doi: 10.1016/S0963-9969(00)00190-3

Teets, A. S., & Were, L. M. (2008). Inhibition of lipid oxidation in refrigerated and frozen salted raw minced chicken breasts with electron beam irradiated almond skin powder. *Meat Science, 80*(4), 1326-1332. doi: 10.1016/j.meatsci.2008.06.010

Tironi, V. A., Lopez, L. B., Pellegrino, N., Añtón, M. C., & Tomás, M. C. (2004). Malonaldehyde-induced microstructural modifications in myofibrillar proteins of sea salmon (*Pseudopercis Semifasciata*). *Journal of Food Science, 69*(7), 519-523. doi: 10.1111/j.1365-2621.2004.tb13645.x

Tokur, B., & Polat, A. (2010). Myofibrillar and sarcoplasmic protein oxidation and degradation of thin-lipped gray mullet (*Liza Ramada*) during refrigerated storage (4C). *Journal of Muscle Foods, 21*(1), 102-118. doi: 10.1111/j.1745-4573.2009.00170.x

Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry, 48*(5), 1473-1478. doi: 10.1021/jf991342v

Tug, T., Karatas, F., Terzi, S. M., & Ozdemir, N. (2004). Comparison of serum malondialdehyde levels determined by two different methods in patients with COPD: HPLC or TBARS methods. *Lab Medicine, 36*(1), 41-44.

USDA. (2014). Cattle and beef. Retrieved Aug 20, 2014, from U.S. Department of Agriculture http://www.ers.usda.gov/topics/animal-products/cattle-beef/statistics-information.aspx#.U_VzNmOKX1o

Utrera, M., & Estévez, M. (2012). Oxidation of myofibrillar proteins and impaired functionality: Underlying mechanisms of the carbonylation pathway. *Journal of Agriculture and Food Chemistry, 60*, 8002-8011.

Utrera, M., & Estévez, M. (2013). Oxidative damage to poultry, pork, and beef during frozen storage through the analysis of novel protein oxidation markers. *Mario Estévez, 61*, 7987−7993.

Utrera, M., Morcuende, D., & Estevez, M. (2014). Fat content has a significant impact on protein oxidation occurred during frozen storage of beef patties. *Lwt-food Science and Technology, 56*(1), 62-68. doi: 10.1016/j.lwt.2013.10.040

Vignoli, J. A., Bassoli, D. G., & Benassi, M. T. (2011). Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material. *Food Chemistry, 124*(3), 863-868. doi: 10.1016/j.foodchem.2010.07.008

Viljanen, K., Kylli, P., Kivikari, R., & Heinonen, M. (2004). Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *Journal of Agricultural and Food Chemistry, 52*(24), 7419-7424. doi: 10.1021/jf049196n

Vivian, J. T., & Callis, P. R. (2001). Mechanisms of tryptophan fluorescence shifts in proteins. *Biophysical Journal, 80*(5), 2093-2109. doi: 10.1016/S0006-3495(01)76183-8

Wang, Y. Q., Zhang, H.-M., & Zhou, Q.-H. (2009). Studies on the interaction of Caffeine with bovine hemoglobin. *European Journal of Medicinal Chemistry, 44*, 2100-2105.

Yen, W.-J., Wang, B.-S., Chang, L.-W., & Duh, P.-D. (2005). Antioxidant properties of roasted coffee residues. *Journal of Agricultural and Food Chemistry, 53*(7), 2658-2663. doi: 10.1021/jf0402429

Yin, S., Faustman, C., Tatiyaborworntham, N., Ramanathan, R., Maheswarappa, N. B., Mancini, R. A., . . . Sun, Q. (2011). Species-specific myoglobin oxidation. *Journal of Agricultural and Food Chemistry, 59*(22), 12198-12203. doi: 10.1021/jf202844t

Zhang, W., Xiao, S., & Ahn, D. U. (2013). Protein oxidation: basic principles and implications for meat quality. *Critical Reviews in Food Science and Nutrition, 53*(11), 1191-1201. doi: 10.1080/10408398.2011.577540

Zhou, J. H., Wu, X. H., Yang, C., Gu, X. T., Zhou, L., Song, K. X., . . . Shen, J. (2007). Spectroscopic studies on the interaction of hypocrellin A with myoglobin. *Spectroscopy-an International Journal, 21*(4), 235-243. doi: 10.1155/2007/503537

Zhou, S., & Decker, E. A. (1999). Ability of amino acids, dipeptides, polyamines, and sulfhydryls to quench hexanal, a saturated aldehydic lipid oxidation product. *Journal of Agricultural and Food Chemistry, 47*(5), 1932-1936. doi: 10.1021/jf980939s