7-2018

Comparison of Traditional and Alternative Ingredients on Meat Curing Reactions Using a Model System

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COMPARISON OF TRADITIONAL AND ALTERNATIVE INGREDIENTS ON MEAT CURING REACTIONS USING A MODEL SYSTEM

by

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Animal Science

Under the Supervision of Professor Gary A. Sullivan

Lincoln, Nebraska

July 2018

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University of Nebraska, 2018

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Five curing systems (3 traditional, 2 alternative) at ingoing nitrite concentrations of 10, 50, 100, 150, and 200 ppm were evaluated in two meat model systems (cysteine, and cysteine with myoglobin). Curing systems evaluated were: Sodium nitrite (SN), sodium nitrite with sodium chloride (NaCl) to equal the salt in celery juice powder (0.5% in solution; SN/NA), sodium nitrite with NaCl and sodium erythorbate (2.76 mM; SN/SE), pre-converted celery juice powder (CP), and pre-converted celery juice powder and acerola cherry powder (2.76 mM ascorbic acid; CP/CH). All solutions were evaluated for residual nitrite, sulfhydryl group concentration, and residual reducing capacity. Myoglobin solutions were evaluated for cured meat pigment.

The CP/CH curing system developed the most cured meat pigment, followed by the SN/SE curing system and then treatments without reducing agents (SN, SN/NA, and CP; $p < 0.001$). Ingoing nitrite concentrations greater than 10 ppm developed the most cured meat pigment ($p < 0.001$) An interaction occurred between ingoing nitrite concentration and curing system for residual nitrite concentration and sulfhydryl groups ($p < 0.001$), where at ingoing nitrite concentrations lower than 50 ppm there were no

differences between curing systems. At ingoing nitrite concentrations above 100 ppm, CP/CH and CP retained the most sulfhydryl groups, followed by SN/SE, and the SN and SN/NA curing systems had the least amount of residual sulfhydryl groups ($p < 0.001$). CP had the most residual nitrite followed by SN and SN/NA, and the treatments with reducing agents had the least residual nitrite ($p < 0.001$). CP/CH had a greater reducing capacity than SN/SE, and CP had similar reducing capacity to SN/NA and greater reducing capacity than SN ($p < 0.001$). Reducing capacity decreased with increasing ingoing nitrite concentration ($p < 0.001$). Both alternative and traditional curing systems develop similar cured meat pigment but differences in the nitrosation of cysteine exist.

Acknowledgments

I must recognize the help and encouragement of many mentors, friends, and family, without which I would not be here today richer in the knowledge of the intricacies of meat. First, I must thank Dr. Gary Sullivan for providing me so many opportunities during my career as his graduate student. I hit the ground running when I came to UNL and cannot enumerate the diverse opportunities I have embraced as his master's student. Each interaction with his contacts in the industry, colleagues in academia, and those he has mentored over the years was inspiring and reflective of the compassion he shows in even simple interactions. I would also like to thank the members of my committee, Dr. Dennis Burson and Dr. Kaustav Majumder for challenging my thinking of meat science and the industry. Dr. Chris Calkins, Dr. Steve Jones, Dr. Ty Schmidt, Dr. Ozan Ciftzi your enriching courses and high expectations of scientific learning sculpted my application of scientific method and thought. I am grateful for every lesson, both in and out of the classroom and with professors and mentors alike.

Learning through scientific experimentation is hard work; made easier by working with those around you to accomplish great things. I need to thank Tommi Jones for pondering preparations with me, organizing the group lab space, and in general providing emotional support when I was troubleshooting chemical protocols. Calvin Shrock was instrumental in ensuring that I had everything I needed to instruct future meat scientists in the processing of meat, and Sherri Pitchie is the glue that holds the office together. My gratitude also extends to the meat science graduate student family: Emery, Kathrine, Ashley, Hope, Kelly, Chad, Felipe, Kellen, Morgan, Lauren, Jessica, Nicolas, Nick, and

Becca. As well as, Jennifer and Kevin for helping with research, all of the students of the ASGSA, and all of the students of ASCI 410.

Finally, I need to thank my family and friends who were there for me on the hard days. Mom, Dad, Matthew, Nathan, and Sarah have reminded me, the last two years, of the importance of culturing relationships and maintaining good communication with those you love. I thank God for the strong group of friends he provided to bolster my faith McKenna, Kevin, Coddy, Jessica, Megan, Alaina, Jamie, and the rest of the Calvary church family.

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1. Introduction

The production of cured meat characteristics is defined by the reactions between nitrogen oxide compounds, components of meat, and other added ingredients. The first cured meat products utilized saltpeter (calcium or potassium nitrate) as a preservation agent (Binkerd & Kolari, 1975). In the late nineteenth century, it was determined that nitrite, produced from bacterial reduction of nitrate, was a more effective and direct curing agent (Lewis, Vose, & Lowry, 1925). Nitrite, in the presence of acidic conditions or reducing agents, forms either nitric oxide or intermediate complexes that can transfer a nitric oxide group to other compounds. In the mildly acidic conditions of a meat system (pH 5.6), sodium and nitrite dissociate into separate ions and nitrite is protonated to form nitrous acid (Honikel, 2008). Dinitrogen trioxide, a powerful nitrosating agent, is formed via a dehydration reaction of two nitrous acid molecules and is found in equilibrium with nitrous acid (Pegg & Shahidi, 1997; Sebranek & Fox, 1985). Reducing compounds can enhance the reduction of nitrite to nitric oxide by forming intermediate complexes with nitrous acid and other nitrosating compounds (Barbieri, Bergamaschi, Barbieri, & Franceschini, 2013).

The reactions of nitric oxide with transition metal complexes, sulfhydryl group containing amino acids, reducing agents, oxygen, reactive oxygen species, secondary amines, halides, and carbonyl groups in meat provide the attributes of cured meat. Haldane (1901) identified the red color of cured meat to be due to the production of nitric oxide bound hemoglobin. Reactions with nitrogen oxides are known to impart a cured meat flavor and aroma. Nitrogen oxide compounds act as an antioxidant reacting with

reactive oxygen species as well as radical products of lipid oxidation, chelating transition metals, and through the production of some volatile compounds not found in meat without nitrite added (MacDonald, Gray, & Lee, 1980.; Shahidi, 1992). Furthermore, nitric oxide is a potent antimicrobial compound preventing or limiting the outgrowth of *C. Botulinum*, *C. Perfringens*, and *L. Monocytogenes* by interacting with enzymes that have thiol-containing amino acids in the active site (Christiansen, 1980; Oleary & Solberg, 1976; Osterbauer et al., 2017).

In the 1970s, it was discovered that the presence of nitrite and secondary amine groups, in acidic or high heat conditions, could form cancerous N-Nitrosamines (Sen, Seaman, & Miles, 1979). Thus, reducing agents, commonly referred to as "cure accelerators" such as ascorbic acid and isoascorbate (sodium erythorbate), are added to cured meat formulations to prevent the formation of nitrosamines, reduce the amount of residual nitrite, and enhance the stability of cured meat pigment during storage in light and oxygen (Izumi, Cassens, & Greaser, 1989; Mirvish, Wallcave, Eagen, & Shubik, 1972). Bacon produced in the United States is required to have a 120 ppm ingoing sodium nitrite and 547 ppm of sodium erythorbate (USDA, 1995)

Consumers, driven by the perception of increased health and wellness, have begun to purchase natural and organic products, and $41 billion was spent to purchase foods labeled as "natural" in 2014 (Ferdman, 2014). From 2011 to 2015, conventional meat posted compound annual sales growth of 4.6%, while products with a natural label posted growth of 14.6% (Nielsen, 2016). According to the USDA 9 CFR 319.2, cured meat products labeled as natural cannot be referred to as cured since they lack either sodium nitrate or nitrite or potassium nitrate or nitrite and if the product is defined as cured by its

standard of identity, it must include the word “uncured (USDA, 2010b)”. Majority of “uncured” products in the marketplace have cured meat characteristics but are produced by using a vegetable source of nitrate that is reduced via bacteria nitrate reductase to produce nitrite. Currently, most processors use a pre-converted vegetable powders which are produced with standardized concentrations of nitrite (Redfield & Sullivan, 2015; Sebranek & Bacus, 2007). While similar cured meat characteristics are found in meat cured with traditional and alternative sources of nitrite at similar ingoing concentrations (Djeri & Williams, 2014; King, Glass, Milkowski, & Sindelar, 2015a; Posthuma, Rasmussen, & Sullivan, 2018), the understanding of the effect on specific meat curing reactions is limited due to the complexity of meat and nitric oxide reactions (Sullivan & Sebranek, 2012).

2. Review of Literature

2.1 Brief History of Curing Meat

Meat is a nutrient dense food rich in protein, lipids, and minerals. As such a source of nutrients and high in moisture, meat can spoil rapidly. The curing of meat, as we know it today, has developed for thousands of years. In primitive times, the first methods of preservation included salt and heat to reduce the moisture in the meat. Earliest written records indicate that salt from the Dead Sea was used by the Jewish nation in 1600 BC, and in 900 BC, Europeans used salt excavated from salt mines (Binkerd & Kolari, 1975). Ancient humans discovered that salt applied to the exterior of meat would dehydrate it, and such a product would not spoil due to microbial growth (Honikel, 2008).

The salt procured from the earth or coalesced and precipitated from seawater was impure and contained potassium and sodium nitrates and nitrites. In the 10th century, it was recorded that the impurity in salt known as "saltpeter" (potassium nitrate) was thought to contribute to the production of a reddish pink color and an extended shelf life. Thus, saltpeter became an essential ingredient in the production of cured meat (Binkerd & Kolari, 1975; Honikel, 2008).

In the 1890s, researchers noted that nitrite, the reduced form of nitrate, was responsible for cured meat characteristics (Lewis et al., 1925). Haldane (1901) identified that the pink pigment of cured meat products was formed by the attachment of nitric oxide to the hemoglobin protein. Hoagland (1908) showed that nitrous acid (HNO_2) or nitric oxide (NO) was the molecule responsible for nitrosylating hemeproteins. Since nitric oxide is not directly added to meat products, research into the reduction of nitrates by nitrate reducing bacteria to nitrites and then to nitric oxide began. The early research investigated the characteristics of cured meat such as color, flavor, antioxidant activity, and antimicrobial activity and showed that nitrite was an essential ingredient to the formation of these characteristics (Sebranek & Bacus, 2007).

2.2 Cured Meat Characteristics

The characteristic pink color of cured meat is the result of the reaction of nitric oxide with the heme portion of the myoglobin protein (Haldane, 1901). Myoglobin, the major pigment protein in meat, contributes to 80% of the color of skeletal muscle (Pegg & Shahidi, 1997). Myoglobin in fresh meat can be found in three main forms depending on the oxidation state of the iron held in the protoporphyrin- IX complex of the protein, the molecule bound to the 6th ligand of the complex, and gaseous environment. A reduced

iron state (ferrous) will contribute to either a dark purplish red (deoxymyoglobin) or a bright red color (oxymyoglobin; oxygen bound) dependent on the absence or presence of atmospheric oxygen. An oxidized iron state (ferric) will contribute to a brown color (metmyoglobin). When nitric oxide is bound to a heme pigment with a ferrous iron, it forms nitrosylmyoglobin which is reddish in color in an anaerobic environment and quickly oxidizes to nitrosylmetmyoglobin in aerobic states. Heating either nitrosylmyoglobin or nitrosylmetmyoglobin in excess of 150 °F denatures the globin portion of the protein resulting in cured meat pigment nitrosylhemochrome (Fox, 1966; Honikel, 2008). As low as 10 ppm ingoing nitrite can create enough nitrosylhemochrome to appear cured, but higher concentrations (40-50 ppm) of ingoing sodium nitrite result in the production of a sufficient amount of nitrosylhemochrome to produce stable cured meat color (Heaton, Cornforth, Moiseev, Egbert, & Carpenter, 2000). Concentration above 100 ppm ingoing nitrite is required to ensure the added safety associated with cured meats (Osterbauer et al., 2017).

Cured meat flavor and antioxidant potential are also believed to be attributed to reactions of the free radical nitric oxide with oxygen compounds and products of lipid and protein oxidation processes. Cured meat products exhibit less than half of the volatile compounds found in uncured meats, and the volatile alcohols, ketones, and phenols can undergo nitrosation reactions (Ramarathnam, Rubin, & Diosady, 1993). Lipid autooxidation can be terminated by nitric oxide as nitric oxide rapidly reacts with oxygen and reactive oxygen species (Miranda, Espey, & Wink, 2000). Like cured meat color, cured meat flavor can be perceived at low ppm of sodium nitrite. In a sensory study, Froehlich, Gullett, and Usborne (1983) found that though trained panelists thought salt

and higher levels of nitrite contributed to a more intense “cured meat flavor,” untrained panelists rated ham samples with 50 ppm and 150 ppm similarly for cured meat flavor and both were above samples prepared without nitrite. Some distinct flavor compounds have been attributed cured meats; hydrocarbons such as 2,2,4-trimethylhexane, 1,2,4-trimethylcyclohexane, and 1,3-dimethylbenzene were detected in cured beef and chicken products but not in their uncured counterparts (MacDonald, Gray, Kakuda & Lee, 1980).

Nitrite added to meat works as an antimicrobial as it is reduced to nitrous acid and nitrosates/nitrosylates compounds within the microorganism. Christensen (1980) explained the effectiveness of nitrite as an antimicrobial is a function of residual nitrite concentration, pH of the product, microbial load, and added reducing agents. Higher concentrations of nitrite (100 ppm or greater) in an acidic environment or in the presence of reducing agents will produce nitric oxide. Added reducing agents, such as ascorbate, chelate metals and enhance the efficacy of ingoing nitrite but also reduce overall residual nitrite concentration by producing gaseous nitric oxide. Temperature abuse of products with a pH above 4.6, concentrations of residual nitrite below 5 ppm, and high inoculation levels will have an outgrowth of microorganisms (Christiansen, 1980). Nitric oxide is also a potent antimicrobial, and at concentrations, such as is produced from nitrite added to meat, will covalently bond with DNA, proteins, and lipids in microorganisms thus inhibiting growth (Schairer, Chouake, Nosanchuk, & Friedman, 2012). The spores of *Clostridium perfringens, Bacillus cereus,* and *Staphylococcus aureus* will germinate in the presence of nitrite, but the outgrowth and replication is halted because membrane proteins undergo modification by nitrosation of thiol-containing proteins by either nitrous

acid or nitrosothiols (Castellani & Niven Jr., 1955; Morris & Hansen, 1981; O'Leary & Solberg, 1976).

2.3 Major Nitrogen Oxide Compounds and Reactions

The characteristics of cured meat are the result of nitrogen oxide compounds reacting with compounds both endogenous to meat and added during processing (Honikel, 2008). The atmosphere around us is comprised of 78% nitrogen, found as the diatomic molecule N_2 which has two triple bonded nitrogen atoms. Nitrogen atoms have three valence electrons and in the correct conditions, will form up to three covalent bonds with other non-ionic atoms. Nitrogen atoms, covalently bonded to oxygen atoms, form a complex group of reactive compounds that are biologically significant because of their oxidizing and reducing potential (Wink & Mitchell, 1998). Nitrogen oxide compounds range from the fully oxidized nitrate, NO_3, to fully reduced ammonia, NH_3. Nitrate and ammonia, being the most oxidized and reduced of the nitrogen oxide compounds, respectively, are relatively inert; it is the intermediate compounds, such as nitrite and nitric oxide, that are more reactive (Lewis et al., 1925).

Nitrogen oxides interact with other chemical compounds by either reduction or oxidation. The direct addition of a nitric oxide to a metal or ionic molecule is termed nitrosylation and results in a nitrosylated compound such as nitrosylmyoglobin. The transfer of a nitric oxide from one compound to another is termed nitrosation and is seen with non-metal molecules such as sulfur, other nitrogen, or carbon (Williams, 2004). Mechanisms for oxidation, reduction, nitrosylation, and nitrosation reactions between nitrogen oxide compounds and other biochemical compounds have been suggested and researched, but complexities still obscure secondary reactions (Honikel, 2008; Sebranek

& Fox, 1985; Williams, 2004). Though some nitrogen oxide reactions have been considered harmful, such as the production of nitrosamines or the formation of methemoglobin, more research indicates that there are several reactions that are beneficial to human health and many compounds act as biological signaling molecules (Schairer et al., 2012). Reactions with nitrogen oxide compounds are also essential to producing safe, high quality, cured meat products (King, Glass, Milkowski, & Sindelar, 2015b). Using modern day technology (such as refrigeration, high-pressure processing, and ingredient technologies) it is possible to produce low safety risk meat products without the addition of nitrogen oxide compounds, but such products will lack the characteristics of cured meat flavor, color, and aroma.

2.4 Nitrosating Nitric Oxide Compounds and the Production of Nitric Oxide in Cured Meat Systems

As discussed in previous sections, traditional methods of producing cured meat involved the addition of sodium or potassium nitrate (and subsequent bacterial reduction to nitrite) or direct addition of nitrite. Sebranek and Fox (1985) summarized the potential reactions in a meat system to produce reactive nitrosating species within meat and curing solutions. Sodium nitrite is typically dissolved in an aqueous solution, whether it is in a brine or in dissolving in the natural water in meat, and the term nitrite refers to both the anion NO_2^- and the reactive neutral nitrous acid HNO_2. The concentration of hydrogen atoms in a solution (H^+) will determine the pH of the solution. The higher the concentration of hydrogen atoms in a solution, the lower the pH and the more hydrogen atoms to associate with the nitrite to form the neutral nitrous acid. The pH of fresh meat ranges from 5.6-6.5 and at that pH, with an acid dissociation constant for nitrous

acid/nitrite equilibrium of 3.98×10^{-4} (pK_a= 3.4), the concentration of HNO_2 is less than 1% of the total nitrite. A lower pH, closer to 3.4, would result in a higher concentration of HNO_2. However, low concentrations of reactive nitrous acid can generate reactive nitrosating compounds (Sebranek & Fox, 1985).

It is possible in strong acidic conditions to form strong nitrosating compounds, such as the positively charged (electrophilic) nitrogen oxide nitrosonium ion (NO^+) or nitrous acidium ($H_2NO_2^+$), by adding H^+ to HNO_2 to produce the nitrous acidium, then a dehydration reaction occurs to form the nitrosonium ion. Though both NO^+ and $H_2NO_2^+$ could exist in a meat solution even at unfavorable pH, it is likely that they are at a very low concentration. Instead, another nitrosating compound is responsible for the reactions seen in meat products (Sebranek & Fox, 1985).

The dehydration reaction of two nitrous acid molecules results in dinitrogen trioxide (N_2O_3) and water (H_2O). The resonance structure (N_2O_3) that can form in an aqueous solution possesses an electrophilic site which will readily react with nucleophiles to form nitrosated compounds. Nitric oxide, the nitrogen oxide compound responsible for the nitrosylation of transition metals and ionic molecules, can be produced by the one-electron reduction of nitrite. Though dinitrogen trioxide can dissociate into a nitric oxide ($NO\cdot$) and nitrite (NO_2), this reaction has been shown to be slow in comparison to the production of nitric oxide via a reducing agent (Pegg & Shahidi, 1997).

Nitric oxide can be produced from nitrosation reactions of reducing agents whether endogenous to the meat system or added (Sebranek & Fox, 1985; Williams, 2004). Nitric oxide interacts directly as a nitrosylating agent in the case of transition metals and oxygen compounds, while a nitrosating intermediate is needed for the

nitrosation of other compounds such as ascorbic acid (and its derivatives), sulfhydryl-containing amino acids, secondary amines, and carbonyl compounds.

2.5 Reactions with Oxygen and Reactive Oxygen Species

Nitric oxide can consume oxygen and radical oxygen species to produce less harmful products and provide an antioxidant effect. Oxidation of nitric oxide by oxygen gas (O_2) is the most well-known and studied reaction of nitric oxide. This reaction takes place in an aqueous solution with a third order rate constant of 5×10^6 dm^6 mol^{-1} s^{-1} at 25°C and is unaffected by pH between 1-13 (Williams, 2003). A more important reaction in meat is between nitric oxide and radical oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical. Superoxide ($O_2\cdot$), a single electron containing radical, can share the electron with nitric oxide to produce peroxynitrite ($ONOO^-$) which can have mutagenic and carcinogenic properties in vivo and is stable at high pH (pK_a=6.5). However, at a lower pH, such as the pH of meat products, peroxynitrite will become protonated with H^+ and isomerize to yield nitrate (Logager & Sehested, 1993). The combination of a peroxynitrite and another nitric oxide molecule will result in the production of two nitrite molecules (Ignarro, 2000). A reaction between hydrogen peroxide and nitric oxide via the peroxynitrous acid intermediate (HOONO) decomposes to form nitrate (Williams, 2004).

Previous research indicates that nitric oxide can act as a primary antioxidant and terminate autooxidation of lipids by reacting with lipid-derived alkyl (L·), alkoxyl (LO·), and peroxyl radicals (LOO; Skibsted, 2011). It is believed the reactions of primary lipid oxidation products and nitric oxide follow similar mechanics to the reaction between nitric oxide and the reactive oxygen species (Skibsted, 2011). Furthermore, nitric oxide

acts to either reduce or chelate transition metals and atmospheric oxygen, thus, functioning as a secondary antioxidant by removing prooxidants from the system.

2.6 Reactions with Transition Metals and Heme Proteins

In meat science, there are few reactions more closely studied than myoglobin chemistry; the oxidation, reduction, and oxygenation of myoglobin as well as the formation of nitrosylmyoglobin, and heat denaturation of the nitrosylmyoglobin to produce nitrosylhemochrome the pink cured meat pigment (Fox, 1966; . Fox & Ackerman, 1968; Kanner, Harel, Shagalovich, & Berman, 1984; Suman & Joseph, 2013). This reaction between nitric oxide and the iron center of the hemeproteins, myoglobin, and hemoglobin, is an example of the nitrosylation reactions that can occur between nitric oxide and transition metals.

Myoglobin specific reactions involve the reversible binding of a nitric oxide molecule to the sixth ligand of the heme iron and either oxidation or reduction of the iron group. Nitric oxide is preferentially bound to a ferrous iron (Fe^{2+}) before a ferric iron (Fe^{3+}) due to the oxidation state of the transition metal (Khade, Yang, Shi & Zhang, 2016). Two mechanisms of nitric oxide binding to oxidized transition metals have been considered. The two-step nitric oxide method involves an oxidized transition metal (ex. Fe^{3+}) to be reduced (ex. Fe^{2+}) before binding a different nitric oxide molecule than the one that was used for reduction. Reductive nitrosylation is the method where HNO is used in a single step reaction to both reduce the transition metal and bind the nitric oxide at the same time (Miranda, 2005).

Many transition metals are used in the active sites of enzymes and it has been suggested that the rate-limiting condition for proteins containing transition metals is the shape and the amino acids in the active site. This is why the rate of nitrosylation in cytochrome c is lower than that of myoglobin, but the stability of the nitrosyl complex in myoglobin is lower than that of cytochrome c (Miranda, 2005). In an anaerobic environment, nitrosylated transition metals are relatively stable, but the introduction of oxygen is likely to oxidize the transition metal and result in the uncoupling of nitric oxide or the oxidation of nitric oxide to nitrite or nitrate (Pegg & Shahidi, 1997)

2.7 Reactions with Sulfhydryl Group-Containing Amino Acids

The nitrosation of sulfhydryl group-containing amino acids in the active sites of proteins is suggested as the mechanism that nitrogen oxide compounds influence the metabolic function of cells. Nitrosocysteine and other nitrosated thiols are produced readily in an aqueous, slightly acidic environment, by the reaction with dinitrogen trioxide (N_2O_3) but nitrosated thiols are less stable than nitrosylated transition metal complexes (Schairer et al., 2012; Williams, 2004). The nitrosation of thiol groups can lead to the release of nitric oxide and create nitrosothiols which can participate in trans-nitrosation of other molecules such as secondary amines and transition metals (Noble & Williams, 2002). The nitrosation of thiol-containing amino acids in the active sites of proteins was found to have a detrimental effect on the outgrowth of several pathogens, from anaerobic spore formers *C. perfringens* and *C. botulinum*, to psychrotrophic bacteria *L. monocytogenes* (Castellani & Niven, 1955; Duncan & Foster, 1968; Oleary & Solberg, 1976; Sofos, 1979). The rate of release of nitric oxide from nitrosothiols can be enhanced by an increased concentration of ascorbic acid which acts as a reducing agent to

regenerate sulfhydryl groups or promote disulfide bond formation (Holmes & Williams, 1998).

2.8 Reactions with Reducing Agents

A molecule or a compound that can donate electrons to a different compound is known as a reducing agent and thus oxidizes itself as it reduces other compounds. In meat products, reducing agents, both endogenous and added, play a pivotal role in maintaining the reduced state of the heme iron in myoglobin and the addition and maintenance of a bound nitric oxide molecule. The ferrous iron protoporphyrin-IX complex of hemoglobin, myoglobin, and ferrichrome C is responsible for much of meat color with myoglobin contributing to 80% of the pigment in muscle tissue (Pegg & Shahidi, 1997). Reducing agents also react with free radicals, or compounds with unpaired electrons, facilitate a faster rate of nitrite reduction to nitric oxide and form intermediate complexes that facilitate the transfer of nitric oxide to other compounds. The general chemical mechanism to produce nitric oxide by using a reducing agent as a catalyst involves the oxidation of the reducing agent by nitrous acid (HNO_2) to produce nitric oxide and water. The nitrosating nitrogen oxide compound dinitrogen trioxide also readily reacts with reducing agents to form reaction intermediates that have yet to be completely identified and quantified (Skibsted, 2011).

2.9 Added Reducing Agents

Ascorbic acid, isoascorbic acid (erythorbic acid), and the sodium salts of both compounds are the most used reducing compounds in meat formulations. Defreitas et al. (1988) showed that sodium erythorbate included at 550 ppm in liver sausage cured with

156 ppm ingoing nitrite helped suppress the outgrowth of inoculated *C. sporogenes*. In several studies, the inclusion of an ascorbic acid based reducing compound reduces the amount of residual nitrite in meat products (Izumi et al., 1989). Additionally, the ability to form an intermediate which can transfer a single electron increases the ability of reducing compounds to scavenge free radicals thus lowering the rate of oxidation in food products (Sanmartin et al., 2000).

2.10 Alternative Curing Systems

Chemical ingredients and even ingredients that sound like chemicals on labels have been perceived as being associated with negative health aspects by consumers. Even though synthetic sodium nitrite has been proven to provide safety, palatability, and quality to cured meat products, the fear of the formation of carcinogenic nitrosamines has led to the use of alternative curing systems. Most alternative curing systems utilize vegetables high in nitrate, bacterial inoculation, and subsequent reduction of endogenous vegetable nitrate to form nitrite. The nitrite formed from these alternative sources is used similarly to synthetic nitrite and produces similar cured meat characteristics. As a cure accelerator, either acidifying compounds or natural sources of ascorbic acid and other reducing agents can be used.

However, since alternative curing agents are not identified as a curing agent, the USDA has ruled that products without direct addition of synthetic sodium nitrite or nitrate, or potassium nitrite or nitrate must be labeled as “Uncured” (9 CFR 319.2). According to 9 CFR 317.17 a product labeled as “Uncured” that also does not meet certain pH, water activity, or thermal processing thresholds to provide additional safety measures, must also include the statement “Not Preserved- Keep Refrigerated Below

40°F At All Times." To prevent the confusion of and possible misleading of consumers, a statement must also be made on the label claiming the inclusion of naturally sourced nitrates or nitrites. Labels accomplish this by stating "Uncured, no nitrates or nitrites added" and then claiming near the ingredient statement "except for those naturally occurring in celery juice powder, sea salt (or another natural source of nitrite)." according to USDA regulation (USDA, 2010a; USDA, 2010b)

Original studies on alternative curing used in meat products began with the addition of a vegetable source of nitrate as well as a nitrate-reducing starter culture. Vegetable powders as a concentrated nitrate source and starter culture containing nitrate reducing bacteria were utilized to make the alternative curing process efficient. Alternative curing processes using nitrate and a starter culture required incubation of the meat at 38°C - 42°C for two hours, prior to smoking and cooking to allow for the development of and subsequent reaction of nitrite with the meat (Sindelar et al., 2007; Terns, Milkowski, Rankin, & Sindelar, 2011). However, the incubation step required to produce alternatively cured meats in this fashion was prohibitive to productivity. This has evolved to the current practice where the ingredient supplier of celery juice powder inoculates and cultures the celery juice or other vegetable juices with the same nitrate reducing bacteria to reduce nitrate to nitrite prior to centrifuging and freeze drying. The subsequent products are a "pre-converted" or cultured celery juice powder that contains nitrite. This eliminated the need for an incubation step if such a product was used (Sebranek & Bacus, 2007).

Several studies have delved into the efficacy of celery juice powder as an alternative curing agent both as a source of nitrate to be fermented, or a pre-converted

source of nitrite. Sebranek and Bacus (2007) determined the obstacles and qualities that would have to be seen in a product that was alternatively cured to prove that it was just as effective as traditional curing methods. The industry began producing products labeled as "uncured" and Sullivan et. al (2012b) examined alternatively cured commercial products on the market. They found that alternatively cured products were more susceptible to microbial outgrowth than traditional control products and speculated that the cause could be lower ingoing nitrite as well as limited options for natural antimicrobials. The first products evaluated in a laboratory setting included the addition of celery juice powder containing nitrate, a nitrate reducing bacterial starter culture, and incubation times of up to 2 hours. Sindelar et. al (2007) determined that treatments containing more than 0.2% of the vegetable juice powder were undesirable due to off vegetable flavors and aromas. Terns et al. (2011) evaluated emulsified sausages with 0.2% nitrate vegetable juice powder, and varying levels of bacterial starter culture and the addition of acerola cherry powder (a source of ascorbic acid) and found treatments with acerola cherry powder as a source of reducing agents produced similar sensory characteristics to traditionally cured sausages. Sullivan et al. (2012a) reported that to prevent the outgrowth of *L. monocytogenes* at ingoing nitrite concentrations from pre-converted celery juice powder and vegetable juice powder with a starter culture of 68 ppm of nitrite, an added antimicrobial was needed. In turkey bologna, it was once again confirmed that addition of nitrite via pre-converted celery juice powder was limited to 0.2% due to vegetable off flavors, however ingoing nitrite concentrations of 156 ppm with 469 ppm ascorbic acid from cherry powder even though from a vegetable source provided similar antimicrobial properties suggesting refinement of the powder (Djeri & Williams, 2014). Redfield and

Sullivan (2015) found that pre-converted celery juice powder could be added to cured deli turkey logs at up to 0.47% of the formulation (100 ppm ingoing nitrite) without vegetable off flavors being detected by consumers. Development of cured meat color was evaluated between traditional and alternative curing systems in a model system where 156 ppm ingoing sodium nitrite was compared to 100 ppm nitrite from pre-converted celery juice powder with and without equivalent molarities of reducing agents (sodium erythorbate and ascorbic acid from cherry juice powder). It was determined that treatments with reducing compounds (regardless of nitrite source and concentration) had greater cured meat pigment and reduced residual nitrite (Posthuma et al., 2018).

King et al. (2015) reported the outgrowth of *C. perfringens* could be mitigated in deli-style turkey breast with low ingoing nitrite concentrations (50 ppm) from pre-converted celery juice powder if added natural antimicrobials were used (acetate and lactic acid from dried vinegar and cultured sugar). When comparing equivalent ingoing nitrite and ascorbate concentrations (100 ppm and 547 ppm respectively) from traditional and alternative sources, less than 1 log of growth was seen in *C. perfringens*, and treatments with 50 ppm nitrite and 500 ppm ascorbate or ≥75 ppm nitrite and ≥250 ppm ascorbate had less than 1 log of growth (King, Glass, Milkowski, & Sindelar, 2015a). *C. botulinum* toxin production was delayed by 3 weeks in inoculated Dijon pork marinated in a sauce with 80 ppm nitrite from cultured celery juice powder (Golden et al., 2017). These results indicate that it is not the source of nitrite, but the ingoing concentration and presence of reducing compounds to produce nitric oxide, that impact the cured meat color (nitrosylation of myoglobin), flavor, texture, and antioxidant and antimicrobial capacity of cured meats.

Celery is one of many vegetables that have studied and used as a nitrate source to produce pre-converted nitrite containing vegetable powders. Though it is high in initial nitrate and is mild in flavor, celery is an allergen of concern in the European Union, where it must be expressed on the label (EU, 2003). Pre-converted swiss chard powder (60,539 ppm nitrite) was standardized to 120 ppm ingoing concentration and used to formulate cured pork patties, ascorbic acid was added at 0.05% of the formulation as a reducing compound and cure accelerator. The patties were found to have similar percentages of cured meat pigment out of total meat pigment, a* values, and sensory characteristics when evaluated over time (Shin et al., 2017). Red beet extracts were also pre-converted using a starter culture and contained 729.28 ppm nitrite and added to a meat emulsion system at 5% and 10% of the meat block and ice weight, and the treatments were evaluated with and without added ascorbic acid 0.05% of meat block. Samples with 10% pre-converted beet extract and ascorbic acid were found to have a higher overall sensory acceptability score than the control treatment of 150 ppm sodium nitrite, but similar to the treatment with 150 ppm nitrite and 0.05% ascorbic acid (Choi et al., 2017).

2.11 Summary

As it has been shown through the history of research on nitrogen oxides and their use in meat, the true function of nitrogen oxide compounds is to either nitrosylate transition metals or nitrosate non-ionic molecules such as oxygen, sulfur-containing compounds, and amine compounds. The production of nitrosylhemochrome contributes to the characteristic pink color of cured meats, the reaction of nitrogen oxide compounds with residual oxygen and flavor molecules contributes to the flavor of cured meats, and

the modification of cysteine and other thiol amino acids in the active sites of proteins inhibits the outgrowth of bacteria. It has been shown that extent of these reactions in cured meats is mostly attributed to the ingoing concentration of nitrite and the use of reducing compounds such as ascorbic acid to produce nitric oxide and other nitrosating compounds. Alternatively cured meats, using vegetable sources of nitrite, have become appealing to consumers.

Evaluating molecule specific reactions between nitrite, endogenous compounds in meat, and added ingredients are difficult due to the complexity of the system. Though several mechanisms have been suggested to explain the characteristics of cured meat, the main reactions are between the radical nitric oxide, nitrosating compounds, and electron donating compounds such as myoglobin (transition metals), cysteine (sulfhydryl group proteins), reducing agents, oxygen, and radical oxygen products (Williams, 2004). A model system may provide clearer tracking of specific meat curing reactions (Sullivan & Sebranek, 2012). Therefore, the purpose of this experiment is to compare two meat curing reactions, nitrosylation of myoglobin and nitrosation of cysteine, in either traditional curing systems or alternative "natural" curing systems with and without added reducing agents, at varying concentrations of ingoing nitrite, using a model meat system with myoglobin and cysteine.

A deeper look into the nitrosylation and nitrosation reactions in alternative curing systems would provide clarity on the importance of ingoing nitrite and reducing compounds. With such knowledge, more effective alternative curing systems can be developed to provide the same characteristics and quality of traditionally cured meats.

3. Materials and Methods

3.1 Development of Model Meat Curing Systems

To compare the nitrosylation and nitrosation reactions of traditional curing systems and alternative curing systems, five model curing systems were designed: sodium nitrite (SN), sodium nitrite with sodium chloride (NaCl) to equal the salt in celery juice powder (0.5% in solution; SN/NA), sodium nitrite with NaCl and sodium erythorbate (SN/SE), celery juice powder (CP; VegStable 504, Florida Food Products, Inc., Eustis, FL), and celery juice powder with acerola cherry powder (CP/CH; VegStable 515, Florida Food Products, Inc., Eustis, FL). Solutions were made to compare nitrite sources: synthetic sodium nitrite, and pre-converted celery juice powder with and without reducing agents (2.76 mM of sodium erythorbate, or ascorbic acid from cherry powder) at ingoing nitrite concentrations of 0.072, 0.362, 0.725, 1.087, and 1.450 mM (equivalent to 10, 50, 100, 150, and 200 ppm added to the final solution). The SN/NA treatment was made to determine if the salt in celery juice powder would have any effect on the reactions of the nitrite with myoglobin and cysteine. Two model meat solutions, one containing only cysteine, and one containing cysteine and myoglobin, were used to evaluate the effect of the curing system solutions on the nitrosylation of myoglobin and the nitrosation of cysteine. The two meat model systems were used to evaluate the order of reactions, and the effects of having both in the solution. Three independent replications of the experiment were conducted.

3.2 Stock Solution Preparation

For each replication, two 0.1 M phosphate (potassium phosphate, monohydrate) buffer solutions were prepared using 13.6 g in 250 mL de-ionized double distilled water

(DDD), adjusted to pH 5.6 and 7.4 with a 0.5 M sodium hydroxide solution, and brought to 1L volume in a volumetric flask. A 0.117 mM stock myoglobin solution was prepared using 0.3 g of myoglobin from equine skeletal muscle (Sigma Aldrich Co., St. Louis, MO) in 150 mL of pH 5.6 phosphate buffer solution. A 20.25 mM stock cysteine solution was prepared with 0.7980 g of L-cysteine hydrochloride anhydrous in 250 mL of pH 5.6 phosphate buffer solution. Cysteine solutions were used immediately following preparation to limit the reduction of sulfhydryl groups due to oxidation and disulfide bond formation.

For each replication, a synthetic nitrite stock solution was made by mixing 1 g of sodium nitrite in 1 L of DDD (14.49 mM; 1000 ppm nitrite) and diluting that stock solution with DDD to 0.288, 1.450, 2.899, 4.348, and 5.798 mM (40, 200, 400, 600, 800 ppm) before addition to the model meat solutions. Similarly, 15.85 g of pre-converted celery juice powder was added to 250 mL of DDD, to obtain a concentration of 1000 ppm nitrite, and dilutions were made to obtain the equivalent of 0.288, 1.450, 2.899, 4.348, and 5.798 mM (40, 200, 400, 600, 800 ppm) sodium nitrite. Prior to experimentation, pre-converted celery juice powder was tested for residual nitrite and that concentration was used to calculate solution concentrations in the experiment.

For each replication, a 55.2 mM sodium erythorbate stock solution was prepared by dissolving 1.2 g sodium erythorbate in DDD and diluting it to 100 mL in a volumetric flask. A 55.2 mM ascorbic acid stock solution was prepared by dissolving 9.7 g of acerola cherry powder in DDD and diluting it to 100 mL in a volumetric flask. Supplier testing of ascorbic acid content (1.1%) of the cherry juice powder was used to calculate the amount required to achieve 55.2 mM ascorbic acid. Finally, a stock sodium chloride

(NaCl) solution was prepared by mixing 5 g of NaCl in 195 mL DDD to obtain a concentration of 0.43 M.

For each replication, Ellman reagent to measure sulfhydryl concentration was prepared with 0.1586 g of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) mixed with 20 mL of pH 7.4 phosphate buffer (20 mM). Reagents to measure residual nitrite concentration, sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NED), were prepared as described in AOAC method 973.31 (AOAC 1990). The stock reagent solution of the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) was prepared to obtain a concentration of 87.2 µM by mixing 0.0344g in 1 L of methanol (Brand-Williams, Cuvelier, & Berset, 1995).

3.3 Model Meat Curing Solution Preparation and Simulated Cooking

To 13 mL test tubes, 2.5 mL of cysteine stock solution and 2.5 mL of DDD (cysteine only meat model), or 2.5 mL of cysteine stock solution and 2.5 mL of myoglobin stock solution (cysteine and myoglobin meat model) was added. Then, 2.5 mL of prepared stock nitrite solution (synthetic nitrite or pre-converted celery juice powder) and either 2.5 mL of DDD, 2.0 mL of DDD and 0.5 mL of reducing agent solution (sodium erythorbate stock solution, or ascorbic acid stock solution), or 2.0 mL of stock NaCl solution and 0.5 mL of DDD were added according to the curing system treatment protocol (Table 1). The tubes were vortexed and capped. The curing solutions were made in concentrations of 0.072, 0.362, 0.725, 1.087, and 1.450 mM (equivalent to 10, 50, 100, 150, 200 ppm) of nitrite in each treatment. Model meat curing solutions were heated in a 40°C water bath for 30 minutes, then in an 80°C water bath for 30 minutes and allowed to cool at 25°C for 15 minutes before being evaluated for cured meat pigment, residual

nitrite, sulfhydryl groups, and remaining reducing capacity. Within a replication, all samples were prepared in duplicate.

3.4 Cured Meat Pigment

Cured Meat pigment, nitrosylhemochrome, was evaluated using a modified method of Hornsey (1956). Briefly, 2.5 mL aliquots were vortexed with 10 mL of acetone, and 0.75 mL DDD and immediately filtered through Fisher Q2 filter paper (Fisher Scientific, Pittsburgh, PA). The absorbance was read at 540 nm (DU 800 Spectrophotometer, Beckman Coulter, Fullerton, CA). Cured meat pigment concentration (ppm) was calculated as $A_{540} \times 290$ (Sindelar et al., 2007). Duplicate measures for each sample were analyzed.

3.5 Sulfhydryl Group Concentration

The model meat curing solutions were evaluated for remaining sulfhydryl groups using a modified Ellman's reaction (Sullivan & Sebranek, 2012). Nitrosation of cysteine was reflected by a decrease in remaining sulfhydryl groups. In duplicate using 13 mL test tubes, 2.97 mL of pH 7.4 buffered phosphate, 0.03 mL of the model solution, and 0.015 mL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were added, vortexed, and the resulting colored solution absorption was measured using a spectrophotometer at 412 nm. A conversion factor of 1.1415 M^{-1} cm^{-1} was used to determine the sulfhydryl group millimolar concentration. Duplicate measures for each sample were analyzed.

3.6 Residual Nitrite

Residual nitrite was measured using the Association of Analytical Chemists (AOAC) method 973.31 with modifications (AOAC, 1990). Briefly, in duplicate for each

sample 3.6 mL of DDD water 0.4 mL of sample were combined in test tubes and 0.22 mL of sulfanilamide reagent was added. The mixture was vortexed and allowed to stand for 5 min before 0.22 mL of NED reagent was added, the tube vortexed, and allowed to stand for another 15 minutes. A solution of 4.5 mL DDD water, 0.25 mL sulfanilamide reagent and 0.25 mL of NED reagent was used as a blank before reading the sample absorbances at 540 nm in a spectrophotometer. A standard curve to calculate residual nitrite concentration was created as described in the original method, and the curve was used to calculate the concentration of residual nitrite in the sample solutions.

3.7 Remaining Reducing Capacity

Remaining reducing capacity of each model solution was determined by the reduction (indicated by the color loss) of the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) by a modified method of Brand-Williams (1995). In duplicate, 0.025 mL of model solution was added to 3.975 mL of DPPH reagent. The solution was vortexed and placed in a dark cooler at 4°C for 20 minutes to allow for a reaction of the reducing agents with the free radical. The absorbance of the solutions, as well as the DPPH reagent, was read at 515 nm on a spectrophotometer blanked with methanol. The conversion factor of 1.25 M^{-1} cm^{-1} was used to determine the concentration of DPPH, and the concentration of each sample solution was subtracted from the reagent concentration to obtain the micromoles of DPPH reduced.

3.8 Statistical analysis

Data were analyzed as a completely randomized design in a factorial arrangement of treatments (5 curing systems, 5 ingoing nitrite concentrations, and 2 model meat

solutions) for interactions and main effects using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). For significant effects ($p \leq 0.05$), LS means separation was conducted using a Tukey adjustment.

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5. Comparison of Traditional and Alternative Ingredients on Meat Curing Reactions Using a Model System

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5.1 Abstract

Consumer's negative perception of "chemical-sounding" ingredients has increased the use of alternative ingredients. The objective of this study was to determine the effect traditional (sodium nitrite with or without sodium erythorbate) or alternative (celery juice powder with or without acerola cherry powder) curing systems with different ingoing concentrations of sodium nitrite using model meat solutions. Curing systems with reducing agents developed the most cured meat pigment ($p < 0.001$). Using more than 50 ppm of nitrite did not further increase cured meat pigment ($p < 0.001$). Curing systems with reducing agents and 50 ppm or greater nitrite had the least residual nitrite ($p < 0.001$). Alternative curing systems had the most residual sulfhydryl groups when formulated with 100 ppm or more nitrite ($p < 0.001$). Treatments with reducing agents had greater residual reducing capacity than treatments without reducing agents ($p < 0.001$). Both alternative and traditional curing systems develop similar cured meat pigment but differences in the nitrosation of cysteine exist.

KEYWORDS- Nitric Oxide, Nitrosylation, Nitrosation, Myoglobin, Cysteine

5.2 Introduction

The production of cured meat characteristics is defined by the reactions between nitrogen oxide compounds, different components of meat, and other added ingredients. The first cured meat products utilized saltpeter (calcium or potassium nitrate) as a preservation agent (Binkerd & Kolari, 1975). In the late nineteenth century, it was determined that nitrite, produced from bacterial reduction of nitrate, was a more effective and direct curing agent (Lewis et al., 1925). Nitrite, in the presence of acidic conditions or reducing agents, forms either nitric oxide or intermediate complexes that transfer a nitric oxide to other compounds.

Haldane identified the red color of cured meat is due to the production of nitric oxide bound hemoglobin (Haldane, 1901). The interaction of nitric oxide with other compounds in the meat system provide the attributes of cured meat. Nitrite is known to impart a cured meat flavor and aroma by acting primarily as an antioxidant reacting with radical oxygen species as well as radical products of lipid oxidation, chelating transition metals, and through the production of some volatile compounds not found in meat with no nitrite (Donald, Gray, & Lee, 1980.; Shahidi, 1992). Furthermore, nitric oxide is a potent antimicrobial, preventing or limiting the outgrowth of *C. Botulinum*, *C. Perfringens*, and *L. Monocytogenes* by interacting with amino acids containing thiol groups in the active site of membrane proteins (Christiansen, 1980; Oleary & Solberg, 1976; Osterbauer et al., 2017).

It was discovered that the presence of nitrite and secondary amine groups in acidic, or high heat conditions could form cancerous N-Nitrosamines (Sen et al., 1979). Thus reducing agents, commonly referred to as “cure accelerators” such as ascorbic acid and

sodium isoascorbate (sodium erythorbate), are added to curing formulations to prevent the formation of nitrosamines, reduce the amount of residual nitrite, and enhance the stability of cured meat pigment during storage in light and oxygen (Izumi et al., 1989; Mirvish et al., 1972).

Consumers, driven by the perception of increased health and wellness, have begun to purchase natural and organic products, and $41 billion was used to purchase of foods labeled as “natural” in 2014 (Ferdman, 2014). From 2011 to 2015, conventional meat had compound annual sales growth of 4.6%, while products with a natural label had growth of 14.6%, (Nielsen, 2016). According to the USDA 9 CFR 319.2, cured meat products labeled as natural cannot be referred to as cured since they lack either sodium nitrate or nitrite or potassium nitrate or nitrite. If the product is defined as cured by its standard of identity, it must include the word “uncured” (USDA, 2010a). Majority of “uncured” products in the market-place have cured meat characteristics but are produced by using a vegetable source of nitrate reduced via bacteria nitrate reductase activity to produce nitrite. Most processors use pre-converted vegetable powders are now produced with standardized concentrations of nitrite (Redfield & Sullivan, 2015; Sebranek & Bacus, 2007). While similar cured meat characteristics are found in meat cured with traditional and alternative sources of nitrite at similar ingoing concentrations, the understanding of the effect on specific meat curing reactions is limited due to the complexity of meat and nitric oxide reactions (Djeri & Williams, 2014; King, Glass, Milkowski, & Sindelar, 2015; Posthuma, Rasmussen, & Sullivan, 2018).

Evaluating molecule specific reactions between nitrite, endogenous compounds in meat, and added ingredients are difficult due to the complexity of the system. Though

several mechanisms have been suggested to explain the characteristics seen in cured meat, the main reactions are between the radical nitric oxide, nitrosating compounds, and electron donating compounds such as myoglobin (transition metals), cysteine (sulfur-containing proteins), reducing agents, oxygen, and reactive oxygen products (Williams, 2004). A model system may provide clearer tracking of specific meat curing reactions (Sullivan & Sebranek, 2012). Therefore, the objective of this study was to compare two meat curing reactions, nitrosylation of myoglobin and nitrosation of cysteine, in either traditional or alternative curing system with and without added reducing agents, at varying concentrations of ingoing nitrite, using a model meat system.

5.3 Materials and Methods

5.3.1 Development of Model Meat Curing Systems

Five model curing systems were evaluated: sodium nitrite (SN), sodium nitrite with sodium chloride (NaCl) to equal the salt in celery juice powder (0.5% in solution; SN/NA), sodium nitrite with NaCl and sodium erythorbate (SN/SE), celery juice powder (CP; VegStable 504, Florida Food Products, Inc., Eustis, FL), and celery juice powder with acerola cherry powder (CP/CH; VegStable 515, Florida Food Products, Inc., Eustis, FL). Solutions were made to compare nitrite sources: synthetic sodium nitrite, and pre-converted celery juice powder with and without reducing agents (2.76 mM of sodium erythorbate, or ascorbic acid from cherry powder) at ingoing nitrite concentrations of 0.072, 0.362, 0.725, 1.087, and 1.450 mM (equivalent to 10, 50, 100, 150, and 200 ppm added to the final solution). The SN/NA treatment was made to determine if the salt in celery juice powder had an effect on the reactions of the nitrite with myoglobin and cysteine. Two model meat solutions, one with only cysteine, and one with cysteine and

myoglobin were used to evaluate the effect of the curing system solutions on the nitrosylation of myoglobin and the nitrosation of cysteine. The two meat model systems were used to evaluate the preferential order of reactions and effects of having the combination in solution. Three independent replications of the experiment were conducted.

5.3.2 Stock Solution Preparation

For each replication, two 0.1 M phosphate (potassium phosphate, monohydrate) buffer solutions were prepared by dissolving 13.6 g in 250 mL de-ionized double distilled water (DDD), adjusting to pH 5.6 and 7.4 with a 0.5 M sodium hydroxide solution, and bringing the volume to 1L in a volumetric flask. A 0.117 mM stock myoglobin solution was prepared using 0.3 g of myoglobin from equine skeletal muscle (Sigma Aldrich Co., St. Louis, MO) in 150 mL of pH 5.6 phosphate buffer solution. A 20.25 mM stock cysteine solution was prepared with 0.7980 g of L-cysteine hydrochloride anhydrous in 250 mL of pH 5.6 phosphate buffer solution. Cysteine solutions were used immediately following preparation to limit the reduction of sulfhydryl groups due to oxidation and disulfide bond formation.

A sodium nitrite stock solution was made by mixing 1 g of sodium nitrite in 1 L of DDD (14.49 mM; 1000 ppm nitrite) and diluting that stock solution with distilled water to 0.288, 1.450, 2.899, 4.348, and 5.798 mM (40, 200, 400, 600, 800 ppm) before adding to the model curing solutions. Similarly, 15.85 g of pre-converted celery juice powder was added to 250 mL of DDD, to obtain an approximate concentration equivalent to of 1000 ppm of sodium nitrite, and dilutions were made to obtain 0.288, 1.450, 2.899, 4.348, and 5.798 mM (40, 200, 400, 600, 800 ppm) sodium nitrite. Prior to

experimentation, pre-converted celery juice powder was tested for residual nitrite and that concentration was used to calculate solution concentrations in the experiment.

A 55.2 mM sodium erythorbate stock solution was prepared by dissolving 1.2 g sodium erythorbate in DDD and diluting it to 100 mL in a volumetric flask. A 55.2 mM ascorbic acid stock solution was prepared by dissolving 9.7 g of acerola cherry powder in DDD and diluting it to 100 mL in a volumetric flask. The supplier recommendation provided the ascorbic acid content (110,000 ppm) of the cherry juice powder that was used to calculate the amount required to reach 55.2 mM ascorbic acid. Finally, a stock sodium chloride (NaCl) solution was prepared by mixing 5 g of NaCl in 195 mL DDD to obtain a concentration of 0.43 M.

Ellman reagent to measure sulfhydryl concentration was prepared with 0.1586 g of 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB) mixed with 20 mL of pH 7.4 phosphate buffer (20 mM). Reagents to measure residual nitrite concentration, sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NED), were prepared as described in AOAC method 973.31 (AOAC 1990). The stock reagent solution of the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) was prepared to obtain a concentration of 87.2 µM by mixing 0.0344g in 1 L of methanol (Brand-Williams et al., 1995).

5.3.3 Model Meat Curing Solution Preparation and Simulated Cooking

To a 13 mL test tube, either 2.5 mL of cysteine stock solution and 2.5 mL of DDD (cysteine only meat model), or 2.5 mL of cysteine stock solution and 2.5 mL of myoglobin (cysteine and myoglobin meat model) was added. Then, 2.5 mL of prepared stock nitrite solution (synthetic nitrite or pre-converted celery juice powder) and either

2.5 mL of DDD, 2.0 mL of DDD and 0.5 mL of reducing agent solution (sodium erythorbate stock solution, or ascorbic acid stock solution), or 2.0 mL of stock NaCl solution and 0.5 mL of DDD were added according to the curing system treatment protocol (Table 6.1). The tubes were vortexed and capped. The curing solutions were made in concentrations of 0.072, 0.362, 0.725, 1.087, and 1.450 mM (equivalent to 10, 50, 100, 150, 200 ppm) of sodium nitrite in each treatment. Model meat curing solutions were heated in a 40°C water bath for 30 minutes, then in an 80°C water bath for 30 minutes, and allowed to cool at 25°C for 15 minutes before being evaluated for cured meat pigment, residual nitrite, sulfhydryl groups, and remaining reducing capacity. Within a replication, all samples were prepared in duplicate.

5.3.4 Cured Meat Pigment

Cured Meat pigment, nitrosylhemochrome, was evaluated using a modified method of Hornsey (1956). Briefly, a 2.5 mL model solution aliquot was vortexed with 10 mL of acetone, and 0.75 mL DDD and immediately filtered through Fisher Q2 filter paper (Fisher Scientific, Pittsburgh, PA). The absorbance was read at 540 nm (DU 800 Spectrophotometer, Beckman Coulter, Fullerton, CA). Cured meat pigment concentration (ppm) was calculated as $A_{540} \times 290$ (Sindelar et al., 2007). Duplicate measures for each sample were analyzed.

5.3.5 Sulfhydryl Group Concentration

The model meat curing solutions were evaluated for remaining sulfhydryl groups using a modified version of Ellman's reaction. Nitrosation of cysteine was reflected by a decrease in remaining sulfhydryl groups. In duplicate 13 mL test tubes, 2.97 mL of pH

7.4 buffered phosphate, 0.03 mL of the model solution aliquot, and 0.015 mL of DTNB were combined, vortexed, and the resulting colored solution absorption was measured using a spectrophotometer at 412 nm. A conversion factor of 1.1415 M^{-1} cm^{-1} was used to determine the sulfhydryl group concentration in millimolar. Duplicate measures for each sample were analyzed.

5.3.6 Residual Nitrite

Residual nitrite was measured using AOAC method 973.31 with modifications (AOAC, 1990). Briefly, in duplicate for each sample, 3.6 mL of DDD water and a 0.4 mL model solution aliquot were combined in test tubes and 0.22 mL of sulfanilamide reagent was added. The mixture was vortexed and allowed to stand for 5 min before 0.22 mL of NED reagent was added, the tube vortexed, and allowed to stand for another 15 minutes. A solution of 4.5 mL DDD water, 0.25 mL sulfanilamide reagent and 0.25 mL of NED reagent was used as a blank before reading the sample absorbances at 540 nm in a spectrophotometer. A standard curve to calculate residual nitrite concentration was created as described in the original method, and the curve was used to calculate the concentration of residual nitrite in the sample solutions.

5.3.7 Remaining Reducing Capacity

Remaining reducing capacity of each model solution was determined by the reduction (and subsequent color loss) of the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH). In duplicate, a 0.025 mL model solution aliquot was added to 3.975 mL of DPPH reagent. The solution was vortexed and placed in a dark cooler at 4°C for 20 minutes to allow for a reaction of the reducing agents with the free radical. The

absorbance of the solutions, as well as the DPPH reagent, was read at 515 nm on a spectrophotometer blanked with methanol. The conversion factor of 1.25 M^{-1} cm^{-1} was used to determine the concentration of DPPH, and the concentration of each sample solution was subtracted from the reagent concentration to obtain the micromoles of DPPH reduced.

5.3.8 Statistical analysis

Data were analyzed as a completely randomized design in a factorial arrangement of treatments (5 curing systems, 5 ingoing nitrite concentrations, and 2 model meat solutions) for interactions and main effects using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). Duplicate measures were averaged within a replication. For significant effects ($P \leq 0.05$), LS means separation was conducted using a Tukey adjustment.

5.4 Results

5.4.1 Cured Meat Pigment

The main effects of the curing system and ingoing nitrite concentration were significant for cured meat pigment ($p < 0.001$) and the least square means can be found in Table 6.2. Curing system solutions without a cure accelerator (SN, SN/NA, and CP) developed less cured meat color than those solutions with cure accelerators (SN/SE, CP/CH).

For the ingoing nitrite main effect, curing system solutions with a greater concentration of ingoing nitrite (≥50 ppm) developed more cured meat pigment than the solutions with only 10 ppm ingoing nitrite. Curing system solutions with only 10 ppm

ingoing nitrite developed only 13.4 ppm nitrosylhemochrome, while solutions with 50 ppm or more of ingoing nitrite, mean values ranged from 17.85 – 21.22 ppm nitrosylhemochrome.

5.4.2 Sulfhydryl Groups

There was an interaction between curing system solution and ingoing nitrite concentration for the concentration of sulfhydryl groups ($p < 0.001$). This interaction is summarized in figure 6.2. At 10 ppm ingoing nitrite concentrations, there were no differences among curing system solutions. At concentrations of 100, 150 and 200 ppm ingoing nitrite, the sodium nitrite curing systems without a reducing agent (SN and SN/NA) had fewer sulfhydryl groups than either of the alternative systems (CP and CP/CH) and the synthetic system with a reducing agent (SN/SE). As the ingoing nitrite concentration increases, the differences between curing systems became more pronounced. At 200 ppm ingoing nitrite, the traditional systems without reducing agents (SN and SN/NA) had the lowest concentration of sulfhydryl groups (1.8 mM, and 1.6 mM, respectively), the sodium nitrite system with a reducing agent (SN/SE) had an intermediate concentration of sulfhydryl groups (2.3 mM), and the alternative curing systems (CP and CP/CH) had the highest concentration of sulfhydryl groups (3.2 mM and 3.5 mM, respectively). The cysteine only model meat solution contained significantly fewer sulfhydryl groups than the cysteine and myoglobin model meat solution (2.9 mM and 3.2 mM, respectively; $p = 0.005$).

5.4.3 Residual Nitrite

There was an interaction between curing system solution and ingoing nitrite concentration ($p < 0.001$) for residual nitrite, where no differences could be discerned between curing system solutions at 10 ppm ingoing nitrite concentration (Figure 6.1). However, as ingoing nitrite concentration increased, differences between curing systems became more pronounced. At 200 ppm ingoing nitrite, the pre-converted celery juice powder treatment without a reducing agent (CP) had the highest residual nitrite concentration (75.6 ppm; $p < 0.001$). Both sodium nitrite treatments without a reducing agent were intermediate in residual nitrite concentration (SN, 54.8 ppm; SN/NA 46.8 ppm) and the two curing systems with reducing agents had the lowest concentrations of residual nitrite (CP/CH, 10.3 ppm; SN/SE, 3.99 ppm). There was a significant difference seen between the two model meat solutions ($p < 0.001$). The cysteine only model solution had a lower concentration of residual nitrite than the system with both cysteine and myoglobin (16.9 ppm and 21.8 ppm respectively).

5.4.4 Residual Reducing Capacity

The main effects of curing system and ingoing nitrite concentration were considered for the result of residual reducing capacity (DPPH neutralized) (Table 6.3), as the interaction of main effects was not significant ($p = 0.3477$). Curing systems with reducing agents (CP/CH and SN/SE) had more residual reducing capacity than those without reducing agents (CP, SN/NA, SN; $p < 0.001$) and CP/CH had the highest reducing capacity of all the treatments. Among the curing systems that had a reducing agent added to them (CP/CH and SN/SE), the CP/CH had 33% more reducing capacity than the traditional system. A similar trend was seen in the curing systems without an

added reducing agent (CP, SN, SN/NA) where the CP treatment had more reducing capacity than the SN treatment, but a similar reducing capacity to the SN/NA treatment.

Overall, residual reducing capacity decreased as ingoing nitrite concentration increased where 10 ppm ingoing nitrite had in the highest reducing capacity 50, 100, and 150 ppm had similar reducing capacity, and 200 ppm ingoing nitrite resulted in the lower residual reducing capacity than 10 or 50 ppm ingoing nitrite ($p < 0.001$). There was no significant difference between meat model system displayed for residual reducing capacity ($p = 0.2789$).

5.5 Discussion

The nitrosylation of myoglobin and subsequent heat denaturation and separation of the nitrosylated heme group from the globin moiety of the protein results in the cured meat pigment nitrosylhemochrome. Cured meat pigment differences between curing system treatments can be attributed to the addition of reducing compounds. The use of reducing compounds as “cure accelerators” has been well documented, even in alternative curing systems, to increase the formation of cured meat pigment (Posthuma et al., 2018; Redfield & Sullivan, 2015; Terns et al., 2011). Stable cured meat color has been documented to be obtained at ingoing nitrite concentrations of 40-50 ppm, (Froehlich, Gullett, & Usborne, 1983; Sindelar, Cordray, Sebranek, Love, & Ahn, 2007). The results of this study concur as no additional cured meat pigment was formed when increasing the ingoing nitrite above 50 ppm.

Cysteine is known to act as a reducing agent with nitrite, forming nitrosocysteine, which in turn can interact with myoglobin to release nitric oxide to the myoglobin heme when necessary (Fox & Nicholas, 1974; Williams, 2004; Sullivan & Sebranek, 2012).

The depletion of sulfhydryl groups can be explained by nitrosation of cysteine by a nitrosylating compound such as dinitrogen trioxide, and subsequent decomposition and release of the nitric oxide to form disulfide bonds (Morris & Williams, 1988; Peterson, Wagener, Sies, & Stahl, 2007). The presence of iron or other transition metals can enhance this decomposition reaction. In previous research, 75.4% of the cysteine sulfhydryl groups were recovered when no nitrite was added, and approximately 40% were recovered in cysteine and myoglobin meat solutions with 200 ppm ingoing nitrite (Sullivan & Sebranek, 2012). In this experiment, more sulfhydryl groups were recovered from the alternative curing system treatments and the traditional curing system with added reducing compounds (CP, CP/CH, SN/SE) than the traditional curing systems without added reducing compounds (SN, SN/NA). Holmes and Williams (1998) reported that low concentrations of ascorbic acid at pH 7.4 enhanced the decomposition of *S*-nitrosothiols. Though the pH of the cooked solutions were much lower than pH 7.4, the higher concentration of reducing compounds, particularly in the alternative curing systems, could have resulted in the decomposition of nitrosocysteine. The difference in sulfhydryl groups between meat model systems, where the cysteine only treatment had less residual sulfhydryl groups than the cysteine and myoglobin treatment, is likely due to the preferential binding rate constant of nitric oxide to sulfhydryl and heme groups of 4.5 x 10^5 and 2 x 10^7 mol^{-1} s^{-1}, respectively (Williams, 2004). Sullivan and Sebranek (2012) came to the same conclusion in similar meat model systems that myoglobin is preferentially nitrosylated before cysteine.

Residual nitrite is considered both beneficial and a potential risk in processed meats. Residual nitrite can be reduced to nitric oxide to maintain quality and safety and

formulating with ingoing sodium nitrite concentrations of 150 ppm or greater was shown to reduce the outgrowth of psychrotrophic microorganisms such as *L. monocytogenes* (Xi, Sullivan, Jackson, Zhou, & Sebranek, 2011). However, residual nitrite is also considered a potential risk because, at a low pH or high heat in the presence of secondary amines, it could form nitrosamines (Sen et al., 1979; Skibsted, 2011). However, the formation of nitrosamines is inhibited or greatly reduced with ascorbic acid or other reducing compounds (Mirvish et al., 1972). In this study, curing systems with an alternative source of nitrite (CP, CP/CH) had higher concentrations of residual nitrite than their traditional curing counterparts (SN, SN/NA, SN/SE). This suggests that in the model system there are chemical compounds within the pre-converted celery juice powder, or in the CP/CH treatment cherry powder which may reduce the formation of nitric oxide, or regenerate nitrite within the medium. However, in deli turkey breast, no difference in residual nitrite was seen between a traditional curing system (synthetic nitrite) and an alternative curing system (celery juice powder) at equivalent ingoing nitrite concentrations (Redfield & Sullivan, 2015). When a reducing compound was included in the curing system treatment, regardless of traditional or alternative source, residual nitrite was reduced significantly. The efficiency of ascorbic acid and isoascorbate as a “cure accelerator” has been established both in traditional curing systems and alternative curing systems as one way to reduce residual nitrite and enhance the formation of cured meat color (Choi et al., 2017; Defreitas, Olson, & Kraft, 1988; Djeri & Williams, 2014; Fox & Ackerman, 1968; Izumi, Cassens, & Greaser, 1989).

Sodium erythorbate (isoascorbate) and ascorbic acid (either synthetically produced or from acerola cherry juice powder) are known to be potent antioxidants,

reducing compounds, and cure accelerators (Choi et al., 2017; Izumi et al., 1989; Li, Shao, Zhu, Zhou, & Xu, 2013) . Furthermore, they are known to react quickly with the stable radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) which would explain the difference between those curing systems with added reducing compounds (SN/SE, CP/CH) and those without (SN, SN/NA, CP; Brand-Williams, Cuvelier, & Berset, 1995; Jayathilakan, Sharma, Radhakrishna, & Bawa, 2007). Other antioxidants such as polyphenols are capable of reacting with DPPH, though at much slower rates than ascorbic acid or isoascorbate (Brand-Williams et al., 1995). In this experiment, 30 minutes was given to allow for color reduction, which has been shown to allow for the complete reaction of DPPH with rapidly reducing compounds (ascorbic acid, isoascorbic acid), and intermediate reducing compounds (tocopherols, and rosmarinic acid; Brand-Williams et al., 1995). Vegetables and fruit are known to be sources of antioxidants which are reducing compounds. Celery is known to contain antioxidant polyphenolic compounds such as caffeic acid *p*-coumaric acid, and ferulic acid, as well as flavonoids such as apigenin, luteolin and kaempferol (Yao, Sang, Zhou, & Ren, 2010). Acerola cherries are known to contain high levels of ascorbic acid, anthocyanins, phenolic compounds, and dietary carotenoids all of which are potent antioxidants (Delva & Schneider, 2013). When dried, these compounds concentrate so adding even small amounts of dried vegetable or fruit powders, such as were used in this study, could result in the differences seen in residual reducing capacity between the traditional curing systems and the alternative curing systems. It would be of benefit to the scientific community to determine the concentration and reaction mechanisms of such reducing compounds in alternative curing systems with nitrite, and the endogenous compounds of meat.

The differences in residual reducing capacity (neutralization of DPPH in this study) of curing systems with different ingoing nitrite concentrations may be explained by the reaction of nitrite with reducing compounds. Nitrite is known to rapidly react with ascorbic acid and isoascorbate to produce nitric oxide. Thus, the reactions would oxidize those reducing compounds and make them unavailable to react with DPPH, resulting in a lower residual reducing capacity with higher ingoing nitrite concentration (Izumi et al., 1989; Sanmartin et al., 2000). Though it has been shown that ascorbic acid or ascorbate can act as a nucleophile to decompose *S*-nitrosothiols, differences between meat model systems were not seen indicating that the reactions between reducing compounds and nitrosated cysteine or nitrosylated myoglobin did not significantly deplete reducing compound reserves (Holmes & Williams, 1998).

In conclusion, there were small differences in reactions with myoglobin and cysteine between traditional curing systems and alternative curing systems utilizing pre-converted celery juice powder with and without added reducing compounds (sodium erythorbate and ascorbic acid from cherry juice powder). Added reducing compounds increased the nitrosylation of myoglobin to produce cured meat pigment, lowered the residual nitrite concentration, and resulted in a greater capacity to act as antioxidants. Alternative curing systems, at higher ingoing nitrite concentrations, had slightly higher concentrations of residual nitrite and higher residual reducing capacity. It is believed that the endogenous phenolic compounds in celery and acerola cherry may contribute to the added residual reducing capacity as well as regeneration of the sulfhydryl groups on the amino acid cysteine.

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6. Figures and Tables

Table 6.1: Model curing system formulation

	Sodium Nitrite (SN)		Sodium Nitrite and Salt (SN/NA)		Sodium Nitrite, Salt, and Sodium Erythorbate (SN/SE)		Celery Juice Powder (CP)		Celery Juice Powder and Acerola Cherry Powder (CP-CH)	
Model Meat System	Cysteine	Myoglobin / Cysteine	Cysteine	Myoglobin/ Cysteine	Cysteine	Myoglobin / Cysteine	Cysteine	Myoglobin / Cysteine	Cysteine	Myoglobin / Cysteine
Stock Solution (mL)										
Phosphate Buffer (pH 5.6)	2.5	-	2.5	-	2.5	-	2.5	-	2.5	2.5
Cysteine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Myoglobin	-	2.5	-	2.5	-	2.5	-	2.5	-	-
Synthetic Sodium Nitrite[1]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	-	-	-
Sodium Chloride (NaCl)[2]	-	-	2	2	2	2	-	-	-	-
Sodium Erythorbate[3]	-	-	-	-	0.5	0.5	-	-	-	-
Pre-Converted Celery Juice Powder[4]	-	-	-	-	-	-	2	2	2	2
Acerola Cherry Powder[5]	-	-	-	-	-	-	-	-	0.5	0.5
De-ionized Double Distilled Water	2.5	2.5	0.5	0.5	-	-	0.5	0.5	-	-
Total solution volume (mL)	10	10	10	10	10	10	10	10	10	10

[1] Contained variable nitrite concentrations, 10-200 ppm in the total solution
[2] Formulated to result in 0.5% NaCl in the total solution
[3] Formulated to result in a concentration of 2.76 mM in the total solution
[4] Vegstable 504 (Florida Food Products, Inc., Eustis, FL) contained variable concentrations to result in 10-200 ppm nitrite ingoing in the total solution
[5] Vegstable 515 (Florida Food Products, Inc.) formulated to result in 2.76 mM ascorbic acid in the total solution

Table 6.2: Least square means for main effects of curing system (sodium nitrite (SN); sodium nitrite and sodium chloride (SN/NA; 0.5%); sodium nitrite, sodium chloride, and sodium erythorbate (SN/SE; 2.76 mM); pre-converted celery juice powder (CP); pre-converted celery juice powder and acerola cherry powder (CP/CH; to provide 2.76 mM ascorbic acid)) and ingoing nitrite concentration (10, 50, 100, 150, 200 ppm) for cured meat pigment.

Curing system	**Cured meat pigment (ppm)**
SN	15.96[b]
SN/NA	18.18[b]
SN/SE	19.23[ab]
CP	16.76[b]
CP/CH	22.57[a]
SEM[1]	0.99
Ingoing nitrite concentration	**Cured meat pigment (ppm)**
10	13.4[z]
50	17.85[y]
100	19.71[y]
150	20.52[y]
200	21.22[y]
SEM[1]	0.97

[1]SEM=standard error of the means

[a,b] and [yz] Means in the same column within a trait with different superscripts are significantly different ($P \leq 0.05$)

Table 6.3: Least square means for main effects of curing system (sodium nitrite (SN); sodium nitrite and sodium chloride (SN/NA; 0.5%); sodium nitrite, sodium chloride, and sodium erythorbate (SN/SE; 2.76 mM); pre-converted celery juice powder (CP); pre-converted celery juice powder and acerola cherry powder (CP/CH; to provide 2.76 mM ascorbic acid)) and ingoing nitrite concentration (10, 50, 100, 150, 200 ppm) for residual reducing capacity (DPPH neutralized).

Curing system	**DPPH[2] neutralized (µM)**
SN	2.98[d]
SN/NA	3.14[cd]
SN/SE	4.98[b]
CP	3.46[c]
CP/CH	6.65[a]
SEM	1.00
Ingoing nitrite concentration	**DPPH[2] neutralized (µM)**
10	4.65[x]
50	4.41[xy]
100	4.18[yz]
150	4.01[yz]
200	3.93[z]
SEM	1.00

[1]SEM=standard error of the means

[2]DPPH= stable radical 2,2'-diphenyl-1-picrylhydrazyl

[a-d and x-z] Means in the same column within a trait with different superscripts are significantly different ($P \leq 0.05$)

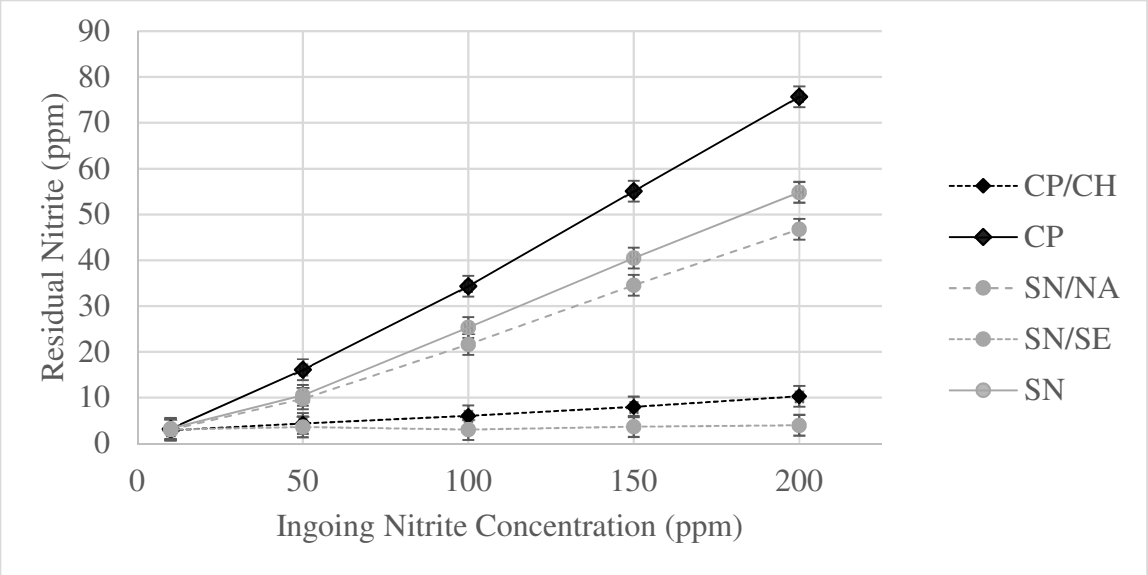

Figure 6.1 Interaction of curing system and ingoing nitrite concentration on residual nitrite concentration. Curing system: sodium nitrite (SN); sodium nitrite and sodium chloride (SN/NA; 0.5%); sodium nitrite, sodium chloride, and sodium erythorbate (SN/SE; 2.76 mM); pre-converted celery juice powder (CP); pre-converted celery juice powder and acerola cherry powder (CP/CH; to provide 2.76 mM ascorbic acid). Error bars indicate ± standard error means.

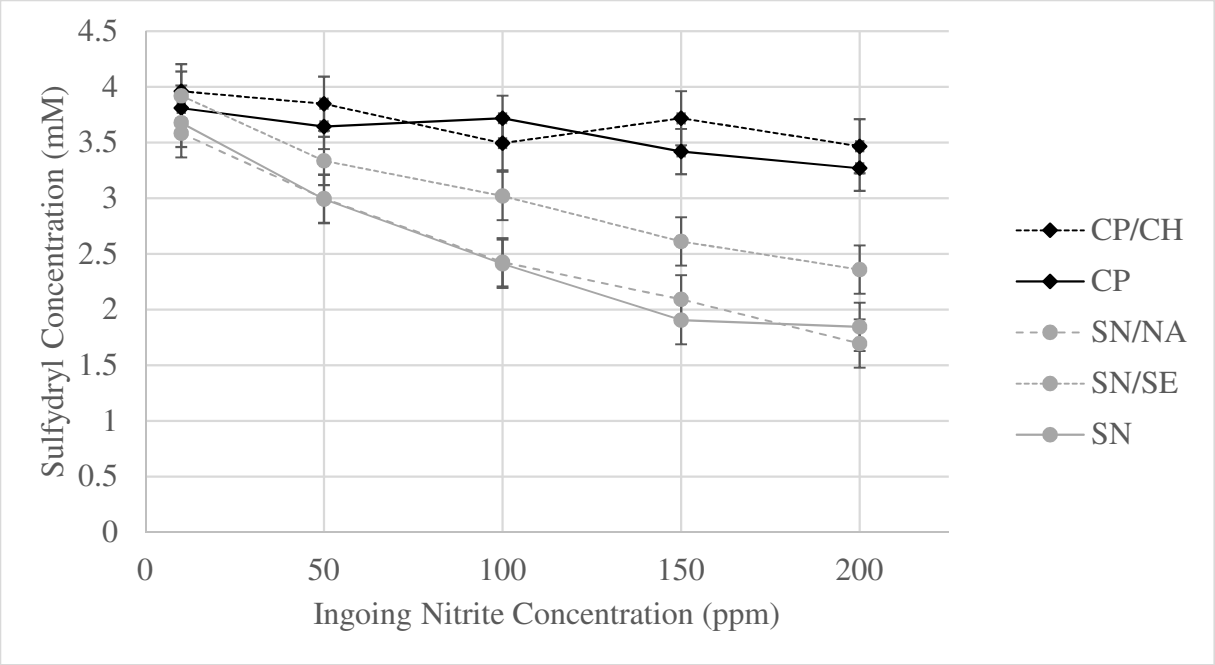

Figure 6.2 Interaction of curing system and ingoing nitrite concentration on sulfhydryl group concentration. Curing systems: sodium nitrite (SN); sodium nitrite and sodium chloride (SN/NA; 0.5%); sodium nitrite, sodium chloride, and sodium erythorbate (SN/SE; 2.76 mM); pre-converted celery juice powder (CP); pre-converted celery juice powder and acerola cherry powder (CP/CH; to provide 2.76 mM ascorbic acid). Error bars indicate ± standard error means.

7. Appendices

7.1 Stock solution preparation

0.5M Sodium Hydroxide (NaOH) stock solution -

1) 0.5M NaOH was used to raise the pH of the potassium monophosphate buffers to 5.6 and 7.4 respectively.
   a) 20g of NaOH was dissolved in approximately 250 mL DDD and then diluted to 500 mL in a volumetric flask

0.1M Potassium monophosphate buffer- pH 5.6 and 7.4 (1L each)

1) Potassium monophosphate buffer 5.6 was used to prepare the equine myoglobin and L-cysteine stock solutions, and the pH 7.4 buffer was used in the Ellman's sulfhydryl groups reaction.
   a) 13.6g KH_2PO_4 (potassium monophosphate) was dissolved in approximately 250 mL DDD
   b) NaOH was added dropwise to adjust the pH to either 5.6 or 7.4 respectively
   c) The volume of the buffered solutions was brought to 1L in a volumetric flask

Sodium Nitrite Stock Solution (1000 ppm)

1) Nitrite solution of 1000 ppm was used to prepare curing system ingoing nitrite solutions and the residual nitrite standard curve.
   a) 1g $NaNO_2$ was dissolved in approximately 250 mL of DDD and then diluted to 1L in a volumetric flask.
   b) A 100 ppm intermediate solution was made by diluting 50 mL of the 1000 ppm stock solution to 500 mL in a volumetric flask with DDD.
   c) A 1 ppm working solution was made by diluting 5 mL of the 100 ppm solution in 500 mL of DDD with a volumetric flask.
   d) Experimental ingoing nitrite ppm stock solutions were prepared according to the following dilutions:

[NO_2] ppm	0	10/20	50/100	100/200	150/300	200/400
mL [1000 ppm NO_2]	0	0.5/1.0	2.5/5.0	5.0/10	7.5/15	10/20
mL ddH_2O	50	49.5/49.0	47.5/45.0	45.0/40	42.5/35	40/30

Sodium Erythorbate Stock Solution -Made day of replication

1) Stock sodium erythorbate solution was prepared to provide a reducing compound for the traditional curing system (2.76 mM sodium erythorbate in the final simulated cooked meat solution)
   a) 1.2g of sodium erythorbate was dissolved in DDD and diluted to 100 mL in a volumetric flask.

2.5% Sodium Chloride (NaCl) stock solution – Made day of replication

1) Stock NaCl solution was prepared to create a treatment with the same level of salt as the alternatively cured system since Celery Juice powder is standardized with salt (0.5% in a 10 mL cook solution as we prepared it).
   a) 5 g of NaCl was dissolved in DDD and diluted to 200 mL in a volumetric flask

Pre-converted Celery Juice Powder Stock solution (formulated for the equivalent of 1000 ppm sodium nitrite)

1) Stock Pre-Converted Celery juice powder solution was prepared to use for dilutions to make the alternative curing systems.
   a) 15.85g Vegstable 504 (pre-tested to contain 13300 ppm nitrite) was diluted in 250 mL of DDD
   b) Experimental ingoing nitrite ppm stock solutions were prepared according to the following dilutions

[NO_2] ppm	0	10/20	50/100	100/200	150/300	200/400
mL [1000 ppm NO_2]	0	0.5/1.0	2.5/5.0	5.0/10	7.5/15	10/20
mL ddH_2O	50	49.5/49.0	47.5/45.0	45.0/40	42.5/35	40/30

Cherry Juice Powder Stock solution (formulated to provide 2.76 mM of ascorbic acid in final solution)

1) Stock cherry juice powder was prepared to provide a reducing compound for the alternative curing solutions.
   a) Based on supplier recommendations 9.67g was dissolved and diluted in 100 mL DDD in a volumetric flask

Model Meat stock solutions- equine myoglobin, and L-cysteine- Made immediately before combining curing solutions and model meat solutions

1) Stock myoglobin solution was prepared to result in 0.177 mM Myoglobin in the final model meat solution
   a) 0.300g equine myoglobin was dissolved in 150 mL of 0.1 M pH 5.6 potassium monophosphate buffer
2) Stock L-cysteine solution was prepared to result in 20.25 mM L-cysteine in the final model meat solution
   a) 0.7980g L-cysteine hydrochloride hydrate was dissolved in 250 mL of 0.1M pH 5.6 potassium monophosphate buffer

Reagent Stock Solution Preparation

1) Ellman's Reaction (Sulfhydryl group quantification)- Ellman's reagent was prepared by:
   a) 0.1586g 5,5'-dithiobis-(2-nitrobenzoic acid) (DNB) dissolved in 20 mL pH 7.4 potassium monophosphate buffer
2) Residual Nitrite- Sulfanilamide and NED reagents were prepared by:
   a) Sulfanilamide
      i) 0.5g sulfanilamide dissolved in 150 mL 15% glacial acetic acid solution
   b) N-(1-naphthyl) ethylenediamine di hydrochloride (NED)
      i) 0.2g NED in 150 mL 15% glacial acetic acid solution
3) DPPH Solution (2,2'-diphenyl-1-picrylhydrazyl) approximately 88.1uM solution to read about 1.1 abs at 515 nm was prepared by:
   a) 0.0344g dissolved in 1L Methanol

7.2 Model Solution Formulations

- Cysteine-only
  - 2.5 mL stock cysteine solution
  - 2.5 mL pH 5.6 buffer

    EITHER
  - 2.5 mL Nitrite Variable Solution and 2.5 mL DDD
  - 2.5 mL Nitrite Variable Solution, 2 mL 2.5% salt solution, 0.5 mL DDD
  - 2.5 mL Nitrite Variable Solution, 2 mL 2.5% salt solution, 0.5 mL Reducing Agent solution
- Cysteine-Myoglobin
  - 2.5 mL stock cysteine solution
  - 2.5 mL stock myoglobin solution

    EITHER
  - 2.5 mL Nitrite Variable Solution and 2.5 mL DDD
  - 2.5 mL Nitrite Variable Solution, 2 mL 2.5% salt solution, 0.5 mL DDD
  - 2.5 mL Nitrite Variable Solution, 2 mL 2.5% salt solution, 0.5 mL Reducing Agent Solution
- Solutions cooked for 30 min at 40C and 30 min at 80C then allowed to cool for 15 minutes

Nitrite Variable Solutions- in 50mL tubes

- Nitrite Solutions

	0	10/20	50/100	100/200	150/300	200/400
mL [1000 ppm NO_2]	0	0.5/1.0	2.5/5.0	5.0/10	7.5/15	10/20
mL ddH_2O	50	49.5/49.0	47.5/45.0	45.0/40	42.5/35	40/30

Final Model Solutions for each ingoing concentration of nitrite (10, 50, 100, 150, 200 ppm):

	Sodium Nitrite (SN)		Sodium Nitrite and Salt (SN/NA)		Sodium Nitrite, Salt, and Sodium Erythorbate (SN/SE)		Celery Juice Powder (CP)		Celery Juice Powder and Acerola Cherry Powder (CP-CH)	
Model Meat System	Cysteine	Myoglobin/ Cysteine	Cysteine	Myoglobin/ Cysteine	Cysteine	Myoglobin/ Cysteine	Cysteine	Myoglobin/ Cysteine	Cysteine	Myoglobin/ Cysteine
Stock Solution (ml)										
Phosphate Buffer (pH 5.6)	2.5	-	2.5	-	2.5	-	2.5	-	2.5	2.5
Cysteine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Myoglobin	-	2.5	-	2.5	-	2.5	-	2.5	-	-
Synthetic Sodium Nitrite[1]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	-	-	-
Sodium Chloride (NaCl)[2]	-	-	2	2	2	2	-	-	-	-
Sodium Erythorbate[3]	-	-	-	-	0.5	0.5	-	-	-	-
Pre-Converted Celery Juice Powd	-	-	-	-	-	-	2	2	2	2
Acerola Cherry Powder[5]	-	-	-	-	-	-	-	-	0.5	0.5
De-ionized Double Distilled Wate	2.5	2.5	0.5	0.5	-	-	0.5	0.5	-	-
Total solution volume (ml)	10	10	10	10	10	10	10	10	10	10

[1] Contained variable nitrite concentrations, 10-200 ppm in the total solution

[2] Formulated to result in 0.5% NaCL in the total solution

[3] Formulated to result in a concentration of 2.76 mM in the total solution

[4] Vegstable 504 (Florida Food Products, Inc., Eustis, FL) contained variable concentrations to result in 10-200 ppm nitrite ingoing in the total solution

[5] Vegstable 515 (Florida Food Products, Inc.) formulated to result in 2.76 mM ascorbic acid in the total solution

7.3 CURED MEAT PIGMENT- Modified Method of Hornsey and Sindelar

(Sindelar et al., 2007)

Sindelar, J. J., Cordray, J. C., Sebranek, J. G., Love, J. A., & Ahn, D. U. (2007). Effects of varying levels of vegetable juice powder and incubation time on color, residual nitrate and nitrite, pigment, pH, and trained sensory attributes of ready-to-eat uncured ham. *Journal of Food Science*, *72*(6), 388–395.

Duplicate aliquots of 2.5 mL cooked model solution was combined with 10 mL Acetone, and 0.75 mL DDD in an aluminum foil covered glass test tube

1) The solution was vortexed and immediately filtered through Fisher Q#2 filter paper into another aluminum foil covered tube and capped
2) The spectrophotometer was blanked with 80% acetone, 20% DDD
3) The absorbance of samples was read at 540 nm
4) Absorbance was converted to ppm nitrosylhemochrome by multiplying the absorbance by 290

7.4 ELLMAN'S SULFHYDRYL GROUPS -Method of Sullivan and Sebranek

(Sullivan & Sebranek, 2012)

Sullivan, G. A., & Sebranek, J. G. (2012). Nitrosylation of myoglobin and nitrosation of cysteine by nitrite in a model system simulating meat curing. *Journal of Agricultural and Food Chemistry*, *60*(7), 1748–1754.

1) Duplicate aliquots of 0.06 mL cooked model solution was combined with 5.94 mL pH 7.4 potassium monophosphate buffer and 0.03 mL of DTNB solution and vortexed
2) The spectrophotometer was blanked with pH 7.4 potassium monophosphate buffer
3) Solutions were read at 414 nm
4) Absorbance was converted to concentration by a factor of 1.414 $M^{-1}cm^{-1}$

7.5 RESIDUAL NITRITE- Modified method of AOAC 973.31, Redfield and Sullivan

(Redfield & Sullivan, 2015)

[AOAC] Association of Official Analytical Chemists. 1990. Nitrites in cured meat. In: Official Methods of Analysis. 15th ed. Arlington, VA: AOAC 973.31. *Official Methods of Analysis* (pp. 938). (15th ed.) Arlington, VA: AOAC International.

Redfield, A. L., & Sullivan, G. A. (2015). Effects of conventional and alternative curing methods on processed Turkey quality traits. *Poultry Science*, *94*(12), 3005–3014. https://doi.org/10.3382/ps/pev299

1) Duplicate aliquots of 0.04 mL cooked model solution were combined with 3.96 mL DDD and 0.22 mL sulfanilamide reagent
2) The solution was vortexed and held for 5 minutes before the addition of NED
3) 0.22 mL of NED reagent was added
4) The solution was vortexed and held for 15 minutes to develop color
5) The spectrophotometer was blanked with a solution of 4.5 mL DDD, 0.25 mL Sulfanilamide, and 0.25 mL NED
6) The method protocol for creating a standard curve was followed after every replication
7) The absorbance of the samples was read at 540 nm and the standard curve was used to obtain the concentration in ppm of residual nitrite

7.6 DPPH DETERMINATION OF RESIDUAL REDUCING CAPACITY – modified Brand-Williams method

(Brand-Williams et al., 1995)

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25–30.

1) Duplicate aliquots of 0.025 mL cooked model solution was combined with 3.975 mL 8.8uM DPPH methanol reagent and vortexed
2) The solution was held for 20 minutes at 4°C to allow for color development
3) The spectrophotometer was blanked with methanol
4) A solution of only the 8.8uM DPPH solution was read at 515 nm for each replication
5) Solution absorbances were read at 515 nm
6) The concentration of DPPH in solution was calculated by:
    a. ABS(515nm)= 12,509*(C(DPPH))-2.58*10^-3 mol/L
7) The concentration of the solution was subtracted from the concentration of the DDPH only solution to obtain DPPH reduced in uM.

8. Future Research Recommendations

This study suggested that the nitrogen oxide reactions that take place with myoglobin and cysteine are similar in many ways between traditional curing systems and alternative curing systems. However, there were some distinct differences revealed in this model system that could prove interesting. First, there was the difference seen in sulfhydryl groups between traditional systems and alternatively cured systems. It was our speculation that the other antioxidants/reducing compounds native to celery juice powder and cherry juice powder modified the reactions of cysteine with nitrosating compounds. Research could be done to quantify the concentration of these extraneous compounds. Secondly, as this was a model system, it would be interesting to see if the results of this research carry over into an actual meat system, where sulfhydryl groups would be quantified. Other reactions that could be considered testing with this model system would be the addition of some lipids and quantification of oxidation. Additionally, as the DPPH method was a rather simplistic way at confirming the residual reducing capacity, it would be intriguing to be able to quantify the exact amount of ascorbic acid or erythorbate still remaining in the system. Such research might help to identify optimum reducing compound concentrations. Finally, this model system was useful for looking at simplistic reactions, it would be great research to continue to use this system to evaluate other small reactions between nitrite containing compounds and myoglobin and cysteine.