APPLICATIONS OF LACTIC ACID AND ITS DERIVATIVES IN MEAT PRODUCTS AND METHODS TO ANALYZE RELATED ADDITIVES IN RESTRUCTURED MEAT

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Doctor of Philosophy

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

APPLICATIONS OF LACTIC ACID AND ITS DERIVATIVES IN MEAT PRODUCTS AND METHODS TO ANALYZE RELATED ADDITIVES IN RESTRUCTURED MEAT

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a candidate for the degree of doctor of philosophy,

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I would like to dedicate to my loving husband, Yongqiang Yang, and my lovely son Allen Yang, and my lovely daughter Alice Yang. I would like to express my gratitude to my revered parents, Fuxing Huang and Xiaoyu Wang. They have supported me continuously through my entire life. I love all of you.

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APPLICATIONS OF LACTIC ACID AND ITS DERIVATIVES IN MEAT PRODUCTS AND METHODS TO ANALYZE RELATED ADDITIVES IN RESTRUCTURED MEAT

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ABSTRACT

The food supply system in the United States is among the safest in the world. Even so, each year, foodborne illnesses affect 48 million Americans and accounts for 17% of the total population in the United States and leads to 128,000 hospitalizations and 3,000 deaths. The “Farm-to-table” movement refers to the stages of food production from harvesting, storage, food processing, packaging, marketing, and preparing it to be eaten. Any stages of the food chain might cause foodborne illnesses if the foods are not handled properly. The strategies for reduction or prevention of foodborne illnesses are needed at various stages. Lactic acid and its derivatives are widely applied to various processed food products for multiple functions; it can be used as an acidulant, flavoring, pH buffering agent and bacterial spoilage and pathogen inhibitor. In this research, the application of lactic acid for antimicrobial study was investigated; the analysis methods for lactic acid by HPLC (High performance liquid chromatography) and FTIR (Fourier transform infrared spectroscopy) were used to analyze the residual amount of lactic acid in different types of meat; quantitative and qualitative properties of sodium alginate in restructured meat were investigated using FTIR.

The objective of the first two stages was to determine if lactic acid could be employed as a shelf life extender in fresh beef products. Lactic acid solution was used for pre-chill and post-chill beef carcass washing. The study was conducted in two phases.

The first phase consisted of application of lactic acid to pure culture strains and application to beef carcasses. Pure cultures of STEC-8 cocktail were exposed to 55 °C and 25 °C of 0.5% lactic acid for 0 s, 15 s and 30 s. The results were very promising. There were 5.7-6.0 log reductions when STEC-8 were exposed to 55 °C lactic acid, regardless of contact time; however, there was only 1-log reduction when the STEC-8 were exposed to 25 °C lactic acid regardless of exposure time. In the second phase, beef carcasses were inoculated with STEC-8, and sprayed with 2.0% lactic acid. The observations from this study showed that there were 1.8 and 1.2 log reductions at 55 °C and 25 °C, respectively. When an electrostatic sprayer was used, there were no significant differences between spray times, even with different temperatures.

The objective of the third phase was to determine the amount of encapsulated lactic acid in restructured meat and to measure residual lactic acid on the meat surface when fish fillets were immersed into different concentrations of lactic acid solutions ranging from 0% to 5%. This study involved two common lactic acid applications. First, immersion of meat pieces into different concentrations of lactic acid solution; Second, addition of encapsulated lactic acid powder into restructured meat. The amount of lactic acid was evaluated and analyzed with different instruments, such as HPLC and FTIR. The results showed as sodium alginate concentration increased, it showed high WHC, low moisture and low pH when compared with control samples. Compared with HPLC, FTIR method cannot provide extract values, and can only predict the possible amounts. However, these two methods could be used to determine lactic acid levels in the meat industry if they are interested in determining the residual chemical level in meat samples or attempting to determine factors that influence meat quality.

The objective of the fourth phase was to compare different effects of hydrocolloids added to a restructured fish product. In this study, rather than lactic acid, encapsulated lactic acid was added into restructured meat. It was used to release free calcium ions from the calcium carbonate and hence develop alginate gelation. Eight types of meat binders were formulated into fish meatballs, and then physical and chemical characteristics were compared to control samples without binders. The eight types of meat binders included cornstarch, commercial meat-binder, carrageenan, methylcellulose, Activa® RM, plasma powder FG+, plasma powder FG and encapsulated lactic acid with sodium alginate and calcium carbonate. The aim of this stage was to compare and investigate the behaviors of different restructured products with different meat binders during chilled storage and after cooking. This study showed that samples treated with Activa® RM and FG+ and FG produced satisfactory binding in fish balls. These three binders can result in higher cooking yield, hardness texture, and maintain both cooked and raw fish ball lightness during storage period. Considering overall parameters evaluated in this study, it is concluded that Activa® RM binder showed the best functionality or performance, following with FG+ and FG treatments. Samples treated with sodium alginate performed at a medium level.

In the final phase of this study, the FTIR analysis method was developed to quantitate sodium alginate levels and quantify properties of the restructured meat with added sodium alginate, which was added to fish samples at concentrations of 0, 0.5, 1.0, 2.0, or 5.0%. In this study, there were two sample pre-treatment methods for FTIR analysis, which included a directly drying method; the samples were hydrated by vacuum oven only; and a chemical preparation method, which included fat removal by acetone,

enzymatic protein degradation, polysaccharide precipitation, and centrifugation. The objective of this research was to develop a useful alternative method for direct quantitation of total sodium alginate in restructured meat. The results showed that a direct drying method could be used to analysis sodium alginate in meat sample. The FTIR spectroscopy combined with PLS and PCA methods at wavenumber of 800 cm^{-1} can be used for the quantitative analysis of control and different concentrations of sodium alginate. FTIR technique also can be used as a screening tool to determine types of polysaccharides that may be added into meat samples.

In summary, this study is important to the food industry. The promising results could contribute to consumer health. The purpose of this project was to provide reference data for food safety, including both shelf life study and chemical residual testing, which may provide solutions for processing added-value meat samples.

CHAPTER 1
INTRODUCTION

The food supply system in the United States is among the safest in the world. Even so, each year, foodborne illnesses could affect 48 million Americans and accounts for 17% of total people in the United States. This leads to 128,000 hospitalizations and 3,000 deaths annually according to the Centers for Disease Control and Prevention (CDC). The “Farm-to-table” concept refers to all the stages of food production including harvesting, storage, food processing, packaging, marketing, and preparing it to be consumed. It is also called “farm-to-fork”. Any of these stages might cause foodborne related illnesses if the foods are not handled properly. The foodborne illnesses pose $77.7 billion in economic burden annually in the United States based on CDC reports. Foodborne illnesses also lead to a huge challenge and economic burden on consumers, government, and the food industry. The foodborne pathogens can cause sickness or even death. About 2% to 3% among these infected patients have developed serious secondary long-term illnesses. According to the Economic Research Service (ERS) of the USDA (United States Department of Agriculture), annual cost for treating illness related to Shiga toxin-producing *E. coli* (STEC O157) was $478 million in 2009. The costs include, but are not limited to medical bills, kidney dialysis, and transplants, time lost from work and premature death. However, zero risk of microbiological hazards in food is impossible, and no single strategy can eliminate all pathogens or related toxins from the food chain. Foodborne illnesses can be serious, or even deadly, but they are preventable. It is very important for all consumers including food manufactures, to practice food-safe handling protocols and to help reduce the risk of food contamination.

Lactic acid has been normally applied for washing meat carcasses. The spraying process was performed by immersing meat pieces in different concentrations of lactic acid for a short period time. HPLC and FTIR were then used to test the residual organic acid levels on the meat surface. When lactic acid solution was used to spray meat samples or powder lactic acid was formulated into the meat production, they reduced pH dramatically. Encapsulated lactic acid, which could slowly release calcium ions into meat products, has been widely applied into restructured meat samples. The amount of organic acid on the meat surface and the amount of encapsulated lactic acid in restructured meat were investigated by HPLC and FTIR in our study.

Besides its antimicrobial inhibitor properties in meat samples, the physical and chemical properties of lactic acid were also evaluated in our study when different types of meat binders and restructured meat samples were treated with encapsulated lactic acid and sodium alginate. Eight types of meat binders were formulated into fish meatball for comparison. The eight types of meat binders include cornstarch, commercial meat-binder, carrageenan, methylcellulose, Activa® RM, plasma powder FG+, plasma powder FG and encapsulated lactic acid with sodium alginate and calcium sources. There are two types of restructured meat products. One is meat made from trimmings and the other is made from the whole muscle. Both show more uniform, shape, more color, and other attractive properties. In this study, the fish balls were prepared by cutting and blending fish fillets with or without binders. These mechanical actions helped to extract myofibrillar protein and promote gel development. The encapsulated lactic acid was used to improve calcium solubility and cohesiveness of restructured meat products. The aim of this stage of study

was to investigate the behavior of different restructured products with different meat binders during chilling storage and after cooking.

According to the Food and Agriculture Organization of United Nations, more than 1.3 billion tons of food and $750 billion are wasted each year. There is an estimated 4.7 billion pounds of edible seafood supply produced per year, including both domestic and imported products. Nearly 2.3 billion pounds of seafood is wasted each year, which accounts for 50% of total seafood supply. Among the seafood waste, an estimated 330 million pounds is lost during distribution chain, and 573 million pounds is wasted due to bycatch. This study can provide a good strategy to reduce seafood waste and show potential ways to increase seafood consumption. This project can also provide solutions for food poverty. Annually wasted seafood protein can feed as many as 10 to 12 million people based on protein demands. Based on data in 2006, more than 75% of fish production was for human consumption. Half is consumed as fresh fish and the other half undergoes fish processing, such as frozen, canned, cured, dried, salted, and smoked etc. In developed countries, processed fish makes up the majority of products. In developing countries, most fish is consumed as fresh fish or by a simple method of preservation such as drying and salting. In the United States, some processors convert fish into fish fillets, canned fish, or other packaged products. Some processors convert fresh or frozen fish products with various seasoning ingredients into final products sold in retail stores and food service, such as smoked food, sushi, salad, sandwiches, seafood entrees, and meals. Fish balls and fish cakes are not common foods in the United States. The objective of this study is to attempt to provide the food industry with technical support and comparison data for different types of meat binders added to fish products.

Many researchers have investigated the physicochemical properties and sensory characteristics of fish samples with addition of various hydrocolloids; no method has been developed to distinguish polysaccharides among meat samples. In this study, sodium alginate quantitation was analyzed by using FTIR. Many research studies have analyzed sodium alginate with FTIR in pharmaceutical research, film development, and materials development; however, no studies have been carried out to quantitate sodium alginate in complex meat matrices. In this stage, FTIR is a tool to quantitate sodium alginate and distinguish it from other types of hydrocolloids. More research should be done to further optimize the extracting method and reduce the noise from samples spectra.

The aims of this whole project are:

1. Investigate whether Shiga toxin-producing *Escherichia coli* (STEC-8) inoculated on pre-chilled beef will decrease its population when treated with lactic acid at different temperatures

2. Investigate whether Shiga toxin producing *Escherichia coli* (STEC-8) inoculation of chilled beef will decrease its population when treated by electrostatic spraying lactic acid at different temperatures

3. Investigate lactic acid added into restructured meat or used for meat washing by HPLC and FTIR

4. Investigate the physical and chemical characteristics of tilapia fish balls treated with cold-set binders, food hydrocolloids, and commercial meat binder.

5. Develop a method to quantitate sodium alginate in restructured meat products by using FTIR

CHAPTER 2
LITERATURE REVIEW

2.1 Microbiological Hazards Associated with Processed Meat

2.1.1 Microbiological Hazards in Meat and Poultry

Potential biological hazards in meat and poultry include bacteria, toxins, viruses, protozoa, and parasites. Among them, the most common are bacteria. Some of these microorganisms can cause human illness; others can lead to food spoilage. A bacterium is a tiny single-cell microorganism and cannot be seen with the naked eye. Large numbers of bacteria can form visible colonies on the surfaces of growth media. Large numbers of bacteria may also be seen or observed on food, for example, the slimy layer on meat surface and abnormal smell (Doyle and Buchanan 2012).

The potential biological hazards can cause one of three types of illnesses, including infection, intoxication and toxin-mediated infection. Infection is one type of illness when people eat food containing harmful microorganisms, which eventually grow in the intestinal tract and cause illness. Infection can be caused by all viruses, parasites and some bacteria. The most common foodborne bacteria that can cause infection are *Salmonella* spp., *Listeria monocytogenes, Campylobacter jejuni, Vibrio parahaemolyticus, Vibrio vulnificus*, and *Yersinia enterocolitica*. The most common foodborne viral agents that can cause infection are Hepatitis A, norovirus, and rotavirus. The most common foodborne parasites are *Trichinella spiralis, Anisakis simplex, Giardia duodenalis, Toxoplasma gondii, Cryptosporidium parvum*, and *Cyclospora cayetanensis*. When people eat food-containing toxin, it could cause intoxication. Viruses and parasites cannot cause foodborne intoxication. Only some bacteria can cause this type of illness. The most common foodborne bacteria that can cause intoxication are *Clostridium*

botulinum, Staphylococcus aureus, Clostridium perfringens, and *Bacillus cereus*. When people consume food containing harmful bacteria, the bacteria might produce toxins, which will lead to illness. The foodborne bacteria that cause toxin-mediated infection are: *Shigella* spp. and Shiga toxin-producing *Escherichia coli,* which include O157:H7 and other Shiga toxin-producing *Escherichia coli* (Doyle and others 2001).

2.1.2 Pathogen Associated With Processed Meat

2.1.2.1 Slaughter for All Species

The Food Safety and Inspection Service (FSIS) announced the zero tolerance policy for visible fecal contamination (USDA-FSIS 1998). Fecal contamination is the primary source of pathogens. The occurrence of pathogens, such as *Salmonella*, *E.coli* O157:H7 and other STECs and *Campylobacter* on carcasses, varies depending on the prevalence and numbers of pathogens on the feathers, skin and the intestinal tract of the animals. It also relies on whether there is any cross-contamination during slaughter and processing (Zhao and others 2001). The pathogens that may come from fecal material or on the body surface or the intestinal tract of an animal can be introduced into the slaughter step if proper sanitation processing procedures are not practiced. The organisms can spread from carcass to carcass, surface of equipment, any other surfaces, or among meat handlers. *E.coli* O157:H7 and other *E.coli* pathogen outbreaks are primarily related to beef products. *Campylobacter and Salmonella* outbreaks are primarily related to poultry products.

2.1.2.2 Ground and Non-Ground Meat Samples

Cutting, chopping or comminuting are very similar meat processing procedures, which produce small sizes of meat particles. If meat processors are regulated by efficient Sanitation Standard Operating Procedures (SSOP) and Good Manufacturing Practices

(GMP) systems and the meat is not ground, but only processed by cutting, it should still show the same level of contamination as the carcass. The meat grinder and bowl chopper are the two primary devices that are used for meat size reduction. At the beginning, the bacteria stick on the meat surface and they can be distributed throughout the meat during grinding. In addition, the grinding step tends to increase meat temperature, which will help the bacteria to grow rapidly. In theory, this step should not introduce new types of bacteria, but the bacteria could grow rapidly, and may cause cross-contamination from the meat surface to any possible places. Therefore, if the plants have sufficient SSOP and GMP, the possible pathogens associated with ground meat are still the same as the slaughter step.

2.1.2.3 Fully Cooked and Not Shelf Stable

For this category, the finished products are not shelf stable and must be frozen or refrigerated for food safety purposes. One example is hot dogs. If present in raw meat or poultry, *Salmonella*, *Escherichia coli* O157:H7 and *Campylobacter* can be destroyed by proper cooking at certain temperatures for a minimal period of times. *Clostridium perfringens* and *Clostridium botulinum* are two gram-positive spore-forming bacteria. The spores are heat-resistance and able to survive during the cooking process. If food cooling is not performed properly, these bacteria can grow rapidly. The majority of outbreaks associated with *Clostridium perfringens* are undercooked meat. It is also called "food service germ" (Clark 2005). Primary outbreaks are due to large quantities of food without following the properly cooling time window and being left in the range of temperatures between 70°F and 140°F for long time. Food poisoning from *Clostridium perfringens* is common. The illness usually lasts for around 24 hours and is rarely fatal. The symptoms of this poisoning are similar to flu symptoms.

Fully cooked meat products have risk of cross-contamination of *Listeria monocytogenes* and *Salmonella,* which could come from environmental or food contact surfaces (USDA-FSIS 2015). *Listeria monocytogenes* is commonly found in soil, water and some animals. Humans can be infected after consumption of contaminated food. *Listeria* bacteria can live in food processing plants for years, causing contamination of food products. *Listeria* bacteria can be killed by cooking or pasteurization. They can grow and multiply in the food even under refrigerated conditions.

2.1.2.4 Fully Cooked and Shelf Stable

This category applies to products that undergo heat treatment processing to achieve food safety. Food processing includes the following steps: curing, drying, or fermentation processing. The finished products are shelf stable and not required to be frozen or refrigerated during food distribution or storage. The examples are summer sausage, jerky, meat sticks, and kippered beef etc. The shelf stable dried meat snacks show low moisture content, which is around 22-24%. They also show low water activity. The water activity less than 0.85 can prevent all pathogen growth. If the product is vacuum packaged, the water activity can be less than 0.91 under anaerobic conditions. Once the packages are opened, the products should be stored under refrigerated conditions, since it is not considered a shelf stable meat product any more. *Salmonella* and *E. coli* O157:H7 outbreaks have occurred in jerky products. Therefore, the beef should be heated to a minimum 160°F and poultry should be heated to a minimum 165 °F in order to destroy dangerous bacteria. If not all bacteria is destroyed after dehydration, the surviving bacteria can become more heat resistant. If these surviving bacteria are pathogenic, they can cause foodborne illnesses associated with jerky products (USDA-FSIS 2015).

2.1.2.5 Thermally Processed Meat-Commercially Sterile Meat

Thermally processed meats are primary canned food, such as Vienna sausages, luncheon meat, Spam, or canned corned beef. Thermal treatments are critical in controlling foodborne pathogens in this type of meat product. Thermal treatments are among the most useful strategies to eliminate or reduce food pathogens to the acceptable levels. The pathogen of concern in this category of product is *Clostridium botulinum. Clostridium botulinum* is a Gram-positive, rod-shaped, anaerobic, spore-forming, motile bacterium with the ability to produce the neurotoxin botulinum. *Clostridium botulinum* is prevalent in soil and marine sediment worldwide. The bacteria form protective spores when the survival conditions are poor. These spores are generally harmless, and can be found anywhere. However, when the spores begin to grow out into active bacteria and produce neurotoxin. They become dangerous, since the toxin can affect the central nervous system. *Clostridium botulinum* can produce seven different types of neurotoxin and they are named as type A to G. The type A, B, E, and F neurotoxin can cause human illnesses and the rest can cause animal illnesses.

Clostridium botulinum organism grows best under low oxygen conditions; it cannot grow when pH is below 4.6. Foods with higher pH, such as meat with neutral pH, must undergo high temperature processing to destroy the spores. If the canned foods are improperly sealed under pressure, the *Clostridium botulinum* bacteria can be destroyed in boiling water, but the *Clostridium botulinum* spores cannot be destroyed. The low oxygen environment encourages the spores to grow into active bacteria. When the canned foods are stored at room temperature, the spores can germinate and produce neurotoxin that cause people to become sick after consuming the food. However, the spores can be killed by boiling the food for about 10 minutes.

2.1.2.6 Not Heat Treated and Shelf Stable

This category applies to food manufacturing that is processed only by curing, drying, or fermentation steps to achieve food safety. Low-level heat may be applied for some processed meat. Various curing processes have been developed to prevent *Clostridium botulinum* growth and toxin formation. These processes include salt curing, dry salting, pickle curing, sweet pickle curing, and smoking. The curing agent can be applied by immersion, injection, pumping or dry salting. Dried whole muscle, such as dried cured hams like prosciutto, parma and country ham, are grouped in this category. Dried intact pieces of meat include dried pork bellies (pancetta), dried pork shoulders (coppa), and dried beef rounds (bresaola, beef prosciutto, and basturma). Dry and semi-dry summer sausages also belong in this category.

2.1.3 Factors Affecting Microbial Growth

The combination of intrinsic and extrinsic factors could determine meat shelf life and influence meat quality. Intrinsic factors include moisture content, pH, nutrient content, biological structure, redox potential, naturally occurring antimicrobials, added antimicrobials, and competitive microflora. Extrinsic factors include types of packaging, the atmospheres around food samples, temperature conditions, storage conditions, and processing steps. Some other factors include the intended end-use of the product, product history, traditional use, and interactions with other factors.

Moisture Content: Microbes require water in a suitable form to grow in food product. This form of water is defined as water activity (a_w). The a_w of pure or distilled water is 1.00 and the addition of solute will decrease water activity to less than 1.00. For example, the water activity is 22% when sodium chloride concentration is 0.85 and water activity is 0.9% when sodium chloride concentration is 0.995. FDA (2005) addressed that

the lower water activity of solution might increase the ability to kill bacteria when compared with higher water activity at the same temperature. For example, the population of *Salmonella typhimurium* will have one log reduction at water activity 0.995 when heated at 60°C for 0.18 minutes. If the a_w lowers to 0.94, it will take 4.3 minutes to cause one log reduction at the same temperature. The water activity of fresh meat is 0.97-0.99, which provides the optimum growth for the most microorganisms. The water activity can be changed by adding salt, sugar, drying, and binding water. The gram-negative bacteria are generally more sensitive to low water activity than gram-positive bacteria. Many pathogens cannot grow if the water activity is lower than 0.85 (FDA 2005). For *Clostridium botulinum* microbes, the lower limit of water activity is 0.92-0.94 depending on the solute content.

pH and Acidity: Meat and fish tend to be more neutral but could be slightly acidic. The pH values can be changed by addition of weak acids, or by a fermentation process. pKa is another term to describe the pH value, which addresses the state of dissociation of the acid. When pKa is the same as pH value, the concentrations of dissociated and undissociated acids are the same. Organic acids are more effective as preservatives in the undissociated state. Therefore, lowering pH in food samples could increase the effectiveness of organic acid as a preservative. Many studies have reported the optimum, minimum and maximum pH for the growth of different pathogenic and spoilage organisms in foods (Jay 1998). Therefore, controlling organisms' growth by adjusting pH in food can increase product shelf life. In addition, microbial growth controlled by both pH and other intrinsic or extrinsic factors could work better at

preventing growth than using only one single factor. In general, most pathogens do not grow or slightly grow at pH lower than 4.6.

Nutrient Content: The nutrients in meat include water, abundant protein and amino acids, minerals, fat and fatty acids, vitamins, and carbohydrates. Foodborne microorganisms can obtain energy from fat, carbohydrates, and protein. In general, the simple carbohydrates and amino acids are used by microbes first, followed by other complex forms of these nutrients. Since meat has a very complex form of nutrients, several microorganisms can grow in food at the same time. The growth rate depends on the availability of essential nutrients.

Biological Structure: Meat has a protective structure, such as hide, skin, and feathers. These natural layers provide excellent protection against microbial invasion. Meat may have pathogenic microorganisms attached to the surface or trapped within surface folds or crevices (FDA 2015). During preparation of meat samples, such as cutting, slicing, chopping, or grinding, the physical barriers might be destroyed, which increases the chance for the microbes to penetrate the barriers, especially in ground meat. The interior of meat could then be contaminated.

Redox potential: The oxidation-reduction or redox potential of a substance is the ratio of the total oxidizing (electron accepting) power to the total reducing (electron donating) power of the substance (Jay 1998). Redox potential can be determined by measuring the gain or loss of electrons in a substance. Based on microbial growth and their redox potential values, there are three types of microorganisms, including aerobes, anaerobes and facultative aerobes. The redox potential values can be changed depending

on pH in food, microbial growth, packaging types and partial pressure of oxygen in the storage environment, ingredients and composition of the food (Jay 1998).

Naturally occurring and supplemented antimicrobials: Numerous naturally occurring antimicrobial agents are present in animal and plant tissues. These natural antimicrobial agents can be useful as a host defense hurdle against invasion by microbes. They might be derived from barks, stems, leaves, flowers, fruits, animal tissues, microorganisms, herbs, spices, fruits, milk, eggs, and lactic acid bacteria. Smoked fish and meat can result in the development of antimicrobial substances on the meat surface (USDA-FSIS 2011). Maillard reaction between sugars and amino acids with additional heating can influence antimicrobial effectiveness. Examples of natural antimicrobial substances that have been approved by USDA include egg-white lysozyme, hydrogen peroxide, ethanol, and nisin (USDA-FSIS 2016a).

Extrinsic factors: Extrinsic factors include types of packaging or atmospheres around food samples, time and temperature conditions on microbial growth during storage, holding conditions, and processing steps. There are different meat package applications, including overwrap packaging, modified atmosphere packaging (MAP), vacuum packaging, and bulk-gas flushing MAP packaging that are currently used in the meat market (Scetar and others 2010). All microbes have their own optimum temperature for growth. There are mesophilic, thermophilic, and psychrophilic types based on microorganism optimal growth temperature. Mesophilic is one type of organism that can grow well usually between 20 and 45 °C. Thermophilic bacteria contain special heat resistant enzymes and can function at high temperature. Psychrophilic bacteria have the optimum growth at refrigerated temperature (Doyle and others 2001).

2.1.4 Strategies to Prevent STEC-8 in Raw Meat Products

Two strategies were commonly used to control microbial growth, to eliminate microorganisms or inhibit their growth. Many decontamination strategies are applied in the meat industry for controlling food spoilage and food pathogens. There are three types of interventions, including physical, chemical, and natural antimicrobials. The physical interventions include but not limited to animal carcass washing, trimming, hot water washing, rinsing, chilling, irradiation such as ultraviolet light, pulsed light technology, gas plasma, pulsed electric field, and electromagnetic radiation such as dielectric or radiofrequency, microwave radiation and infrared, electrolyzed water, high pressure processing and ultrasonic, and cold antimicrobial treatments such as pre-chill spray or dip, chiller water immersion, and post-chill spray or dip (Midgley and Alison 2006; Zhou and others 2010). Other physical methods, such as moist heat, dry heat, autoclave, boiling, steam pasteurization, steam vacuums, and other types of heat treatments, are not suitable for keeping raw meat samples fresh and away from pathogens contamination. In the meat industry, the most common interventions include spray-washing with different types of organic acids or hot water (Beyaz and Tayar 2010; Bosilevac and others 2006; Castillo and others 2001b; Ellebracht and others 1999; Hamby and others 1987; Snijders and others 1985; Anderson and Marshall 1989; Anderson and Marshall 1990; Anderson and others 1991; Berry and Cutter 2000; Brackett and others 1994; Andrews and others 2002). Many countries prohibit the use of some organic acid sprays, especially chlorine for reducing cross-contamination on poultry meat.

Chemical interventions involve the application of food grade chemicals to the animal or carcass surface to inhibit the growth of microbes. Midgley and Alison (2006) summarized the mode of action of these chemical antimicrobials. Some organic acid

antimicrobials such as lactic acid or acetic acid can alter the pH of the meat surface to achieve preservative purpose. The authors addressed two side effects of chemical interventions. First, resistance may develop to lead to spoilage or pathogenic bacteria possibly being able to survive environmental stresses. This will cause chemical antimicrobial to become less effective over time. Another disadvantage of chemical intervention is the potential effect on employees, corrosive equipment, and sensory effect on meat. Two types of chemical interventions are commonly used in the meat industry. Addition of processing aids is one of them, which should not have residual effects. Another way is to add chemical into meat samples as food additives. The application methods include needle injection, still marinating, and vacuum marinating.

Chemical interventions include but are not limited to chlorine (chlorine dioxide, cetylpyridinium chloride (CPC), sodium hypochlorite, stannous chloride, timsen, acidified sodium chlorite, sodium chlorite), organic acids (lactic acid, acetic acid, citric acid, propionic acid, peracetic acid, lauric acid) and salt of organic acid (sodium lactate, sodium sorbate, sodium citrate, potassium lactate, buffered sodium citrate), inorganic phosphates (trisodium phosphate, sodium tripolyphosphate, acid sodium pyrophosphate); bacteriocins (nisin, magainin; EDTA-nisin), oxidizers (hydrogen peroxide, ozone, quaternary ammonium) and other chemical compounds such as sodium metasilicate, sodium nitrite, sodium nitrate, lauric acid and its salt lauric arginate (Huang 2010; Ma and others 2013; Becerril and others 2013; Buchanan and Bagi 1994). My research will be to investigate the effects of these chemical interventions on STEC-8 growth in food.

Another strategy to prevent STEC or *E.coli* O157:H7 growth is natural antimicrobial, which is made from plants, animals, and microorganisms. Natural

antimicrobials extracted from plants could prevent or inhibit microbial growth and extend shelf life. The presence of both antioxidant and antimicrobial properties in a single compound could be a key in providing an effective food preservative. Most of the plant antimicrobials showed better inhibition effect against gram-positive than gram-negative bacteria. Some plant antimicrobials could inhibit both types of bacteria, which may be due to the antibiotic compound or metabolic toxins (Hayek and others 2013). Herbs or spices and their extracts are the most commonly used natural antimicrobials in the food industry, due to presence of essential oils (Indu and others 2006). These types of antimicrobials include, but are not limit to extracts from clove, kaffir lime peels, cumin, cardamom, coriander, nutmeg, mace, ginger, garlic, holy basil and kaffir lime leave, oregano, thyme and bay. Research showed that these extracted essential oils can produce significant bacteriostatic and bactericidal properties and these components can irreversibly damage *E. coil* O157:H7 cells within minutes (Hayek and others 2013). In general, fruits and vegetables contain phenolic and organic acids that possess antimicrobial activity. Pomegranate juice could inhibit the growth of *E. coil* O157:H7, in addition, guava extracts also have a great inhibition effect against the growth of *E. coil* O157:H7 and *Salmonella*. Xoconostle pears extract could be used as an antimicrobial against *E. coil* O157:H7. Garlic extract, due to presence of allicin, can act as a growth inhibitor for both gram-positive and gram-negative bacteria, such as *E. coil* O157:H7 and *Salmonella* (Avato and others 2000). Chinese chives extract containing sulfur compounds showed strong antimicrobial effect against *E. coli* and yeast (Mau and others 2001).

Some antimicrobials come from plant seeds and leaves extract; some antimicrobials come from animal origin. Pleurocidin was investigated and showed effects

against several foodborne organisms (Burrowes and others 2004). Lactoperoxidase, coming from milk, is an abundant enzyme that showed a strong antimicrobial effect against bacteria, fungi, and viruses (Van Opstal and others 2003). Antimicrobial peptides, found from living organisms including bacteria, fungi, plants, and animals, demonstrate strong antimicrobial activities. Chitosan, a natural linear polysaccharide obtained from seafood has been observed to have antimicrobial effect against *S. aureus* and *E. coli.*O157:H7 (Goy and others 2015).

Microorganisms such as bacteria, fungi, and mold produce different compounds that could be active against other microbes. Many microorganisms can produce antimicrobial compounds, which are widely used in the food industry. These antimicrobial compounds include organic acids, hydrogen peroxide, ethanol, diacetyl, fatty acids and bacteriocins (Hayek and others 2013; Rai and Bai 2014). The antimicrobial effects of organic acids were reported as following order from strong to weak: formic acid, lactic acid, acetic acid, propionic acid. These organic acids are more effective against gram-positive bacteria than against gram-negative bacteria (Hayek and others 2013). Hydrogen peroxide shows strong antimicrobial activity since it produces strong oxidization on the bacterial cell and causes damage on protein molecular structure. Its antimicrobial effect has been demonstrated on both vegetative and spore forms of bacteria, molds and yeast (Hayek and others 2013; Rai and Bai 2014). Bacteriocins are primarily produced in different species of lactic acid bacteria. Bacteriocins are cationic peptides that target the bacterial membrane. These types of antimicrobials have been widely used in food application as biopreservatives. The most important bacteriocins are nisin, diplococcin, acidophilin, pediocin, bulgarican, helveticin, lactacin, and plantaricin.

Diacetyl shows antimicrobial activity by producing citrate compounds and lowering pH values in food samples. Ethanol can cause bacteria protein denaturation and damage. Some microorganism species produce suitable chain length fatty acids demonstrating antifungal properties (Rai and Bai 2014; Hayek and others 2013).

2.2 Lactic Acids and Its Derivatives in Meat Application

2.2.1 Lactic Acid

Lactic acid is a processing aid agent and widely used in the meat industry as an antimicrobial for decontamination of carcasses. It has been approved for use in a HACCP (Hazard Analysis and Critical Control Points) plan to reduce pathogens on the surface of carcasses, primal trimming, and poultry meat (USDA-FSIS 2016a). It is Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA). USDA-FSIS approved usage applied up to 5% at 55^{0}C by continuous spray, mist, fog, small droplet rinse or a dip as an antimicrobial agent applied prior to fabrication, offal, and variety meats (USDA-FSIS 2016a). The antimicrobial mechanism of lactic acid is being able to reduce pH levels, which will prevent bacteria from initiating growth (Mcclure 2009).

2.2.2 Encapsulated Lactic Acid

Unencapsulated acids can react with food ingredients to produce undesirable sensory characteristics, such as decreased shelf life, flavors, or color changes. Encapsulated food acids with formulated coating can dissolve or melt at specific temperatures, which can solve the sensory defect issues. The encapsulated coatings comprises of maltodextrin and hydrogenated vegetable oil. The encapsulated acids will not be active when below certain temperatures. After adding encapsulated acids, further grinding process should be avoided due to potential breakage of coating, which might result in denaturing meat protein, color, and texture. Encapsulated organic acids are one

type of meat processing aid, especially for cured meat products, such as dry and semi-dry sausages (Nedovic and others 2011). In the past years, lactic acid was formulated into summer sausages, pepperoni, and hard salami to develop flavor and reduce pH. However, the products have inconsistent flavor, color, and texture. Uncoated lactic acid or citric acid could not be applied in the production of cured meats due to the instantaneous reaction with meat, rendering it unsuitable for further processing. Encapsulated lactic acid could overcome the product defects since it could be controlled to delay its release under smokehouse temperatures and it can also be used as an alternative to using a starter culture (Poncelet and others 2011; Nedovic and others 2011). The encapsulated lactic acid can develop the desired pH needed for fermentation in a shorter time than starter culture method. The encapsulated acids were added into shelf-stable meat products, which can develop desired aroma and flavor. The encapsulated lactic acid should be added into meat samples after grinding steps, since the grinding process can damage the capsule and the released lactic acid can denature protein and affect meat quality. The intact coating prevents the acid interaction during holding storage. During cooking processes, such as smoking and boiling, the coating will melt and release lactic acid into meat and allow consistently meat quality and achieve the targeted pH and flavor.

2.2.3 Lactic Acid Bacteria

Lactic acid bacteria (LAB) have an important role in food industry due to the production of antimicrobial substances. It can be used as starter culture. In the meat industry, there are three basic applications, including raw fermented sausage, raw cured hams, and pasteurized and sliced prepackaged meats. It also can be used as protective cultures in prepackaged, refrigerated sliced bologna-type sausage and cooked ham to prevent *Listeria* growth. *Lb. sakei* and *Lb. curvatus*

are the predominant LAB in dry-fermented sausage. The main purpose of LAB is to convert fermentable sugars in meat sausage to lactic acid and produce shelf-stable products. During the process, small amounts of acetic acid, ethanol, carbon dioxide, etc. are produced. These
compounds can produce aromatic substances and effect sensory attributes of meat products (Krockel 2013). LAB can produce a buffer condition of a certain pH due to conversion to organic acids. This can alter product texture and reduce stickiness of meat samples. LAB can also be used as protective cultures during the ripening of vacuum-packaged raw beef and it may be useful for reducing *E. coli* O157:H7 in frozen ground-beef patties (Krockel 2013). In some fermented meat products, the starter cultures are used as protective cultures, especially with respect to acid-sensitive bacteria. It may produce bacteriocins inhibitory to *Listeria* and undesired LAB. *Heterofementative* LAB of *Carbobacerium, Leuconostoc* and *Weiseellagenera* are usually more involved in meat spoilage than the homofermentative genera (Krockel 2013; Pothakos and others 2015; Castellano and others 2008). Pothakos and others (2015) summarized the LAB catabolites such as organic acids, volatile fatty acids, ethyl esters, sulfur compounds, ketones, aldehydes, alcohols, ammonia, and other molecules that are responsible for off-flavor of meat spoilage.

2.2.4 Lactic Acid Salt

Sodium, calcium, or potassium lactates are salts derived from the neutralization of lactic acid. Mcclure (2009) pointed out that application of lactates in the meat industry started around the early 1980s, and was initially proposed by Oscar Mayer Foods Corporation due to food safety concern regarding uncured poultry products. Sodium lactate ($C_3H_5O_3$ Na) is a GRAS ingredient. Potassium lactate and calcium lactate share

similar properties and are GRAS. They showed the ability to extend shelf life in processed meat products by controlling the growth of aerobes and anaerobes in meat. The exact mechanism of this property is unclear. There are three proposed mechanisms. First, lactate can change meat water activity. Second, lactate passes through the cell membrane and lowers the intracellular pH value. Third, adding lactate may affect cellular metabolism by inhibition of ATP generation (Mcclure 2009). Lactate shows improvement on fresh meat color stability, meat tenderness, and acts as an antioxidant, which could decrease growth of microorganisms. Some researchers stated that sodium lactate resulted in darkening cooked meat. The possible explanation could be that the myoglobin color may be affected by lactate ion concentration. Once the cellular ion concentration reaches a certain level, the myoglobin pigment can be denatured. Once the concentration passed the threshold, the color would not be able to change regardless of amounts of lactate (Mcclure 2009).

2.3 Meat Binders

Hydrocolloids, also known as gums, come from various sources. Most of these hydrocolloids are not digestible in the human digestive system (Feiner 2006). Carrageenan, sodium alginate, and agar come from various seaweeds, while guar gum, locust bean gum, cellulose, starch, and pectin come from plants. Xanthan gum comes from fermentation by bacteria, and gelatin comes from animal collagen. These gums may be added into meat products for different purposes. They can develop gel and act as a thickener. They could increase water-holding capacity, which will reduce cooking loss and purge loss. The formation of gels can improve meat texture, and the meat can become juicer and moister. In addition, gums do not interfere with the activation of protein within meat products (Feiner 2006).

2.3.1 Xanthan Gum

Xanthan gum is an anionic polysaccharide. It can be used to modify the rheological properties of food products. It is obtained by extraction from the saccharide fermentation with bacterium *Xanthomonas*. It has a high molecular weight and has cold swelling properties. The basic structure of xanthan gum is a polymer of D-glucose units with a trisaccharide side chain. The trisaccharide molecules of mannose and glucuronic acid attach at every second glucose of the polymer chain. The carboxyl groups in this compound may ionize to create negative charge and increase the viscosity of liquid solution in food products (Msagati 2012). Xanthan gum is fully soluble in cold water and the negatively charged carboxyl groups (COO-) on the side chains of the molecules with water molecules develop a highly viscous fluid (Feiner 2006). The xanthan gum is very stable at low pH and high temperature. Xanthan gum added into injection brine for ham can delay the sedimentation of other ingredients within the brine solution. It can keep all brine solute well dispersed for a long period time. When xanthan gum is combined with locust bean gum or guar gum, they show synergistic effects. At 65 °C or higher, the viscosity of xanthan gum decreases as temperature increases (Feiner 2006).

2.3.2 Guar Gum

Guar gum is another polysaccharide that is soluble in cold water. It is composed of molecules of galactose and mannose in a ratio of 1:2. The backbone is a linear chain of β 1,4-linked mannose. The 1,6-linked galactose is bound to every second of mannose to form short side branches (Feiner 2006; Msagati 2012). It is hydrophilic. When added to a water solution at a very low concentration, it forms a thick, viscous gel. It shows similar properties to xanthan gum, but is relatively cheaper. It shows up to ten times thickening capacity than that of starch. It is widely used in meat marinade solution. Since it can be

fully dissolved in cold water, guar gum can hold the brine within the meat product after injection and reduce the purge loss in uncooked meat product during storage.

2.3.3 Carrageenan

The chemical structure of carrageenan is comprised of a polymer chain of sugar units of galactose and anhydrogalactose with conjunction of sulfate group (SO_4^{2-}) (Msagati 2012). Unlike sodium alginate, carrageenan is extracted from red seaweed. There are three main types of carrageenan, including kappa, iota, and lambda carrageenan. The differences among the three types are the positions and number of ester sulfate groups within the molecule. These differences determine the functional properties of each type of carrageenan. An increased numbers of ester sulfate groups within the molecules make that type of carrageenan more soluble at lower temperature.

High sulfate carrageenan shows less gel strength. K-carrageenan contains one sulfate group within the molecule and forms very firm and brittle gels. It shows the strongest gel with the presence of potassium (K+) ions. If there are three ester sulfate groups within its molecule, it cannot from a gel at all (Feiner 2006). The higher amount of potassium cations in carrageenan can result in a firmer gel. The limitation of potassium chloride added into k-carrageenan is around 27-30% within the blend. Exceeding the amount of this limit can decrease the gel strength (Feiner 2006). K-carrageenan applied into meat processing requires temperature control between 68-70 °C in order to fully solubilize the material. Within this temperature range, the carrageenan solution develops a random coil. When the temperature decreases, the random coil develops into a double helix, and the gel network forms when the double helices align with each other. The water molecules are held within the network. Cations (K+) mainly from potassium chloride play an important role in the formation the alignment of helices. The number of

ester sulfates determines how tightly the helices align each other and influence the final firmness of gel. The function of potassium helps to connect or link the coils upon cooling. Therefore, there is no gel system forming without the presence of cations. During cooling period, the meat containing carrageenan should avoid mechanical forces or other external forces, since they will not allow the gel forming properly (Feiner 2006).

According to the production process, carrageenan may be grouped as refined or semi-refined. Both are widely applied in the meat processing industry (Feiner 2006). Refined carrageenan contains around 1-3% insoluble matter, while semi-refined carrageenan contains up to 15% insoluble matter. Refined carrageenan shows smoother, more elastic, and higher gel strength than the semi-refined type. The price of the refined type is significantly higher than the semi-refined type. The semi-refined carrageenan produces a less clear gel than refined type. Once the brine solution is injected and incorporated within the meat matrix, the difference is hardly detectable by consumers in the finished product.

Iota (i-carrageenan) type has two sulfate groups within the molecule. It forms a soft, elastic gel, and is fully soluble between 50°C to 55°C. This type of carrageenan is mainly for development of a gummy texture in meat products, and it is not widely added into meat products. It will from the strongest gel in the presence of Ca2+ ions.

Lambda (λ-carrageenan) type has three sulfate groups within the molecule. It also does not undergo conformational ordering. Therefore, this chemical is cold swelling and does not develop a gel at all. It will act as a thickener only.

2.3.4 Starch

Starch is found the most abundant in plant tissue. It is a polysaccharide and does not taste sweet. The most common sources are potato, wheat, rice, tapioca, and corn.

Depending on the starch types, it might contain protein ranging from 0.1% to 0.7%. The thin layer of protein covers each starch granule (Feiner 2006). It consists of two types of glucose polymers including amylose and amylopectin. Different types of starch show different ratios of amylose to amylopectin. Amylose is essentially linear and amylopectin is highly branched. However,

Coultate and others (2002) addressed that amylose chains are not fully linear and they contain a very small amount of branching characteristics of amylopectin. The length of amylose varies, but it contains about 200–15,000 glucose units, which are bound together with hydrogen bonds. Amylopectin is a larger molecule, containing about 1 million glucose units per molecule.

Amylose is responsible for the firmness of the gel strength of the starch system. The linear chain of glucose units can align in a parallel way. The alignment avoids the access of water and enzyme activity. It also explains why starches containing high amylose require higher temperature to gelatinize and longer times for enzyme digestion. Amylose is unstable in aqueous solutions. The intermolecular and intramolecular interactions increase the viscosity, retrogradation and even precipitation in amylose particles (Feiner 2006)

When starch solutions combine with hot cooked meat matrices, the amylose particles move freely within the hot matrix and the liquid solution is immobilized. During the process of cooling, the linear structure of amylose will force water out. Some of this water is previously bound within the gel. However, cooling too quickly can also cause the retrogradation as the amylose particles do not have enough time to set up the well-organized gel structure. Due to these reasons, meat products containing starch should be

cooled as quickly as possible to avoid the retrogradation phenomenon. However, the level of retrogradation depends on the type of starch. And with the order tapioca > potato > maize > wheat, the wheat starch shows the greatest tendency towards retrogradation (Feiner 2006)

The second major component of starch is amylopectin. It consists of a glucose chain, but the molecule is highly branched unlike the amylose's liner chain. The branches open up the molecules, so they are not packed together as closely and tightly as amylose. Amylopectin is responsible for the elasticity and viscosity of starch gel. Starch that contains more amylopectin develops viscosity better, cooks more easily, and develops gelatinization at a lower temperature than starch with higher amounts of amylose. The higher amylopectin starch also has lower tendency to retrograde.

2.3.5 Methylcellulose

Carboxymethyl cellulose (CMC) or cellulose gum is one of the most abundant food grade hydrocolloids in nature. It is mainly used as a food stabilizer and thickener. Cellulose is a carbohydrate, one of the most common natural carbohydrates in plants. The term methylcellulose means that cellulose has been treated with methyl chloride. The methyl group attaches to the cellulose and forms carboxymethyl gum backbone. Msagati (2012) summarized there were three factors in determining the functional properties of cellulose, including the degree of substitution of carboxymethyl gum by sodium atoms, the chain length of the cellulose backbone structure and the degree of clustering of the carboxymethyl substituent.

Cellulose is a biopolymer, which is made up of glucose units linked in a linear chain formulation. Each hydroglucose unit has three hydroxyl groups (OH groups) that can provide a reactive center that is convenient for various chemical reactions to take

place. Recently, chemically modified polymers have been extensively investigated. Cellulose itself is not soluble in water or organic solvent. By chemical modification, the hydroxyl groups may convert the cellulose into a water-soluble compound or become soluble in a specific organic solvent. The important classes of modified polymers are cellulose ethers such as methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), hydroxyethyl cellulose (HEC) and carboxymethyl cellulose (CMC) (Nasatto and others 2015). All of these derivatives have been commercialized. One well-known company that produces cellulose derivatives is DOW Chemical Company. MC is one of the most important commercial cellulose ethers. It is the simplest cellulose derivative, where methyl groups (–CH3) substitute the hydroxyls at C-2, C-3 and/or C-6 positions of anhydro-D-glucose units (Nasatto and others 2015). The MC is a gelling agent and it will form a gel when it is hot and become liquid when it becomes cool. When combined with other hydrocolloids, such as xanthan gum, the texture of foam is improved.

2.3.6 Sodium Alginate

Alginates are polysaccharides, carbohydrate polymers, and hydrocolloids. They are water-soluble biopolymers that are extracted from brown seaweed. The first research regarding this ingredient was investigated in the late 19th Century by British chemist E.C. Stanford. It took 50 years to get it commercialized. Alginates are comprised of two urinate sugars, the salts of mannuronic and glucuronic acid. When extracting alginate from plants, the uronic acids are converted into the salt forms of mannuronate (M) and guluronate (G) through a neutralization step (FMC-Biopolymer 2016). Alginic acid is a linear, 1, 4-linked copolymer of β-D-mannuronic acid (M unit) and its C5 epimer, α-l-guluronic acid (G unit). The G and M units are joined together in homopolymeric and heteropolymeric sequentially alternating blocks, which affect the strength of gel formed

in restructured food products. The carboxyl groups present at C-6 of the G and M uronic acid units stabilize the glycosidic bonds from acid hydrolysis (Rourke and others 1993). The proportion, distribution, and length of G and M blocks influence the chemical and physical properties of the alginate molecules. G blocks provide gel-forming capacity. Different sequences, and the ratio of G and M blocks provides variable of the uronic acid chains. Therefore, the alginate gel forming ability is determined by the proportion and length of G blocks in its molecular structure. FMC Company addressed the mechanism of gel forming properties. The G blocks in one alginate molecule linked to a similar region in another alginate molecule by means of calcium ions or other multivalent cations. The divalent calcium cation fits into the G blocks and develops the egg box structure. The interaction of two alginate polymers connecting with calcium develops junction zone and results in solution gelation. In addition, alginates are soluble in cold water and no heat or cooling is required for forming a gel. By choosing different food grade alginates, food manufacturers can apply them into different food products and develop a range of structure types from being firm and brittle to being soft and flexible (FMC-Biopolymer 2016).

Alginate is very suitable as a binding agent for meat. It interacts with muscle particle to produce a thermostable gel at temperature below 30ºC. Alginate has been widely used in meat products but not in fish products (Moreno and others 2008). Alginates and alginic acid are widely used in the food industry, biotechnology, and medicine because of their gel-forming capacity. Alginates are polysaccharides obtained from marine brown algae. They are the mixed salts of sodium, potassium, calcium and magnesium, but used mainly in the form of sodium alginate (Msagati 2012). Although

alginates are widely used in food systems as stabilizers and used to change the rheology of food, the most common usage in meat products is forming gelation. It differs from other gel forming hydrocolloids. It forms thermostable gels without thermal treatment (Moreno and others 2008). The alginate gels are formed by intermolecular association of polyvalent cations, such as calcium, with guluronic acid block regions of the polysaccharide molecule. Alginate gel interacts with myofibrillar proteins extractedfrom muscle food. These interactions are mainly because of electrostatic influences between anionic groups on the alginate chain and the positively charged group of the muscle protein. However, little information is provided regarding the molecular mechanism of the change in functionality of myofibrillar protein when it interacting with alginate. Moreno and others (2008) pointed out that the interaction between alginate and protein are mainly determined by the hydrocolloid concentration and calcium ion sources. The interactions between the two chemicals can develop thermostable gels, which are capable of binding minced, ground muscle food. The ratio of sodium alginate and the calcium ion and its source, as well as the setting time and reaction time are all variables that can produce different types of meat texture.

2.4 Instrumental Analysis

2.4.1 HPLC Application in Meat Samples

High Performance Liquid Chromatography (HPLC) is a powerful tool used to analyze the sample mixture or analyte in a solvent at high pressure through a column. Similar to other types of chromatography, such as paper, thin layer, or gas, it is a separation technique and can be used for both quantitative and qualitative analysis of a mixture. Basically, HPLC consists of six main components, the mobile phase (liquid solvent), stationary phase (solid) with column, pump, sample injector, detector and

recorder device, which is also called an integrator (Charde and others 2014). The mobile phase is pumped to the stationary phase under high pressure. The components are separated based on their interaction with the stationary phase. Once the component is separated and is passed the detector, it sends a signal to the recording device.

Three primary characteristics of chemical compounds can be used for separation using HPLC. They are polarity, electrical charge, and molecular size. When separation is based on polarity, there are two primary separation modes: normal phase and reversed-phase chromatography. For normal phase type, the stationary phase is polar and the mobile phase is non-polar. For reverse phase type, the stationary phase is non-polar and the mobile phase is polar. Typically, a strong solvent is non-polar such as chloroform and gasoline, and a weak solvent is polar, such as water and alcohol. Therefore, for separations based on polarity, like is attracted to like and opposites may be repelled (Waters 2016). When separation is based on charge, such as ion-exchange chromatography, like may repel, while opposites are attached to each other. Cation exchange is used to retain and separate positively charged ions on a negative surface, anion exchange is used to keep, and separate negatively charged ions on a positive surface. for separations based on size, such as size-exclusion chromatography or gel-permeation chromatography, the larger molecules elute first and smaller molecules penetrate more of the pores and travel more slowly and eluate later (Waters 2016).

Some other commonly used strong solvents in HPLC from weakest to strongest are methanol, acetonitrile, and tetrahydrofuran (Crawford Scientific 2016). One of the most common stationary phases used in reverse phase is C18. This column consists of endcapped octadecylsilane ($C_{18}H_{37}$) chemically bonded to high purity spherical silica

(SiO_2). When the mixture enters into reverse phase HPLC, the more non-polar chemical reacts with the column pack, and the more polar chemical passes through with the polar mobile phase. Therefore, the more polar substance elutes from the column first, and the more non-polar substance elutes last. As long as there are different polarities between two substances, in theory, they can be separated.

HPLC is able to separate and identify chemical compounds that are present in any sample that could dissolve into a liquid in ranges of concentrations as low as ppm. Sample retention times vary and change depending on the interaction with the stationary phase, the analyzed molecules, solvent types, and concentrations. Many studies have been carried out using HPLC to test different chemicals substances that might be added into meat samples. The majority of these methods involved complicated preparation steps, such as remove fat, protein precipitation procedures after or before extraction or using reversed-phase cartridges to remove sample matrix interferences. The application of HPLC in meat industry includes quantification of specific components, such as food additives, sugars, salt, soy, hydrocolloids, cholesterol, tocopherol, nitrite and nitrate; or natural ingredients in meat, such as lactic acid, protein, and carbohydrate; or adulterated components, such as chemical residues, or carcinogen produced during meat processing.

2.4.2 FTIR Application in Meat Samples

FTIR is a technique that is used to obtain a spectrum of absorption and emission. The main purpose of this analytical method is to measure the amount of light absorbed by a sample at each wavelength. It can be used for qualitative and quantitative analysis purposes. It is a nondestructive measurement of physical and chemical components. It is a simply and rapid method. It can detect functional groups (Hsu 1997). The spectrum

contains important information about the chemical composition of the substance. It works on the basis of functional groups and provides information in the peak areas. The spectrum determination combined with multivariate data methods can be used as rapid food additive screening, concentration comparison, structure change, physical difference, such as moisture, and microbial enzyme changes. Many studies have been performed by using FTIR, for example, the cooked and raw meat adulteration screening tests to ensure the manufacturers have not adulterated their products , since some of them would like lower their cost and illegally replace premium quality meats with cheaper ones (Rahmania and Rohman 2015; Rohman and others 2011; Papadopoulou and others 2011; Zhao and others 2014a; Kurniawati and others 2014; Deniz and others 2015; Lamyaa 2013; Al-Jowder and others 2002; Zhao and others 2014b); monitoring oxidization process in meat samples (Gedikoglu and others 2016); and investigating and monitoring the fresh minced pork meat spoilage (Papadopoulou and others 2011; Nicolaou and others 2012; Kodogiannis and others 2014). FTIR also can be used to predict the content of main chemical components, such as crude protein and intramuscular fat in wet and dry meat samples. More and more researches should be further investigated for other physical, chemical, and microbiology changes related to meat samples compositions, storage and processing.

CHAPTER 3
REDUCTION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* INOCULATED ON PRE-CHILLED BEEF WITH LACTIC ACID AT DIFFERENT TEMPERATURES

3.1 ABSTRACT

Organic acid rinses have been proposed as effective, inexpensive carcass interventions. Lactic acid is the most commonly used compound in beef carcass decontamination. However, application for decontaminating warm carcasses inoculated with eight Shiga toxin-producing *Escherichia coli* strains (STEC-8) and the study of factors that may affect such decontamination have not been fully addressed. In this study, 35 s lactic acid sprays at 55 °C and 25 °C were applied to pre-chilled warm briskets, which had been inoculated with a cocktail of STEC-8. In pure culture experiment, it demonstrated that there were 5.7-6.0 log reductions when STEC-8 was exposed to contact with 55 °C 0.5% lactic acid for 0 s, 15 s and 30 s, while only 1.0 log reduction was achieved when the STEC-8 was exposed to contact with 25 °C 0.5% lactic acid for 0 s, 15 s and 30 s. In contrast, when lactic acid was sprayed on actual beef carcasses inoculated with STEC-8, the reductions observed were only of 1.8 and 1.2 log cycles for 55 °C and 25 °C lactic acid sprays, respectively. These data indicate that the lactic acid might be applied for STEC-8 pathogen reduction in beef carcass processing during pre-chilling, but the temperature of the solution may be a critical factor.

3.2 INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a gram-negative, facultative anaerobe, also referred to as verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC) (CDC 2012). *E. coli* consists of a diverse group of bacteria. *E. coli* bacteria normally live in the intestinal tract of animals and human beings. Most *E. coli* strains are harmless, and play an important role in a healthy intestinal tract.

E. coli strains are used as indicator organisms of fecal contamination. Pathogenic *E. coli* strains are grouped into pathotype, which can cause illnesses, such as diarrhea, vomiting, or other intestinal tract diseases. The pathogenic diseases can be transmitted through contaminated water, food, contact with contaminated animals and persons.

There are six pathogenic types of strains that are associated with diarrhea, including Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC). The STEC pathogens are the most commonly reported in the news, and are one of the most commonly associated with foodborne outbreaks. These groups include *E. coli* serotype O157:H7 and non-O157 serogroups, both of them are responsible for major foodborne outbreaks in the United States. Not all STEC serotypes are pathogens, only relatively small numbers in the entire family of STEC are pathogenic (Farrokh and others 2013a). Since 1982, more than 250 different O serogroups of *E. coli* have been shown to produce Shiga toxin, more than 100 serogroups of these STEC have been associated with sporadic and epidemic human disease (Hughes and others 2006). More and more studies have demonstrated that several non-O157 STEC serogroups were related to outbreaks of foodborne illnesses. The non-O157 STEC infection ranges from mild diarrhea to Hemolytic Uremic Syndrome (HUS) and even death. Non-O157 associated illnesses were not less severe than disease caused by *E. coli* O 157:H7 (Hughes and others 2006). Hughes and others (2006) summarized the global identification of non-O157 STEC identified serogroup in 17 countries. The authors pointed out that in the United States, the most common non-157 STEC groups include O26, O45, O103, O104, O111, O119, O1212, and 0145. In

Argentina, Australia, and Germany, the non-O157 serogroups outbreaks were higher than that of O157. In Germany, non-O157 STEC represented more than 80% of total outbreaks. The most important difference between non-O157 and O157 STEC infections were the illness symptoms. Non-O157 infection had longer diarrhea duration and less bloody diarrhea than O157. The rate of abdominal pain, vomiting, and fever could not be used to distinguish the infection types.

E. coli O 157:H7 can cause disease at a dose of 5-50 cells. The mechanisms of STEC disease broadly include bacterial and host process (Jaeger and Acheson 2000). Producing Shiga toxin is a critical point for the pathogenic category. These types of outbreaks can be categorized as both O157 and non-O157 pathogens. Shiga toxins include Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Shiga toxin must be absorbed into the host's intestinal epithelial cells membrane, then enter into the blood circulation and act on various host cells such as kidney and brain cells, and cause pathological hallmarks of systemic disease (Hughes and others 2006; Thorpe 2004). However, there is no evidence to explain how the absorbance process occurs during STEC infection (Thorpe 2004). Studies supported the theory that the O serotypes associated with human disease had greater adherence ability than the O serotypes isolated from food associated with infection (Jaeger and Acheson 2000).

STEC are noninvasive. Most strategies to prevent HUS related diseases involve preventing further consumption of addition Shiga toxin. Many medicines have been developed to attempt to cure the illnesses. However, no clinical trials could prove that these drugs could prevent the diseases. Unfortunately, once HUS has been developed, there are no strategies or treatments to prevent further damage. Blocking the induction of

cytokine production may have advantages to ameliorate the HUS. Taking antibiotics can cause cytokine induction, which may increase the Shiga toxin expression. Several strategies for therapy have been studied including the use of antibiotics and vaccination. However, there is no specific treatment for *E. coli* O157:H7 infection and the use of antibiotics may be contraindicated. Currently, treatment is mainly supportive to limit the duration of symptoms and prevent systemic complications. Washing hands is currently the most effective way to reduce transmission (Hughes and others 2006; Lim and others 2010).

Each year, an estimated 265,000 STEC infections occur in the United States. Serotype O157:H7 alone takes accounts of 36% of these infections, while non-157 serotype outbreaks account for 64% of outbreaks, Together, these account for 39% of total foodborne illnesses (3.6 million) (Zhao and others 2014b; Brooks and others 2005; CDC 2012; CDC 2014 ; Kaper and O'Brien 1998; Mathusa and others 2010). *E. coli* O157:H7 had been first identified in 1982, it was considered as the most common cause of STEC infection (Farrokh and others 2013b; Gyles 2007). Data from the CDC collected from 1983-2002 showed that 70% of non-O157:H7 STEC infections in the Unites States were caused by one of six major serogroups, which include *E. coli* O26, O103, O111, O121, O45 and O145 (Farrokh and others 2013b; Brooks and others 2005). More than 100 serotypes of STEC have been associated with human diseases reported by Brooks and others (2005). Many non-0157 STEC serogroups were identified, however, not all of them have been shown to cause illness. Data showed that the estimated annual number of illness due to STEC in the United States was 231,157. The estimate cost of each case of O157:H7 human infection was $ 10,048, while non-O 157 STEC of each case was an

estimated cost of $1334 (Scharff 2012). The economic burden for O157 and non-O157 were $635 and $154 million per year, respectively.

In 2012 to 2015, $25 million grant through USDA's Agriculture and Food Research Initiative (AFRI) and administered through USDA's National Institute of Food, Agriculture (NIFA) had been awarded to a multi-institutional, multi-disciplinary team of land-grant Universities and government agencies lead by the University of Nebraska-Lincoln. A subgroup of seven STEC serotypes, including O26, O45, O103, O111, O121, O145, and O157:H7 have been considered as adulterants in ground/non-intact raw beef. Later, O104:H4, which caused a large outbreak in Europe from bean and seed sprouts, was added. The research targets to reduce eight serogroups (STEC-8) across the beef chain. In this research, the cocktail of eight serotypes strains were studied, including O26: H11, O111: H, O103:H2, O121:H19, O45:H2, O145: NM, O157: H7/NM and O104:H4.

Beef carcasses are initially sterile. During the processing of live animals to convert into meat, microbial contamination could occur from the exterior of the live animals or from the processing environment to meat surfaces. In a healthy animal, after killing and evisceration processes, ideally, the inner layers of the carcass are free of contamination from the environment. However, cross-contamination can occur during removal of the intestine, and contact with tools or surfaces such as knives, hooks, walls, as well as human operations. The primary source of bacterial contamination comes from the hide, hair, and hooves of the animal. During the cleaning process or fabrication process, the human workers and surfaces of equipment may spread bacteria from the hide to the product due to cross-contamination. Integration of sanitation methods can be a good way to reduce the cross contamination and improve the meat safety. The strategies

include, but are not limited to hot water spray, organic acid spray, and steam pasteurization, which can reduce the cross-contamination and improve the safety of carcasses after slaughter (Rodriguez 2007; Castillo and others 1999; Castillo and others 1998; Pipek and others 2005).

Organic acid rinses have been proposed as effective, inexpensive carcass interventions. They are used to wash, rinse, and spray to clean carcasses and to reduce numbers of microorganisms. Lactic acid is the most commonly used chemical compound in beef carcass decontamination. Several reports have demonstrated that lactic acid can be used as a sanitizing spraying agent for carcass decontamination. Anderson and Marshall (1990) addressed that lactic acid present in small amounts in blood and animal muscle could lower the pH values of meat surface and inhibit proteolytic bacteria. The mechanism of antimicrobial effectiveness is different with acetic acid, which could produce toxicity of undissociated acid molecules. Application of lactic acids as sanitizing sprays for carcass contamination showed that they could effectively reduce the pathogenic bacteria. Anderson and Marshall (1989) pointed out that there was no discoloration to be observed at concentrations up to 1%, while there was no off-flavor to be detected at concentrations up to 2%. However, the antimicrobial effectiveness of lactic acids could be influenced by temperature. Anderson and Marshall (1989) also discussed that dipping the lean beef muscle into 3% lactic acid at 70 °C was the most effective treatment compared with lower concentrations and lower temperatures of lactic acid application. The authors concluded that compared to the concentration of lactic acid, the temperature is a more significant variable to harm the organisms. Castillo and others (2001 a) reported that using 4% lactic acid solution at 55 °C had significant bacterial

reduction on cold carcass surface and in subsequently ground beef products. Castillo and others (2001b) studied lactic acid sprays on cold beef and ground beef. Castillo and others (1999) decontaminated the beef carcass surface with lactic acids combined with other techniques. Scott and others (2015) concluded that a combination of lactic acid and citric acid solution could reduce the *E. coli* O157:H7, non-O157 Shiga toxin-producing *E. coli*, and *Salmonella.* However, application of lactic acid for decontaminating warm carcasses inoculated with eight Shiga toxin-producing *Escherichia coli* strains (STEC-8) and the study of factors that may affect such reduction have not been fully addressed. No research was published regarding the antimicrobial effectiveness of lactic acid on STEC-8. The objective of this study was to develop a lactic acid spraying system that is suitable for treating pre-chilled beef carcass surfaces to evaluate its effectiveness in reducing the population of STEC-8.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Cultures and Revival Procedures

Eight serotypes of STEC, including O26: H11, O111: H, O103:H2, O121:H19, O45:H2, O145: NM, O157: H7/NM and O104:H4, were obtained from the culture collection of the Food_Microbiology Laboratory in the Department of Animal Sciences at Texas A & M University, college station, TX. These *E. coli* isolates were revived from -80 °C cryogenic storage via triplicate consecutive transfer into sterile 10 ml tryptic soy broth (TSB), followed by overnight aerobic incubation at 37°C. Following revival, cultures were aseptically streaked onto slants of tryptic soy agar (TSA), incubated at 37 °C for 24 h, and maintained on slants at ambient temperature until required for use. One loopful of each culture was transferred to fresh TSA slants every seven to ten days, and incubated at 37°C for 24 h, separately.

3.3.2 Bacterial Inoculum Preparation

For inoculations in the pure culture study, eight individual strains were transferred from TSA slants to tryptic soy broth (TSB) (Difco, Fish Scientific) and growth at 37 °C for 18-24 h separately. Cell cultures (10 ml) of each strain were harvested individually by centrifugation (Eppendorf model 5810 R, Brinkmann Instruments Inc., Westbury, NY; 4,629×g, 15 min, 4°C) at 4,000 x g for 15 minutes, re-suspended into 10 ml phosphate-buffered saline (PBS) for a total of three times. At the last centrifugation step, 10 ml PBS was added and a concentration of 8-9 log CFU/ml was obtained. Inoculum was then prepared by adding 10 ml of the respective strains within each inoculum type together and vortexing to make a cocktail. Stationary-phase cultures were used in this study since previous research demonstrated that stationary-phase cultures have greater heat resistance than log phase culture. The inoculum was used within 3-5 hours for pure culture and the beef project. Preliminary tests (data are not shown) demonstrated that the cocktail strains would not significantly change in population for up to 6 h when placed at ambient temperatures. Cocktail was placed onto TSA media to determine total *E. coli* counts. A 0.1 ml inoculum was added into 9.9 ml 0.5% lactic acid that was kept at 55 °C in a water bath. Samples were collected at 0 s, 15 s and 30 s contact time, separately. Following, the plate counts were carried out by preparing a serial ten-fold dilution, and spread plating on TSA plates. Plates were then incubated at 37 °C in an aerobic incubation chamber for 18 to 24 hours prior to enumeration. The counts were read after 24 h. All counts were recorded as colony forming units per cm^2 (CFU/cm^2).

3.3.3 Bacterial Strains and Preparation of Inoculum in Beef Brisket

For Inoculations in the beef study, eight individuals' strains were transferred to selective media (TSB plus rifampicin (100µg/ml)) and grown at 37 °C for 18-24 h

separately. Rifampicin resistant cultures of each strain were mixed and made into the cocktail. Stationary-phase cultures were used in this study. The eight-strain cocktail was plated onto selective media (TSA plus rifampicin (100µg/ml)) to determine total inoculated count. The inoculum was aseptically transferred into a sanitized misting bottle (VWR). Inoculum was sprayed three times and delivered a total volume of 2.8 ml inoculum.

3.3.4 Lactic Acid and Water Treatments Application

Lactic acid (pH=2.2) was prepared from 88% L-lactic acid (Purac, Inc., Arlington Heights, IL). Following a preliminary study with the comparison of three brands of lactic acids, including Sigma, Birko and Purac (unpublished), a brand was selected. The results showed that there were no significant difference for STEC-8 log reduction at temperature of 55 °C and 25 °C for 3 brands. The Purac brand was selected. Its antimicrobial efficacies were tested at different temperatures on STEC-8 in pure culture and on pre-chilled beef meat surfaces. Two different lactic acid temperatures, as well as two different water temperatures at 55 °C and 25 °C, were evaluated and compared. Distilled water was used to prepare the lactic acid solution. The 0.5% lactic acid was used in the pure culture experiment and the 2% of lactic acid solution was used for spraying on pre-chilled beef. All the test solutions were prepared fresh on the day of the treatment. Delivering the 500 ml lactic acid volume in 35 seconds was calibrated with a graduated cylinder.

3.3.5 Inoculation of Subprimals and Carcass Sanitizing Treatments

Warm briskets before chilling were purchased from ABF, Stephenville, Texas. Each brisket was equally divided into two samplings. One sampling was used for the 55 °C 2% lactic acid treatment; another sampling was used for the 25 °C 2% lactic acid

treatment. The divided samplings were transferred into individual biohazard bags. The plating samples were collected by excising surface area of 30 cm^2 using a borer. The temperatures and pH values of the beef surface were taken prior to inoculation. The pH values of post lactic acid intervention were taken as well.

The subprimals were inoculated with the STEC-8 cocktail. Using a palpation glove, the cocktail was sprayed three times with the spray bottle from a distance of 0.3 meter to the untrimmed fat side of each brisket, which was inoculated with STEC-8 cocktail, followed by a 30 minutes wait to allow bacterial attachment. Three pieces of trimming samples were collected by excising and placed into 0.1 % peptone water. The pH value of the post-treatment meat surface was measured.

Prior to lactic acid treatment, positive control (untreated) samples were taken by aseptically excising three pieces of surface from inoculated beef into peptone water. After 30 minutes attachment, each subprimal was sprayed with warm (55°C) or room temperature (25°C) 2% L-Lactic acid for 35 seconds using a conventional hand pump sprayer with insulation. Lactic acid was applied in a fine mist for 35 seconds from a distance of 0.3 meter from the carcass surface region. The negative samples were removed from the surface area prior to inoculum strains by excising three 10 cm^2 samples (2 mm in depth) using a sterile stainless-steel borer, scalpel, and forceps and compositing them total 30 cm^2 area to establish data on the possible natural presence of the STEC strains. After inoculation, the briskets were let to stand for 30 min to allow bacterial attachment. After the attachment time, immediately prior to treatment, three 10 cm^2 areas were obtained in the same manner to collect data on the true inoculation level of STEC-8. Immediately following the lactic acid treatment, three 10 cm^2 areas were excised to

collect data on the post treatment level of STEC-8. Negative, untreated, and post-treated samples were collected and placed into stomacher bags with 100 ml of peptone water. The samples were homogenized in a stomacher at normal speed for 1 minute, then a series of ten-fold dilutions was taken and spread plating on TSA plates with 0.01% Rifampicin. Plates were then incubated at 37 °C in an aerobic incubation chamber for 18 to 24 hours prior to enumeration. The counts were read after 24 h. All counts were recorded as CFU/cm^2.

3.3.6 Statistical Analysis

Both pure culture and beef experiments were run in triplicate, with the entire experiment repeated twice for a total of six samples per treatment. All bacterial counts were converted to log value before conducting statistical analysis of the data. Data were analyzed by analysis of variance (ANOVA) and general linear model (GLM) procedures. Log reduction was obtained by subtracting the log count (log CFU/cm^2) post treatment STEC-8 population from the log count prior to treatment for each sample. Main effects of temperature and contact time were analyzed. Statistical analysis was conducted using the Tukey of SAS 9.4. $P < 0.05$ was considered statistically significant.

3.4 RESULTS AND DISCUSSION

For cell culture experiments, all 0.5% LA (lactic acid) 55°C treatments significantly reduced the population of STEC-8 from their initial level (ca. 7.8 log CFU/ cm^2, data are not shown) to a level near or below the detection limit of 1.7 log CFU/ cm^2. However, when the temperature of lactic acid reduced to room temperature (25°C), there was only about 1.0 log reduction as seen in Table 3-1. Data on Table 3-1 and Figure 3-1 showed the antimicrobial effect of 0.5% lactic acid at different temperatures. These results demonstrated that the exposure time of lactic acid with STEC-8 cocktail for 0 s,

15 s and 30 s at a temperature of 55°C could cause 5.7- 6.0 log reductions. Compared with different temperatures of distilled water treatments, all experiments including both water treatments at 55°C and 25°C, there was only 1.0 log reduction. From these data, the conclusion was drawn that lactic acid at 55 °C shows significant log reduction for STEC-8 strains compared to lower temperature treatments. When compared with water treatments, once again it confirms the hypothesis that lactic acid at high temperature has better antimicrobial properties against STEC-8, which agreed with the results of Anderson and Marshall (1990). They concluded that lean beef muscle dipping into 2% lactic acid at temperature of 55°C and 70°C caused 0.8 and 1.0 log reduction *E. coli* O157:H7 respectively, which were significantly higher than 40 °C and 25°C lactic acid treatments. However, there were no significant ($P<0.05$) log reduction difference between 55°C and 70°C treatments. As seen in Table 3-1, there were no significant ($P<0.05$) log reduction differences between exposure times for higher concentrations of lactic acid. It showed the same trend at low lactic acid treatments; there was no significant ($P<0.05$) log reduction difference for contact time of 0 s, 15 s, and 30 s. All water treatments had a similar log reductions ($P<0.05$) no matter the temperature of water and the exposure time. The average pH value of 0.5% lactic acid at 55°C is 2.47, the pH value at 25°C is 2.66. The pH values of distilled water at 55°C and 25°C are 7.11 and 7.34, respectively. From the Table 3-1, it can be concluded that the pH change was not the critical factor for lactic acid to reduce the STEC-8 populations. The 25°C lactic acid treatments could not reduce the population drastically. It confirmed that the temperature of lactic acid is one of the main factors that showed a better antimicrobial effect on STEC-8.

As seen in Figure 3-1, it is clear that lactic acid at a higher temperature shows better antimicrobial properties. For lower temperatures of lactic acid treatments, the antimicrobial effects are the same as water treatments no matter its temperature, such as 55°C and 25°C. Data were collected from two trials and the results were based on the average of the trials combined. For the pure culture project, the results demonstrated the higher temperature of 0.5% lactic acid at 55 °C could cause 5.8, 5,7 and 6.0 log reductions when the contact times of strains with lactic acid are 0 s, 15 s and 30 s, while the contact time at 25 °C treatments only could cause an average 0.9 log reduction. From the data in Figure 3-1, it is concluded that lactic acid is a temperature sensitive organic acid. It has better antimicrobial effectiveness at 55°C for STEC-8 cocktail strains.

Table 3-1. Mean Log Reductions (Log CFU/ cm^2) of STEC-8 by 0.5% Different Temperature Lactic Acid Treatments on Cell Culture

Temperature	Treatment	Log reduction (CFU/cm^2) ±SEM[a]
55°C	H_20 [b], 0 s	1.0 ± 0.087 B [c]
55°C	H_20, 15 s	1.0 ± 0.029 B
55°C	H_20, 30 s	1.1 ± 0.087 B
25°C	H_20, 0 s	0.9 ± 0.082 B
25°C	H_20, 15 s	1.0 ± 0.064 B
25°C	H_20, 30 s	1.0 ± 0.121 B
55°C	0.5% LA[d], 0 s	5.8 ± 0.374 A
55°C	0.5% LA, 15 s	5.7 ± 0.410 A
55°C	0.5% LA, 30 s	6.0 ± 0.436 A
25°C	0.5% LA, 0 s	1.0 ± 0.248 B
25°C	0.5% LA, 15 s	0.8 ± 0.241 B
25°C	0.5% LA, 30 s	0.9 ± 0.104 B

[a] Log reduction = ($\log_{10}$ CFU/ cm^2 before treated with sanitation) - ($\log_{10}$ CFU/ cm^2 after treatment). SEM, standard error of the mean

[b] H_20, stand for autoclaved distilled water

[c] Numbers within columns followed by same letter are not significant different ($p < 0.05$)

[d] LA stands for 0.5% lactic acid which was prepared by 88% Purac lactic acid

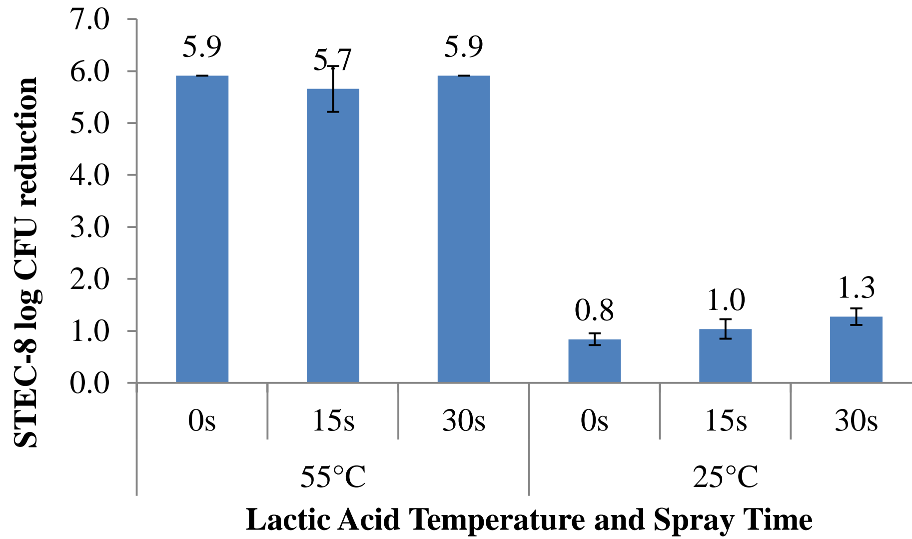

Figure 3-1. STEC-8 Log Reductions in Pure Culture

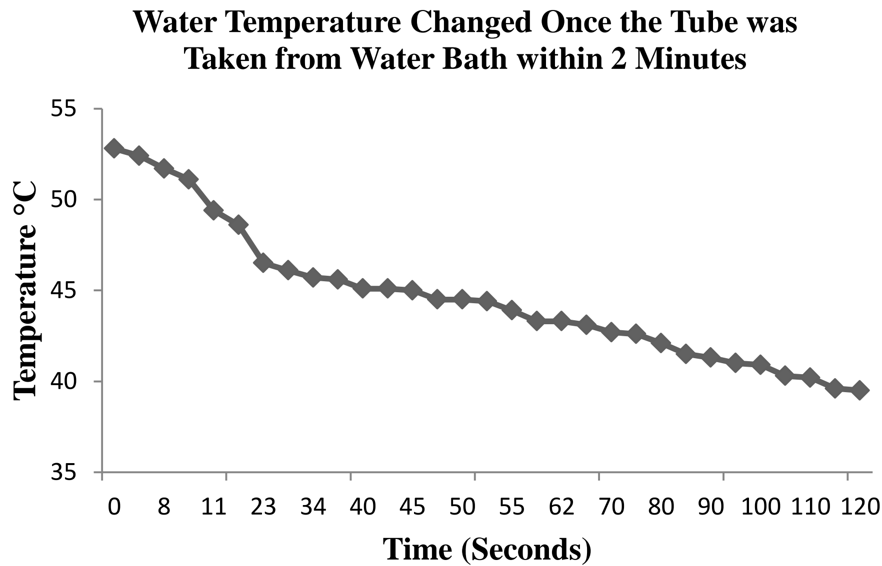

Figure 3-2. Temperature Change Trend of Water within 2 Minutes (The temperature changes by adding 1 mL room temperature water into 9 ml 0.5% lactic acid. The tube was taken out from 55°C water bath before adding).

Figure 3-2 indicates the temperature changed within 2 minutes, when a tube containing 9 ml 0.5% lactic acid was taken out from a 55°C water bath, and immediately

added to 1 mL room temperature water. This process was trying to mimic the culture experiment that a tube containing 9 ml 0.5% lactic acid was taken out from 55°C water bath, then immediately added 1 ml inoculum. This could explain the reason that the log CFU reduction of the preliminary test of 0 s and 15 s were not the same as 30 s contact. In order to reduce the temperature change variable, two tests were done by adding 0.1 ml inoculum into 9.9 ml 0.5% lactic acid that was kept at 55°C in a water bath. Compared with the data from two trials and preliminary tests, it concluded temperature maintenance for the effectiveness of lactic acid is significant.

Data were collected from two trials and the results are based on the mean counts obtained from these two trials. For the warm brisket project, the results demonstrated that the higher temperature of lactic acid at 55 °C resulted in 1.8 log reductions. While spraying lactic acid at 25°C caused 1.2 log reductions. The difference between the log reduction of the beef experiment and the pure culture experiment could be explained by the contact surface temperature of the meat. When the meat was treated with lactic acid, the average surface temperature of meat was 30 °C. Therefore, the contact temperature of lactic acid with meat was lower than the pure culture contact temperature.

Table 3-2. Mean Log Reductions (Log CFU/ Cm^2) of STEC-8 by 0.5% Different Temperature Lactic Acid Treatments on Beef Briskets

Temperature	Treatment	Log reduction (CFU/cm^2) ±SEM [a]
55°C	2% LA[c]	1.8 ± 0.1473 B [b]
25°C	2% LA	1.2 ± 0.1796 B

[a] log reduction = ($\log_{10}$ CFU/ cm^2 before treated with sanitation) - ($\log_{10}$ CFU/ cm^2 after treatment). SEM, standard error of the mean

[b] Numbers within columns followed by same letter are not significant different ($p < 0.05$)

[c] LA stands for 0.5% lactic acid which was prepared by 88% Purac Lactic acid

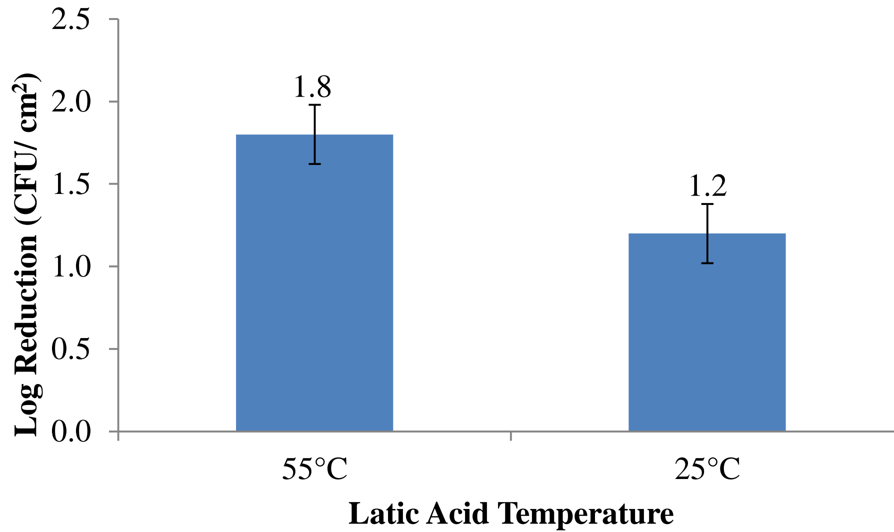

Figure 3-3. STEC-8 Log Reductions in Warm Brisket

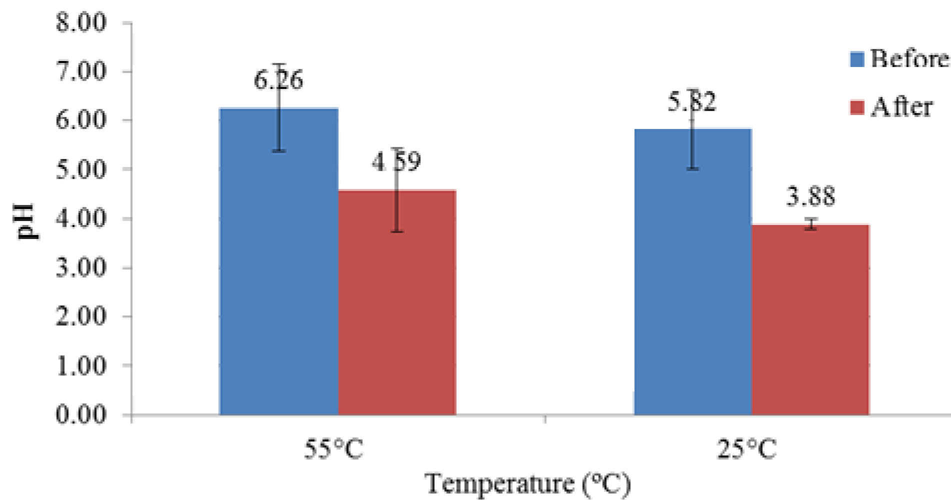

Figure 3-4. The pH Changes Before and After Treated with Lactic Acid (x axis represent lactic acid temperatures and spray times; y axis represent average pH values before and after treatments).

Figure 3-4 describes the meat pH changes before and after being treated with lactic acid. There were significant ($P<0.05$) pH value changes before and after treatments. As mentioned previously, the average pH value of 0.5% lactic acid at 55 °C was 2.47, the average pH value at 25 °C was 2.66. While the pH value of 2.0% lactic acid was about

2.0. The mean pH values of the fatty side of beef surfaces were from 5.82 to 6.26. After the beef was sprayed with 2% lactic acid, the pH values of fatty side dropped to the range of 3.88 to 4.59. The pH values of all treatments dropped 1.7 to 2.0 units after being treated with 2.0 % lactic acid, which was significant ($P<0.05$).

3.5 CONCLUSION

In conclusion, a substantial STEC-8 bacterial count reduction to below the detection limit can be obtained during a pure culture experiment when the cocktail is in contact with 55 °C 0.5% lactic acid for 0 s, 15 s and 30 s. When 55 °C 2% lactic acid was applied to pre-chilled beef, it showed 1.8 log reductions, while there were only a 1.2 log reductions for 25 °C 2% lactic acid. These data indicated that lactic acid might be applied for STEC-8 pathogen reduction in beef carcass processing during pre-chilling.

CHAPTER 4
REDUCTIONS OF SHIGA TOXIN PRODUCING ESCHERICHIA COLI (STEC-8) INOCULATION OF CHILLED BEEF BY ELECTROSTATIC SPRAYING LACTIC ACID AT DIFFERENT TEMPERATURES

4.1. ABSTRACT

In order to avoid or prevent foodborne illness outbreaks, many new strategies to eliminate pathogenic organisms on food have been developed. Electrostatic spray technology (ESS) has shown many advantages compared with a conventional spray method. ESS studies demonstrated that it could improve droplet size, uniform liquid distribution, and is environmentally friendly. In this study, the chilled beef was thoroughly warmed up in a water bath before spraying. ESS was used to spray the warmed brisket with 2.0% lactic acid at 55 °C and 25 °C respectively. The sprayer times were 30 s, 60 s, and 90 s. Preliminary tests were conducted before two independent trials. Blue color dye solutions were used to spray and attempted to cover the fatty and lean sides of the brisket surfaces. The surface temperature and pH of warmed carcasses before and after treatment with 2% lactic acid were measured. Both fatty and lean sides of carcasses were evaluated and analyzed. The log reduction results showed that there were no significant differences between the fatty and lean side. The pH value results demonstrated that the pH values of the fatty and lean sides before treatment had no significant difference ($P>0.05$), while after treatment there were significant reduction ($P<0.05$) of pH values compared with on both sides.

4.2 INTRODUCTION

In 2011, FSIS considered that raw, non-intact beef products or raw, intact beef products that are contaminated with Shiga toxin-producing *Escherichia coli* (STEC) O26, O45, O103, O111, O121, and O145 are adulterated and it is the same for *E coli* O157:H7

(Federal Register 2012). Each year, the CDC (Centers for Disease Control and Prevention) estimates that roughly 1 in 6 Americans (48 million people) get sick, 128,000 are hospitalized and 3,000 die of foodborne infection. Foodborne disease is a common, costly, and preventable public health challenge.

Organic acid rinses are one of the most efficient and common decontamination strategies that have been widely used in the meat industry for years. The effectiveness of organic acids as antimicrobial agents depends on their hydrophobicity and how much completely undissociated acid is present in solution (Bartek Ingredients Inc 2016). The antimicrobial effectiveness improves with hydrophobicity, e.g. sorbic acid and benzoic acid are more potent antimicrobial agents than fumaric acid and propionic acid, while fumaric acid and propionic acid are more potent than lactic acid and acetic acid in many applications. Lactic acid and acetic acid are more potent than tartaric, citric acid and malic acids. Due to the presence of lipids in the microbial cell wall, hydrophobic organic acids can interact with the cell lipid and then disrupt the microbial activity (Bartek Ingredients Inc 2016). Another factor that can influence the antimicrobial potency is the level of completely undissociated acid, which can be calculated from the pKa value. Dissociated acids are less effective because they are less hydrophobic, and the level of completely undissociated acid increases as the pH value decreases. Weak organic acids are most effective at low pH (Bartek Ingredients Inc 2016; Van Beilen and others 2014).

Lactic acid has natural antimicrobial properties and it naturally exists in cheese, pickled vegetables, and meat. It is widely used in the United States for beef processing but was not approved for import into the EU until 2013. The EU insisted that using hot clean water could remove surface contamination and cause log reductions similar to

chemical washing. Commission regulation (EU) No 101/2013 allows the application of lactic acid as a pathogen reduction treatment on beef carcasses, half-carcasses and beef quarters in the slaughterhouse (Commision Regulation 2013). This approval showed great benefit for US beef marketing and demonstrated the significant progress of for science-based meat processing (USDA-FSIS 2016b).

Recently, more and more studies have been performed to compare the antimicrobial reduction of conventional sprayer and electrostatic spraying systems. Electrostatic spraying technology has advantages in improving droplet size, size distribution and deposit rate on targets, absorption and uniformity (Esehaghbeygi and others 2010; Jia and others 2013). Large electrostatic sprayers have been widely used in China for controlling the plant and forestry industry. The small electrostatic sprayers (ESS) were developed by Electrostatic Spraying Systems, Inc. Based on the product description, the heart of the ESS sprayer system is the Max-Charge induction electrostatic nozzle. The electrostatic charges induced by the nozzle cause the droplet to move toward to any direction and cover the target surface with small droplets, which are 30 to 40 microns in diameter. The air and liquid go into at the rear of nozzle separately. Before the air and liquid leave the nozzle, the air hits the liquid and develops many tiny droplets. Then thousands of tiny droplets enter the charge ring, and become charged droplets once coming out of the nozzle. Since the droplets can move in any direction and cover the surface of target, due to the gravity of droplet, the charged droplet can move to the hidden side of target. This process causes uniform spray coverage on hidden surfaces, while the conventional sprayers cannot achieve the purpose.

The droplets are electrically charged when leaving the nozzle; the droplets attract to the target surface instead of drifting away. The coverage of tops, sides, and bottoms is increased 70 times when compared with conventional sprayers. The electrical charging causes an attraction force between the droplets and the target surface. The electrical charge of the droplet is small when compared with the force of attraction to the surface, which is strong. The electrical forces that push the droplet toward the surface is 40 times greater than gravity force. In other words, when the droplets move to the target surface, due to gravity force, the droplets will change moving direction, and move upwards against gravity (MaxSpray International Corporation 2013).

Therefore, due to the fast speed of droplet and high charges produced by the machine, it creates a 360-degree wrap-around coverage phenomenon.

With an ESS system, a high electrical charge is applied to liquid by electrode. As the charged liquid is sprayed, it forms small particles of about 30 to 60 microns in size. The charge caused attraction between the droplets and the target surface. The charge on the droplets pulls the spray towards the target at 75 times the force of gravity, which can explain the reason that the deposition on the target surface could be evenly distributed and coat the entire surface of the objective. The advantages of ESS technology are improving the fine droplet size; higher deposit rate on targets and even size distribution. In addition, it is environmentally friendly, less chemical is used, it is more effective, and shows 300% better spray penetration and coverage on hidden areas of plants (ESS Europe Ltd 2014). The important parameters of ESS are determined including charge-to-mass ratio, flow rate, and the distance of nozzle to target.

Traditional spraying is an inefficient approach for application of multiple antimicrobials, since it requires higher amounts of liquid to cover the target. ESS has been used to spray pesticides and used in the food industry for years. ESS combined with organic and inorganic acids have been studied to apply to vegetables to reduce the populations of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 (Massey and others 2013; Ganesh and others 2010; Ganesh and others 2012). Using the ESS technique in the meat industry has been recently studied, Nam and others (2011) concluded that ESS spray of ascorbic acid on the surface of ground beef can be an efficient and economical approach to prevent lipid oxidation, maintain meat color, and oxidative volatiles during storage. Phebus and others (2014) published results regarding optimum ESS parameters to calibrate devices, test chemical and test the distribution rate on meat carcass surface when using fluorescent dye to spray. However, no research has been conducted on the decontamination of the STEC-8 cocktail strains with ESS. This study determined the effects of 2% lactic acids electrostatically sprayed on STEC-8 inoculated warmed beef brisket. The objective of this research was to evaluate the STEC-8 log reduction on both fatty and lean sides with 2.0% lactic acid at 55°C and 25 °C separately. The fatty and lean surfaces meat pH values were collected and compared.

4.3 MATERIALS AND METHODS

4.3.1 Bacteria Cultures and Revival Procedures

Eight serotypes including O26: H11, O111: H, O103:H2, O121:H19, O45:H2, O145: NM, O157: H7/NM and O104:H4 were obtained from the culture collection of the Food Microbiology Laboratory in the Department of Animal Sciences at Texas A & M University, College Station, TX. *E. Coli* strains isolates were revived from -80 °C cryogenic storage via triplicate consecutive transfer into sterile 10 ml tryptic soy broth

(TSB), followed by overnight aerobic incubation at 37°C. Following revival, cultures were aseptically streaked on to slants of tryptic soy agar (TSA), incubated at 37 °C for 24 h, and maintained on slants at ambient temperature until required for use. Every 7 to 10 days, a loopful of each culture was transferred to fresh TSA slants and incubated at 37°C for 24 h, separately.

4.3.2 Bacterium Inoculum Preparation

For inoculations in the pure culture study, eight individual strains were transferred from TSA slants to tryptic soy broth (TSB) (Difco, Fisher Scientific) and growth at 37 °C for 18-24 h separately. Broth was centrifuged at 4,000 x g for 15 minutes, and pellets were re-suspend into 10 ml phosphate-buffered saline (PBS). Cell cultures (10 ml) of each strain were harvested individually by centrifugation (Eppendorf model 5810 R, Brinkmann Instruments Inc., Westbury, NY; 4,629×g, 15 min, 4°C), re-centrifuged, and added into PBS to obtain a concentration of 8-9 log CFU/ml. Inocula were then prepared by adding 10 ml of the respective strains within each inoculum together and vertexing to make the cocktail. Stationary-phase cultures were used in this study. Previous studies demonstrated that the cultures at stationary phase had greater heat resistance than log phase cultures. The inoculum was used within 3-5 hours for pure culture and beef project. Preliminary tests (data are not shown) demonstrated that the cocktail strains would not significantly change the population for up to 6 h when placed at ambient temperatures.

4.3.3 Bacterial Strains and Preparation of Inoculum in Beef Brisket

For the inoculations of beef study, eight individuals' strains were transferred to selective media (TSB plus rifampicin (100µg/ml)) and growth at 37 °C for 18-24 h separately. Rifampicin resistant cultures of each strain were mixed and made the cocktail. Stationary-phase cultures were used in this study. Cocktail was plated onto selective

media (TSA plus rifampicin (100μg/ml)) to determine total inoculated count (TIC). Inoculum was aseptically transferred to a sanitized misting bottle (VWR). The misting bottle should be sprayed a fine mist of liquid. The bottle was sprayed three times and calibrated to deliver the volume 2.8 ml inoculum on the front sides (fatty sides) and back sides (lean sides), respectively. A waiting of 30 minutes was allowed to provide time for bacteria attachment at room temperature.

4.3.4 Electrostatic Sprayer Parameters

The brisket subsections (n=3 subsections/ treatment) were spray treated using an electrostatic spray system with 2% lactic acid. The flow rate was 130 mL/min. The charge to mass ratio should be range at least -5mC/kg to -12 mC/kg or higher. The distance between the sprayer nozzle and target was 80-100 cm. The spray time was designed for 30 s, 60 s and 90 s. Lactic acid (pH=2.2, prepared from 88% L-lactic acid, Purac, Inc., Arlington Heights, IL) was used. All the test solutions were prepared fresh on the day of the treatment. The solution was kept warm with insulation material around the tank.

4.3.5 Subprimals Inoculation and Sanitation Treatments

The cold briskets were purchased from a local grocery store, Texas. The briskets were warmed with in a water bath. Each warmed brisket was equally divided into three samplings. These three samplings per brisket were used for three treatments, 30 s, 60 s and 90 s spraying separately. Six briskets were used for each experiment. There were 18 samplings in total. Three briskets were randomly selected for 55 °C 2% lactic acid treatments. Another three briskets were used for 25 °C 2% lactic acid treatments. Samplings were transferred into biohazard bags. The plating samples were collected by excising a surface area of 30 cm^2 using a borer from front and back sides of each

sampling. The temperatures of the beef surface were taken prior to spraying. The surface pH values were taken from the back and front sides of brisket before and after lactic acid treatments. The inoculation procedures were the same as the description above. Both front sides and back sides were inoculated which follows a standard inoculation method developed at Texas A&M University. The negative samples were excised from 6 briskets separately. The untreated and post-treated samples were placed into bottles with 100 ml of peptone water. The electrostatic spray and samples were taken at the Food Safety Center of Texas A & M University. After taking samples for microbiological analysis, the samples were transferred back to the Meat Microbiology lab of Texas A & M University. Preliminary testing was evaluated to compare difference between log reductions (data not shown) data of different samples. The treated meat samples were put into stomacher bags without peptone water and treated samples were put into peptone water immediately after excising. The results showed that the latter method was better because contacting the treated meat sample with peptone water could immediately neutralize the lactic acid. The samples with peptone water were poured into stomacher bags and homogenized at normal speed for 1 minute. Following serial 10-fold dilutions, spread plating on TSA plates with 0.01% Rifampicin. Plates were then incubated at 37 °C in an aerobic incubation chamber for 18 to 24 hours prior to enumeration. The counts were read after 24 h. All counts were recorded as colony forming units per cm^2 (CFU/cm^2).

4.3.6 Statistical Analysis

The ESS experiments were run in triplicate, with the entire experiment repeated twice for a total of six samples per treatment. Data were analyzed by analysis of variance (ANOVA) and general linear model (GLM) procedures. Microbiological count data were

transformed into logarithms before obtaining means. Log reduction was obtained by subtracting the post recovery STEC-8 population from the initial inoculum level into sample. The main effects of temperature, contact time were analyzed. Statistical analysis was conducted using the Tukey of SAS 9.4. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

4.4 RESULTS AND DISCUSSION

The pH of the meat surface changes after it has been sprayed with lactic acid for different spraying times and spraying liquid temperatures. The results are shown in Table 4-1. For the fatty side, the values immediately and significantly dropped between 4.0 and 4.4 after sanitizing with different temperatures of lactic acid solution and with different spraying times ($P < 0.05$). Before sanitization, there was no significant difference between any of the meat surface samples. In theory, all these meat samples were inoculated with the same amount of STEC-8, so the pH values of these samples were expected to be the same. After spraying with 2.0% lactic acid, the data demonstrated that the pH of the fatty side of the meat dropped 1.0 unit. For the 55°C treatments, there was a significant drop in pH regardless of the spray time of the fatty sides. However, for the lean sides the drop in pH was not significant ($P < 0.05$). Before treatment, all the samples had the same surface pH value. Except for 30 s and 60 s at 55°C, the pH dropped significantly between 4.5 and 5.2. Table 4-1 data also demonstrate that the pH values before and after sanitization were not significantly different from each other. This was true for both the fatty and lean sides of the meat surface. The mechanism of lactic acid decreasing the pH of meat surfaces has been well explained (Ganesh and others 2012; Anderson and Marshall 1990). Lactic acid, a commonly used organic acid, is a GRAS substance, and it has been considered as strong antimicrobial agent. It is present at a

small amount in blood and muscle of animals, and can reduce the pH of meat. The reduction of pH can inhibit proteolytic bacteria (Anderson and Marshall 1990; Massey and others 2013). Because of its small molecular weight of lactic acid (90.08 g/mol), lactic acid can easily invade the bacteria cells and change the internal pH of meat.

Data in Table 4-2 show the STEC-8 log reduction calculation obtained from fatty and lean sides of meat with 55°C and 25°C at 2.0% lactic acid spray for different spray times. Preliminary testing of cell cultures showed that the 55°C lactic acid could significantly reduce the STEC-8 for 6.0 log reductions. However, in this study, there was only less than 1.0 log reduction for all treatments. When preparing the lactic acid solution, the solutions were measured at 55°C and 25°C, separately. However, the ESS XT-3 has a 7.62-meter hose length. During the spraying process, the temperature is significantly reduced. In addition, in order to wrap up all the sides of the meat, the distance from nozzle to the target was set at 80 to 100 cm. After the droplet is sprayed from nozzle, the charged droplet develops a fine mist, it absorbs the energy from the environment, and it rapidly reduces the temperature of the lactic acid particles. When the particle reaches the meat surface, the temperature of meat surfaces was around 21-23 °C, it was a similar temperature for all treatments, regardless of the initial lactic acid temperature. These results were in agreement with previous studies that had shown the ineffectiveness of lactic acid sprays on reducing bacterial counts on cold beef surfaces (Castillo and others 2001a)

However, the log reduction data supported the hypothesis that ESS can spray the antimicrobial solution evenly over both sides. It supported that there were no significant log reduction for both sides after treatment with different times of lactic acid. Statistically,

there was no difference, however, except for 60s ESS spraying, all treatments of the fatty side showed a greater bacterial log reduction than the log reduction on the lean side. A similar finding was summarized by Hardin and others (1995). They addressed that organic acids showed more effective and greater log reduction from both pathogenic and nonpathogenic bacterial populations when applied to adipose compared to lean tissue.

Table 4-1. Mean pH Values of Front and Back Brisket Before and After Treatment with Lactic Acid Applied with an Electrostatic Sprayer (N = 6 Values Per Mean)

	Treatment Temperature \ Spray Time (Seconds)					
	55 °C			25 C°		
Position	30 S	60 S	90 S	30 S	60 S	90 S
Fatty Side Before	5.39^{bx}	5.68^{ax}	5.51^{abxy}	5.33^{bx}	5.34^{bx}	5.50^{abx}
Fatty Side After	4.39^{ay}	4.24^{abz}	4.18^{abz}	4.16^{abz}	3.98^{abz}	3.91^{bz}
Lean Side Before	5.48^{ax}	5.60^{axy}	5.62^{ax}	5.35^{ax}	5.54^{ax}	5.49^{ax}
Lean Side After	5.48^{ax}	5.24^{aby}	5.18^{abcy}	5.02^{bcy}	4.85^{cdy}	4.52^{dy}

a–c Means in same row with different superscripts differ significantly ($P < 0.05$).
x-z Means in same column with different superscripts differ significantly ($P < 0.05$).

Table 4-2. Mean Log Reduction of Front and Back Brisket after Treatment with Lactic Acid Applied with an Electrostatic Sprayer (N = 6 Values Per Mean)

	Treatment temperature \ Spray time (Seconds)					
	55°C			25°C		
Position	30 S	60 S	90 S	30 S	60 S	90 S
Fatty Side Log Reduction	0.67^{ax}	0.41^{ax}	0.35^{ax}	0.61^{ax}	0.39^{ax}	0.95^{ax}
Lean Side Log Reduction	0.30^{ax}	0.67^{ax}	0.31^{ax}	0.22^{ax}	0.57^{ax}	0.42^{ax}

a Means in same row with different superscripts differ significantly ($P < 0.05$).
x Means in same column with different superscripts differ significantly ($P < 0.05$).

Electrostatic spraying is a technique where a charged particle is attracted to the oppositely charged target. Due to the Coulomb's forces, the charged particles follow electrical field lines, wrap around, and coat all sides. This experiment was designed based on the Coulomb's law. ESS can take advantage of these physical principles to allow the lactic acid to wrap around all sides of briskets, and reduce the amount of chemical used

compared with conventional spraying systems (Castillo and others 1999; Castillo and others 2001a) which sprayed 500 ml for 30 s for one side. However, there was only 65 ml lactic acid used on both sides of meat surface when the ESS was applied. In addition, the lactic acid solution can reach the hidden places of the meat surface.

4.5 CONCLUSION

In general, the data revealed that the pH of brisket before and after lactic acid treatment had the similar values. After treated with lactic acid, the pH values of the front (fatty side) and back (lean side) of briskets decreased significant ($P<0.05$). From the data, it also revealed prior to treatment, the pH values of the fatty sides and lean sides were significantly different. After being treated with lactic acid, there were significant differences between the fatty sides and lean sides. There is not much significantly a difference of pH values between the two different temperatures. There are also no significant differences between the different spray times at 30 seconds, 60 seconds, and 90 seconds (Table 4-1). The log reduction of front and back after treated with lactic acid with an electrostatic sprayer had no significance even with different temperatures and different spray times (Table 4-2). Further research should be designed to increase the environmental temperature when using ESS with lactic acid solution. The future research should investigate using different antimicrobials that are not greatly influenced by temperature.

CHAPTER 5
USE OF HPLC AND FTIR AS A TOOL FOR ANALYSIS OF LACTIC ACID ADDED INTO RESTRUCTURED MEAT AND MEAT WASHING WITH LACTIC ACID

5.1 ABSTRACT

Lactic acid has been added to numerous meat samples for several functions. It has antimicrobial characteristic and can be used as a washing agent for meat products. It can reduce pH values and provide binding assistance to calcium carbonate. In this study, a modified method has been developed to determine the amount of lactic acid in fish samples. The HPLC apparatus was equipped with a UV absorbance detector set at 210 nm, and the column temperature was set at 55 °C. Chromatographic separation was performed on an Aminex HPX-87C column. The mobile phase consisted of acetonitrile (6%) and 0.045 N H_2SO_4, with a flow rate of 0.5 ml/min. Centrifuge and filter methods were used to extract lactic acid. The results showed that this method is suitable for fish or meat products. It can determine the amount of lactic acid in different applications. This method is simple, fast and can be used in quality control of the meat industry. It also can be used to analyze other types of food. The lactic acid extracted from meat samples also was evaluated by FTIR method. FTIR combined with PLS (Partial Least Squares Regression) based prediction method is capable of perceiving the change of the lactic acid concentrations. This method can be considered as a potential tool for monitoring food safety and quality control in the meat industry.

5.2 INTRODUCTION

Lactic acid is also called α-hydroxypropionic acid, or 2-hydroxypropanoic acid. It was first found and isolated in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele (Kompanje and others 2007b). It is an organic acid compound belonging to the group of carboxylic acids, which is present in plants, animals, the human body, and soil.

In 1808, another Swedish chemist, Jons Jakob Berzelius (1779–1848), isolated lactic acid from the fluid or purge extracted from meat products (Kompanje and others 2007a). Pure and anhydrous lactic acid is a white crystalline solid with a very low melting point. Lactic acid has two optical isomers, L (+) and D (-). Figure 5-1 shows the chemical structures of two isomers. L (+)-lactic acid is the biological isomer as it is naturally present in the human body; it is the normal intermediate in metabolism. The D (-)-lactic acid is primarily produced by bacterial, plants and some types of algae. The consumption of the D-isomer in humans must be controlled and the WHO recommends that daily intake be about 100 mg kg^{-1} body weight (Vargas and others 2016). High levels of D-isomer are harmful to humans (Theron and Lues 2010).

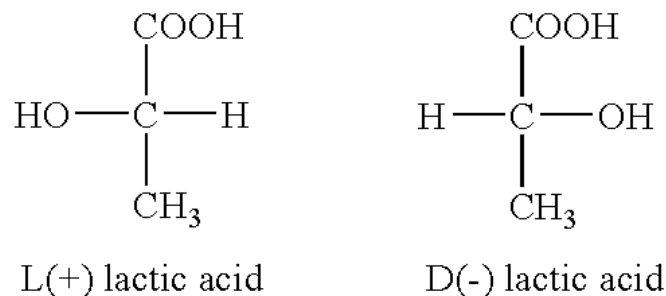

Figure 5-1. L (+)-Lactic Acid and D (-)-Lactic Acid Chemical Structures

L (+)-lactic acid has been widely used in the food industry. It can be used as a flavoring agent and preservative in cheese, salad dressing, pickles, and beverages. Lactic acid also can be produced naturally by lactic acid bacteria. L-lactic acid can be found in many fermented dairy products, pickled products and cured meats and fish (Vargas and others 2016). The determination of lactic acid amount is of great interest in both the food industry and pharmaceutical clinical diagnosis. In the food industry, the control of lactic acid in fermented food, such as beer, yogurt, cheese, milk and other dairy products is critical to ensure food quality. In addition, L (+)-lactic acid inhibits the growth of *E.coli*

better than D (-)-lactic acid. It has been used as a hurdle technology to control bacterial growth and avoid cross-contamination.

Berge and others (2001) investigated the margination in lactic acid solution as softening and flavoring in meat products. The function of lactic acid is to decrease the resistance of meat, and soften the collagen rich tough muscle. By injecting lactic acid solution into muscle meat, samples can be reduced in mechanical strength and increased in meat tenderness. In addition, many experiments have been conducted for decontamination of muscle food with lactic acid by either the spraying method or using the immersion method. Beyaz and Tayar (2010) ascertained that spraying 2.0 % lactic acid on sheep carcasses immediately after slaughter could significantly reduce the numbers of Coliform and *E. coli* and increase shelf life. Bosilevac and others (2006) addressed that in the meat industry, 2.0 % lactic acid spraying applied to pre-evisceration carcasses can reduce aerobic plate count and *Enterobacteriaceae*. Carpenter and others (2011) indicated that 2.0 % lactic acid or acetic acid is a commonly used in industry for decontamination. It has been suggested as one critical control point in hazard analysis and has been considered as a critical limit to reduce pathogens; however, more studies should be further investigated a combination of organic acids to reduce microbial growth. Many researchers studied decontamination of muscle meat, such as beef trimming (Castillo and others 1998), beef carcass surface (Castillo and others 1999), chilled beef carcass (Castillo and others 2001b), cold carcass and subsequent ground beef products (Castillo and others 2001b), with lactic acid alone or in combination with other antimicrobials and reduction of the total microbial population on beef tissue (Anderson and Marshall 1989). They also studied lactic acid alone or in combination with other antimicrobials on lean

beef (Anderson and others 1991); Lactic acid in combination with other antimicrobials to extend the shelf-life of raw beef with vacuum packaging (Ariyapitipun and others 1999); Lactic acid and combination of other antimicrobials on *Listeria* monocytogenes on raw beef with vacuum packaging (Ariyapitipun and others 2000); Effects of lactic acid and antioxidants on the shelf life of beef steak (Djenane and others 2003); Reduction of pathogens on beef trimming (Ellebracht and others 1999); Effect of lactic acid on pork quality (Grajales-Lagunes and others 2012); Inhibition of *Brochothrix thermosphacta* growth in vacuum packaged meat by lactic acid (Grau 1980); Effect of lactic acid on the aerobic growth of psychrotrophic pathogens and spoilage bacteria on lean and fatty pork products (Greer and Dilts 1995).

Another lactic acid application is to add into restructured meat technique. Schmidt and Means (1986) first formulated sodium alginate and calcium into restructured beef in raw state. The meat particles were bound together by reacting sodium alginate and calcium carbonate to form an insoluble algin/ calcium gel system. Encapsulated acids are small beads of acid surrounded by a lipid coating. The acid should be gently mixed and blended into the algin/ calcium gel system near the end of final mixing. Extra mixing after adding acid could disrupt the lipid coating. While adding non-encapsulated lactic acid or acetic acid during the mixing processing can cause the meat protein to coagulate, and negatively impact the meat texture (CookingInn 2015). Mukherjee and others (2009) developed a meat model system for restructured beef products, including salt/phosphate, algin/calcium, Activa® RM, and Fibrimex with or without lactic acid. The authors attempted to compare the destruction levels for inactivation of *Escherichia coli* O157:H7 in ground beef. Ren (2013) a study to compare the effect with different lactic acid sources

on restructured fish with alginate systems. The lactic acid sources include lactic acid bacteria, encapsulated lactic acid, and powder lactic acid. Rahman (2007) compared the encapsulated organic acids, such as lactic, citric and Glucono delta-lactone (GDL), which was added into restructured products for in the development of color and flavor in meat emulsions. The encapsulated lactic acid can control the pH decrease and prevent unexpected protein the binding during blending process.

USDA-FSIS (2016a) updated the list of the approved substances and antimicrobial interventions that are safe and suitable ingredients to add into meat samples. A hydrochloric acid, phosphoric acid, and lactic acid aqueous solution mixture can be used in raw and ready to eat (RTE) meat products and in water used in poultry processing, while lactic acid alone cannot exceed 5%. An aqueous solution of 4% sodium diacetate, 4% lactic acid, 2% pectin, and 0.5% acetic acid can be added into cooked meat products. The solution weight cannot account for more than 0.5% of the finished products. The solution of calcium sulfate and lactic acid can be applied into raw poultry carcasses, parts, giblets, and ground poultry. The concentration of lactic acid cannot be over 5%, when applied as spray and dip methods at 55°C. The raw meat and organ meat can be sprayed or dipped into a blend of lactic acid (45-60%), citric acid (20-35%) and potassium hydroxide (>1%) solution and the total amount of solution cannot be more than 2.5% by weight. The blend of salt, lactic acid, sodium diacetate, monoglycerides, and diglycerides can be used in any type of meat samples, but the total amount of combination cannot exceed 0.2% of total weight of meat samples. Lactic acid also can be applied to carcasses, parts, subprimals, and trimmings up to 5%; for head and tongue meat, 2.0% to 2.8% of lactic acid solution can be applied into a washer cabinet system.

Washing with lactic acid method can be used on beef carcasses. The solution of lactic acid, propionic acid, and calcium sulfate within a pH range of 1.0 to 2.0 can be sprayed into various RTE products prior to packaging. The solution of calcium sulfate and 85-95,000 ppm of lactic acid with a pH range 0.35 to 0.55 can be added into raw beef during grinding process to lower the pH of comminuted beef products. The solution of calcium sulfate, lactic acid, and sodium phosphate with a pH range of 1.45 to 1.55 can be used to spray onto raw whole muscle beef cuts, cooked roast beef products for up to 30 seconds, and spray onto cooked poultry for 20 to 40 seconds. The solution of calcium sulfate, lactic acid, and disodium phosphate with a pH range at 1.0 to 2.0 can be sprayed onto beef jerky product for up to 30 seconds. Lactic acid, combined with propylene glycol or phosphoric acid can be used in poultry processing water. Besides the antimicrobial functions, lactic acid can be applied as flavor enhancer when added into pork fatty tissue, the amount of lactic acid cannot be over 0.37% of fatty tissue prior to dehydration. The lactic acid contents in meat products could influence the meat flavor, stability, and quality. The quantitative determination of lactic acid in these meat applications is required for quality control purposes, and meets required laws and regulation as well as meeting the label requirements.

To date, the present analytical methods for the determination of lactic acid in foods include colorimetric methods (Barker and Summerson 1941), gas chromatography method , and HPLC method . Although some of these methods are accurate, these assays might have major drawbacks. For example, colorimetric methods can be time consuming and lack specificity; GC methods require derivatization of acid before analysis. Some methods may be expensive or cannot provide rapid results. There is a large amount of

research being carried out to determine the organic acids in fruits and fruits juices by HPLC. There were limited reports to quantitate the amount of lactic acid in fish products. Yoshida and others (1999) analyzed organic acids from liquefied raw fish meat by HPLC. The fish was hydrolyzed under supercritical condition. The aim of this study was to determine the amounts of lactic acid in restructured meat and fish fillet that had been washed with different lactic acid solutions. Another objective of this study was to evaluate the feasibility of using FTIR to quantify the lactic acid in meat products and to develop a rapid methodology for monitoring the meat quality.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Swai fillets (Beaver Street Fisheries Inc., FL, USA) were purchased at a local grocery store. The fish were farm raised and produced in Vietnam. The fillets were individually vacuum packed and sold in a frozen state (-18°C). The fillets were thawed before analysis. The 0.45 μm membrane filters were purchased from Fisher Scientific. L-lactic acid (88%) was purchased from Sigma Chemical Corporation (Sigma-Aldrich, St. Louis, USA). HPLC grade water was obtained from Fisher Scientific. Acetonitrile and 95-98% H_2SO_4 with high purity solvent were purchased from Sigma Aldrich. All the other reagents used in this study were analytical grade. Stock solutions of lactic acid were prepared with HPLC water. The following lactic acid concentrations were used for generating a standard curve: blank, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml, and 6.0 mg/ml. The mobile phase consists of 0.6% acetonitrile and 0.045 N H_2SO_4.

5.3.2 Sample Pretreatment

The lactic acid was separated from the meat samples by centrifugation and filtration methods. Ten grams of fish fillet or samples were placed into a stomacher bag (Fisher Scientific, Pittsburgh, PA). Then, 90 ml distilled water was poured into the stomacher bag. The stomacher bag was manually massaged for two minutes. The slurry was filtered through cheesecloth twice, filtered with a buchner funnel and metallic vacuum trap with Whatman® Grade 1 Qualitative Filter Paper. This procedure was repeated several times until the desired amount of liquid was collected. The filtered solutions were kept under refrigerated conditions. Using a pipette transfer technique, the filtrate was transferred into 2 ml tubes for centrifugation. The 2 ml tubes were centrifuged at 13,700 rpm at room temperature for 15 minutes. After centrifugation, the upper phase of liquid was filtered through a 0.45µm filter before being analyzed by HPLC.

For restructured meat sample preparation, fish samples were thawed under refrigerated conditions overnight. The semi-thawed fillets were cut into small pieces and transferred to a food processor (Cuisinart® Prep 9™ 9-Cup Food Processor, Model DLC-2009CHBM), and blended for 1 minute. There were four treatments, which include the control (without sodium alginate system), 0.5% sodium alginate, 1% sodium alginate and 2% sodium alginate of meat weight with the same ratio of sodium alginate: calcium carbonate: encapsulated lactic acid = 1.0 : 6.0 : 1.5. The samples were set at refrigeration temperature overnight.

5.3.3 HPLC Separation and Quantifications

All chromatographic separations were carried out at ambient temperature. The High Performance Liquid Chromatograph (HPLC) consists of pump, column and auto

sampler injection, UV detector and a Galaxie Chromatography software system to calculate the amount of chemicals. The HPLC apparatus was equipped with a UV absorbance detector set at 210 nm, and the column temperature was set at 55 °C. Chromatographic separation was performed on Aminex HPX-87C column. The mobile phase consisted of acetonitrile (6%) and 0.045 N H_2SO_4, with a flow rate of 0.5 ml/min. 20 µl aliquots of the individual standards with series of lactic acid concentrations were auto injected into the column and the retention time of lactic acid was determined. The standards of concentrations were injected into HPLC and their chromatograms were obtained. The calibration curves were determined by Galaxie Chromatography software. After the injection of the pretreated samples, chromatographic peaks were identified by comparing the retention times of the samples with those of the known standards. The quantities of organic acids were estimated and calculated from the peak areas with equations, which were obtained from standard curves.

5.3.4 pH Analysis

Ten grams of raw fish fillet or samples were placed into a stomacher bag (Fisher Scientific, Pittsburgh, PA). Then, 90 ml of distilled water was poured into the stomacher bag. The stomacher bag was manually massaged for two minutes. The slurry was filtered through cheesecloth twice. The pH of meat slurry was determined by using a Fisher Accumet Model 230A pH/ion meter (Fisher Scientific Inc., Salt Lake City, UT). The cooked meat samples were blended with a blender for 15 seconds. The pH measurements of both raw and cooked samples were determined. The pH meter was calibrated using pH buffers 4.00 (SB 101-500, Fisher Scientific, Fair Lawn, NJ) and 7.00 (SB 107-500, Fisher Scientific, Fair Lawn, NJ). The probe was placed into the sample homogenate and

allowed to equilibrate for one minute before the pH reading was recorded. All pH readings were performed in triplicate.

5.3.5 Moisture Analysis

Moisture content determination was determined by following Association of Official Analytical Chemists (Yoshida and others 1999) method with modifications. Three grams of raw paste and cooked fish samples were placed in an aluminum tray and dried in a vacuum oven at 80°C for 24 hours at 23 kPa, and cooled to room temperature in a desiccator prior to taking final weights. Three samples per treatment were measured. Moisture (%) was calculated using the following equation:

$$Moisture\ \% = 100 * (1 - \frac{W2}{W1})$$

W1 represents the weight before drying, g

W2 represents the weight after drying, g

5.3.6 WHC Analysis

Ten grams minced cooked samples were placed into 40 ml tubes containing 30 ml of distilled water and vortexed (Vortex Geniz 2 TM Cat. No. 12-812 Model G 250, Fisher Scientific, McGaw, IL) for 1 minute each tube to ascertain even distribution. The tubes were placed into a 4°C refrigerator for 15 minutes prior to centrifugation. The centrifuge machine (Sorvall RC-5B, Beverly, MA) was turned on 30 minutes prior to measuring in order to cool to 4°C. The tubes were centrifuged at 7000 rpm at 4°C for 15 minutes. After centrifugation, the liquid lost (supernatant) during centrifugation was collected, and the sample was allowed to stand for 1 min so the liquid could drain. Only the liquid was decanted, and solid meat particles were kept in the tube. The WHC of cooked sample was

calculated as the ratio of the water remaining after centrifugation to the initial content of meat sample. WHC (%) was determined by using the following equation:

$$WHC\ \% = 100 * \left(\frac{W1 - W2}{W3}\right)$$

Where W1 represents solution added into the sample, g

W2 represents solution removed after, g

W3 represents the meat samples mass, g

5.3.7 Recovery Studies

A standard addition technique was employed in order to determine the percent recovery rate of the lactic acid in both restructured meat and immersion lactic acid meat samples. This method can be used to verify the effectiveness of the extraction step and to confirm the accuracy of the method used in this experiment. The recovery studies were carried out by injecting known standard lactic acid concentrations into the extracted samples. The percentage recovery was calculated using the following equation:

$$\%\ recovery = \frac{lactic\ acid\ found\ in\ the\ standard\ added\ sample}{lactic\ acid\ found\ in\ extracted\ sample + lactic\ acid\ added\ into\ sample}$$

5.3.8 FTIR Spectroscopy Measurements

FT-IR analysis was carried out using a Thermo Nicolet 380 FT-IR spectrometer (Thermo Electron Corporation, Madison, Wis.) The FTIR spectra using Attenuated Transmission and an internal reflection accessory made of Composite Zinc Selenide (ZnSe) and Diamond crystals. Each spectrum was scanned from 4000 to 400 cm^{-1} . The FTIR spectra were acquired for each treatment at room temperature. Each spectrum was composed of an average of 32 separate scans at a resolution of 4 cm^{-1}. The software Delight Version 3.2.1 (D-Squired Development Inc., LaGramde, OR, USA) was used in

data analysis. FTIR spectra data were converted to trt format. Data pre-processing algorithms including polynomial substrate and Gaussian smoothing was used to subtract the baseline shift and eliminate high frequency noise from the instrument. The partial least square (PLS) model, as multivariate statistical regression model, was used to predict analyte concentrations in tested samples. The number of PLS latent variables was optimized based on the lowest root mean square error of prediction (RMSEP) values to avoid overfitting of spectral data.

5.3.9 Statistical Analysis

One-way analysis of variance was done using the General Linear Model (PROC GLM) of Statistical Analysis System (SAS) computer package (SAS Institute Inc., 2005). Subsequently, Tukey's range tests were conducted to separate means.

5.4 RESULTS AND DISCUSSION

The calibration graph obtained was linear from 0.25 mg/ml to 6.0 mg/ for lactic acid with a correlation coefficient of 0.9999 and 1.0 (n=6) for two trials (Figure 5-2 and Figure 5-3). The LOD (limit of detection) and LOQ (limit of quantification) were determined based on calibration curves. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. Based on the standard deviation of the response and the slope,

$$LOQ = \frac{10\sigma}{S}$$

$$LOD = \frac{3.3*\sigma}{S}$$

Where σ = the standard deviation of response

S= the slope of the calibration curve

In this study, the slope S is estimated from the calibration curve of the analyte. The estimate of σ is calculated based on the residual standard deviation of a regression line. The LOD was 0.016 mg/ml. the LOQ is 0.05 mg/ml. The relative standard deviation (RSD) was 2.0% for determination of 4.0 mg/ml lactic acid standard solution (n=8). These values provided the reproducibility or repeatability of this method. The accuracy was measured based on the recovery rate. The range of recovery rate was 85.6% to 125.3%. The control samples were not spiked with any lactic acid. Due to lactic acid accumulation during harvest, it makes sense that the recovery rate exceeds 100%.

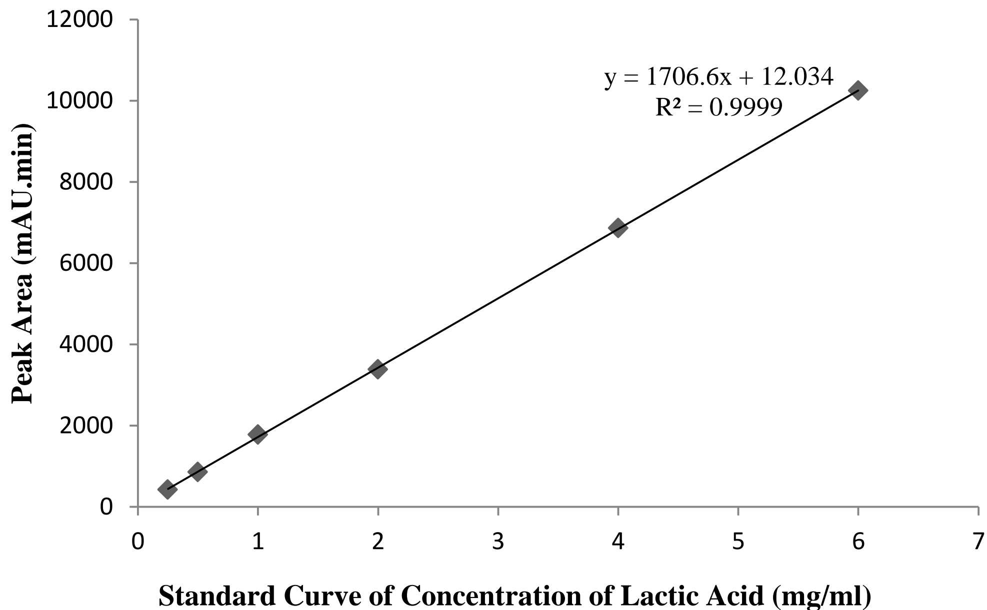

Figure 5-2. Lactic Acid Standard Curve for HPLC Analysis on the First Trial

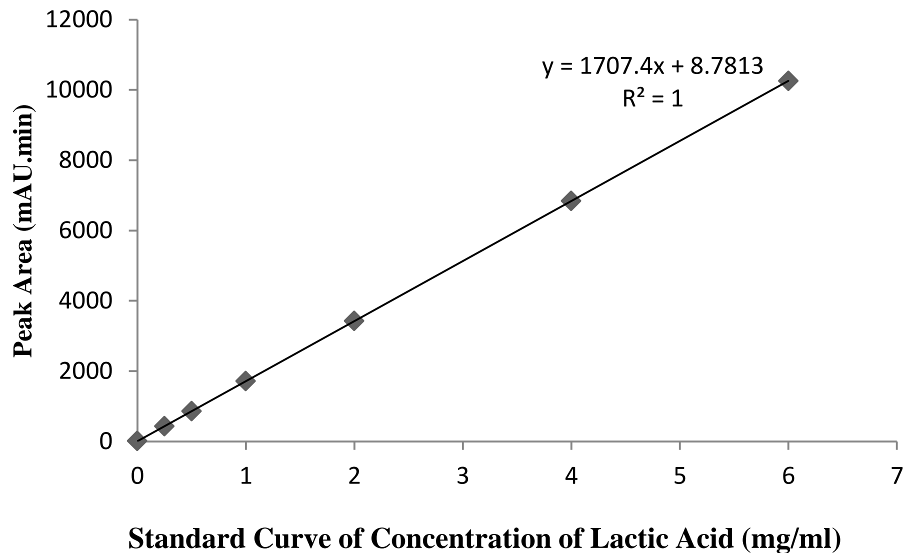

Figure 5-3. Lactic Acid Standard Curve for HPLC Analysis on the Second Trial

5.4.1 Determination of Lactic Acid in Restructured Meat Samples

At the pre-treatment step, the ratio of 1:10 was made in order to extract lactic acid. Lactic acid is present normally in living animal muscles and accumulated more during harvest. The results (Figure 5-4) showed that the average amount of lactic acid contained in raw fish was 9.81 mg/g. It was assumed that control samples did not present any lactic acid. There was a positive linear relationship between amounts of tested and spiked lactic acid. The following equation was developed to show the relationship: $y = 0.465x + 0.0367$, $R^2 = 0.9549$. It is concluded that higher concentrations of lactic acid in meat sample resulted in higher results from HPLC system. Ren (2013) determined the purity of encapsulated lactic acid powder, which was 39% with HPLC. In this study, the purity of encapsulated lactic acid was calculated based on the HPLC results and the proposed lactic acid concentration; the average purity of encapsulated lactic acid powder was 32%. The results also further confirmed that centrifugation and filtration was suitable

extraction method and HPLC can be used to quantitate lactic acid, or encapsulated lactic acid added to meat samples.

Table 5-1.The Formulations of Restructured Meat Treatments

Treatment	Meat (g)	Sodium Alginate (g)	CaCO3(g)	Encapsulated Lactic Acid (g)
Control	300	0	0	0
0.5% SA	300	1.5	0.25	0.375
1.0% SA	300	3	0.5	0.75
2.0% SA	300	6	1	1.5

*SA: Sodium Alginate

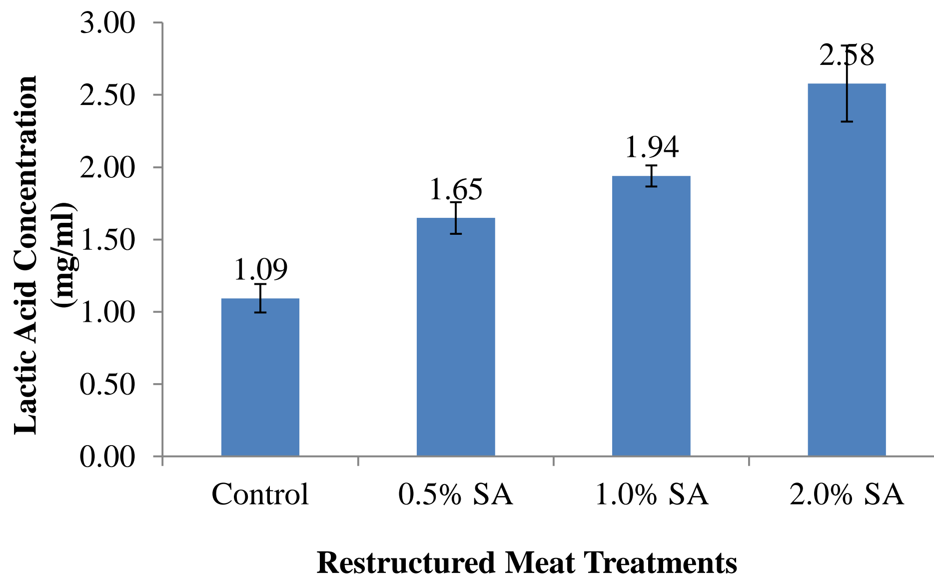

Figure 5-4. The HPLC Results of Lactic Acid Concentration in Restructured Meat Treatments *SA: Sodium Alginate

5.4.2 Determination of Lactic Acid in Immersion Samples

The results from Figure 5-5 demonstrate that when the meat was immersed into the higher concentrations of lactic acid, a higher amount of residual chemical was expected on the surface of meat sample. There was a positive relationship among these treatments. The results also confirmed that this HPLC method could be suitable for

testing organic acids. Especially, the extraction method can be used to analyze lactic acid in all types of meat samples.

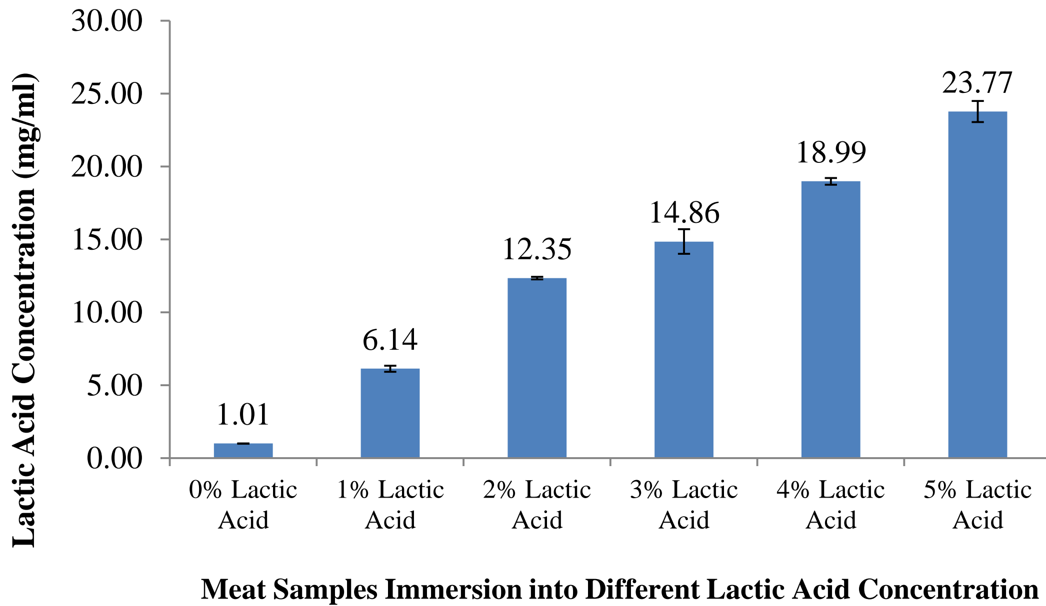

Figure 5-5. The HPLC Results of Lactic Acid Concentrations with Immersion Meat Treatments

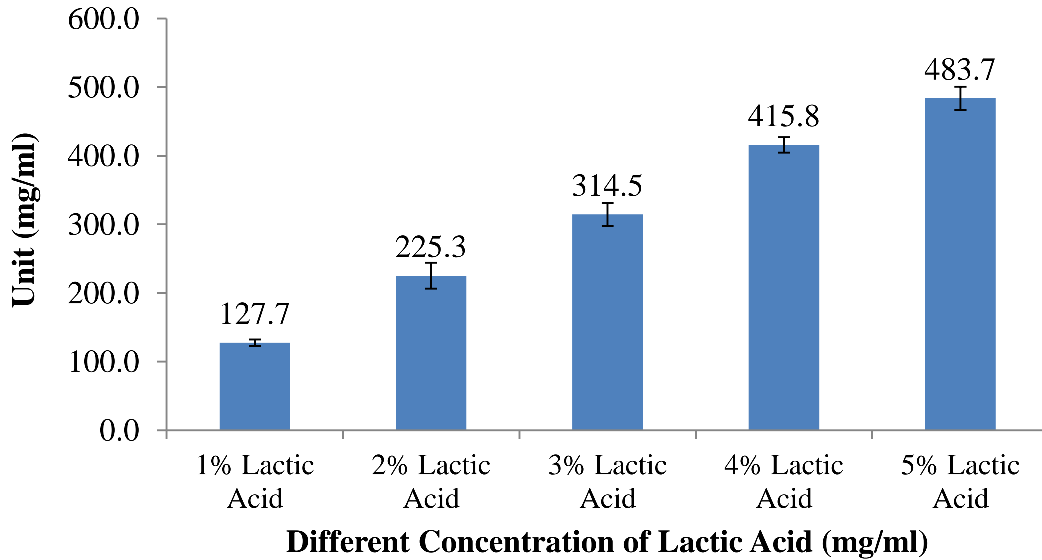

Figure 5-6. The HPLC Results of Lactic Acid Concentration

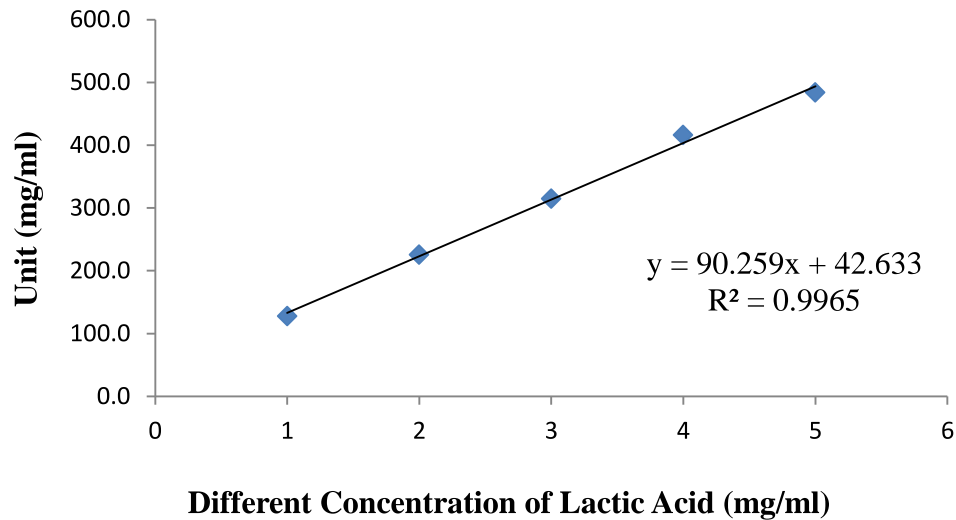

Figure 5-7. The Calibration Curve of HPLC Results of Lactic Acid Concentrations

In this study, the meat samples were immersed into different concentration of lactic acid for specific time. The concentrations of 1% to 5% lactic acids were analyzed by HPLC. The results are shown in Figure 5-6. Figure 5-7 displayed that there was a linear relationship with Y=90.259X+42.6 and R^2=0.9965 between the amounts of actual lactic acid and spiked lactic acid. However, when analyze 5% lactic acid with HPLC, the data did not show exactly values due to out of range. Based on USDA regulation, the lactic acid application on meat either in spray or marinated method based on meat weight, cannot be over 10% of total meat weight. This further confirms that this is suitable method to test the amount of lactic acid or encapsulated lactic acid added to meat.

Table 5-2.The Recovery Rate of Lactic Acid Spiked in Fish Samples

Treatment	Recovery Rate (%)
1% Lactic Acid	105.7
2% Lactic Acid	112.7
3% Lactic Acid	93.8
4% Lactic Acid	99.1
5% Lactic Acid	104.4

The Table 5-2 data demonstrate the recovery rate when fish slurry samples were injected with different concentrations of lactic acid from 1% to 5%. The recovery rates range from 93.8% to 105.7%. Figure 5-8 shows an example of a standard chromatogram of lactic acid, the retention time was determined to be around 14.77 minutes.

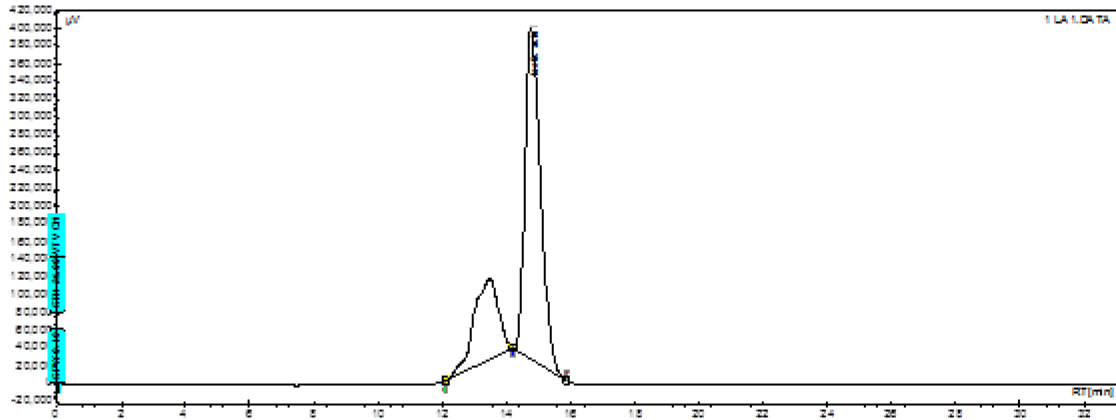

Figure 5-8. Standard Chromatograms for Lactic Acid (Retention Time=14.77 minute)

5.4.3 WHC and Moisture of Two Different Types of Samples

The data demonstrate that meat pH values decrease as the concentration of sodium alginate levels increase. The pH value of the control sample and samples treated with sodium alginate were 8.03, 7.92, 7.89, and 7.83, respectively (Table 5-3). As the sodium alginate levels increased, the encapsulated lactic acid levels increased and the pH values decreased. The pH values of the control samples were significantly higher than samples treated with sodium alginate ($P<0.05$). There was no significant difference among sodium alginate treated samples. The control sample had higher moisture than samples treated with sodium alginate (Table 5-3). The higher moisture of the control sample may be due to the water binding capacity of sodium alginate.

The results of water-holding capacity (WHC) measured with sodium alginate are shown in Table 5-3. There were significant differences in WHC among treatments. The

WHC for all sodium alginate treatments were consistently higher (P>0.05), when compared to the control treatment. No difference (P>0.05) was detected between samples treated with 1.0% and 2.0% sodium alginate. The WHC increased as the sodium alginate levels increased. Therefore, compared with control samples, sodium alginate treated samples had higher WHC, lower moisture and lower pH values than control samples.

Table 5-3. The Attributes of Restructured Meat on WHC, Moisture, and pH Values

Treatment	WHC	Moisture	pH
0% Sodium Alginate	8.80^{c}	85.32^{a}	8.03^{a}
0.5% Sodium Alginate	31.21^{b}	82.37^{b}	7.92^{b}
1.0% Sodium Alginate	39.36^{a}	83.50^{ab}	7.89^{c}
2.0% Sodium Alginate	46.77^{a}	82.85^{b}	7.83^{c}

5.4.4 FTIR Analysis

Different concentrations of lactic acid solutions spiked into fish sample solutions were analyzed by FTIR. The most prominent peak of lactic acid was at about 1132 to 1150 cm-1, which is present in the FTIR spectra collected from second derivation of spiked lactic acid samples and the powder L-lactic acid fingerprint (Figure 5-16). Second derivative transformation can be used to separate over-lapped peaks, eliminate baseline effects, and enhance spectra resolution. It could be used as a common tool to analyze the spectra. Figure 5-9 and Figure 5-10 show the second derivative transformation of FTIR from different concentrations of spiked lactic acid and extracted from restructured meat, which were treated with different concentrations of sodium alginate and lactic acid. Figure 5-9 clearly shows the difference between the control (no spiked lactic acid) and higher concentrations of lactic acid around 1132 cm^{-1} . Figure 5-10 shows the bands range from 1050 to 1250 cm^{-1} . The samples without lactic acid treatment can be easily differentiated from the samples treated with lactic acid and sodium alginate. Yoshida and

others (1999) pointed out the most prominent peak of L-lactic acid was at around 1127 cm^{-1}. In this study, wavenumber 1132 cm^{-1} was used as the most prominent peak of L-lactic acid in spiked fish.

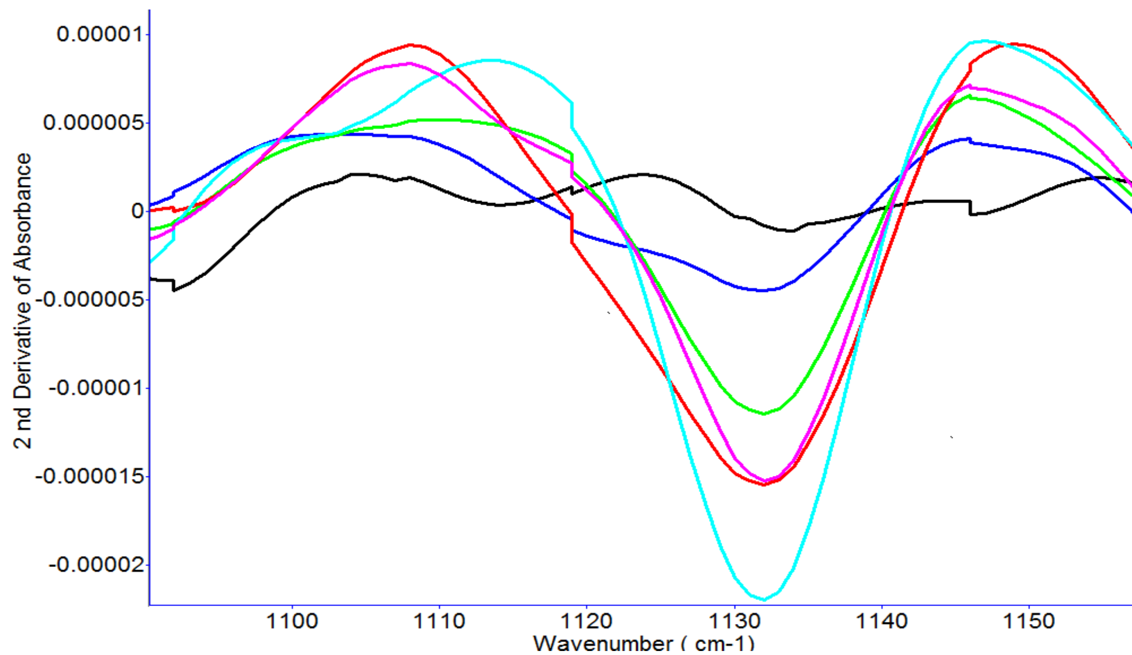

Figure 5-9. Second-Derivative FT-IR Spectra of Spiked L-Lactic Acid into Fish Supernatant. The Final Spiked Concentrations of L-Lactic Acid were 0 (Black), 0.1% (Sky Blue), 0.2% (Green), 0.3% (Pink), 0.4% (Red) and 0.5% LA (Light Blue) at Wavenumber 1132 cm^{-1}.

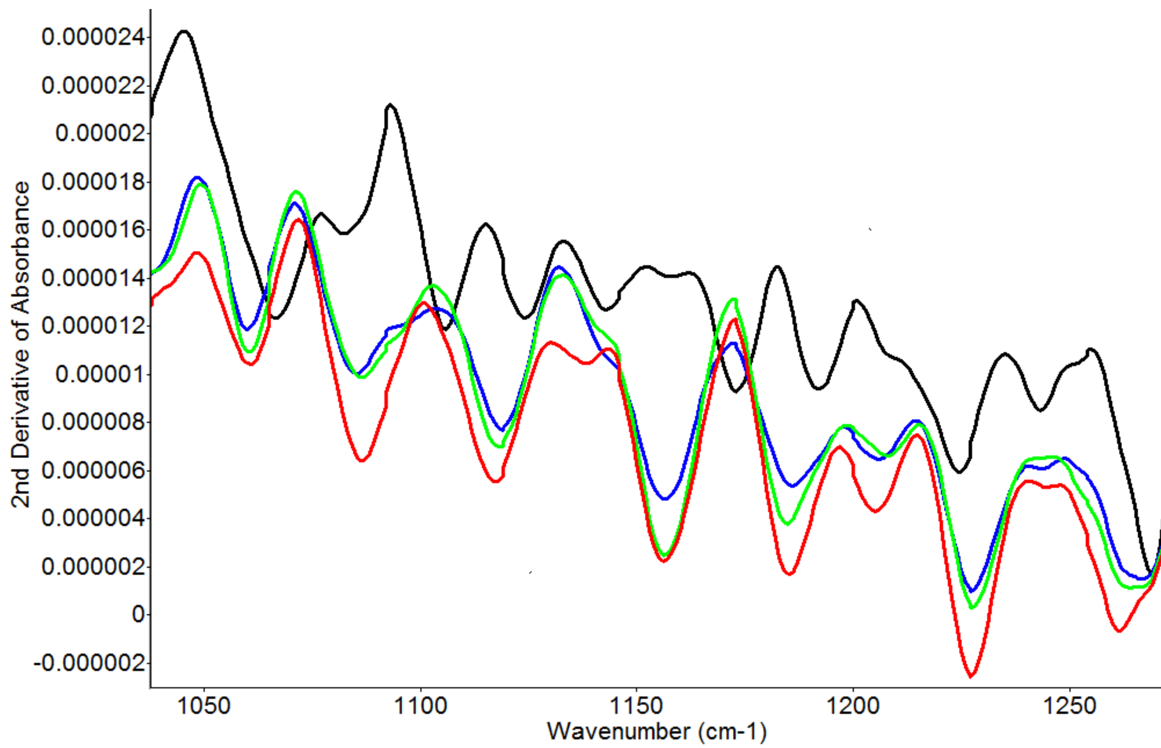

Figure 5-10. Second-Derivative FT-IR Spectra of Extracted Lactic Acid from Restructured Meat with Different Concentration. 0 (Black), 0.5 SA treatment (Sky Blue), 1% SA treatment (Green), 2% SA treatment (Red) at Wavenumber at Range between 1250 to 1050 cm^{-1}

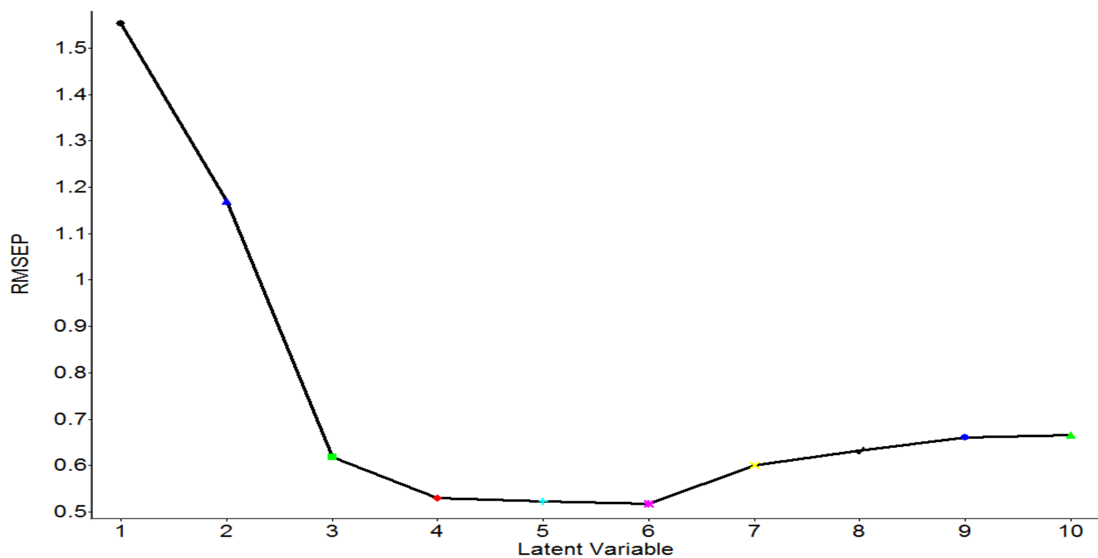

Figure 5-11. Root Mean Square Error of Prediction (RMSEP) Values Obtained from the Partial Least Squares (PLS) Models with Different Latent Variables

RMSEP values obtained from the PLS models with different latent variables are shown in Figure 5-11. The data was processed with smoothing at 4 cm^{-1} and 2nd order polynomial subtraction and second derivative at 12 point in the entire spectral region from 4000 cm^{-1} to 400 cm^{-1}. The lowest RMSEP values were obtained when the four latent variables were used (Figure 5-11), therefore, the optimal number of latent variables to perform PLS model was four. Figure 5-12 shows the PLS predication results with concentration from 0.25-6.0 mg/ml, X axis represents the actual lactic acid concentration and Y axis represents the predicted lactic acid concentration. The prediction results were achieved with R = 0.969 and RMSEP = 0.5179. Figure 5-13 showed the PLS prediction results with concentrations of lactic acid ranging from 1% to 5%. The prediction results were achieved with R = 0.9706 and RMSEP = 0.5074.

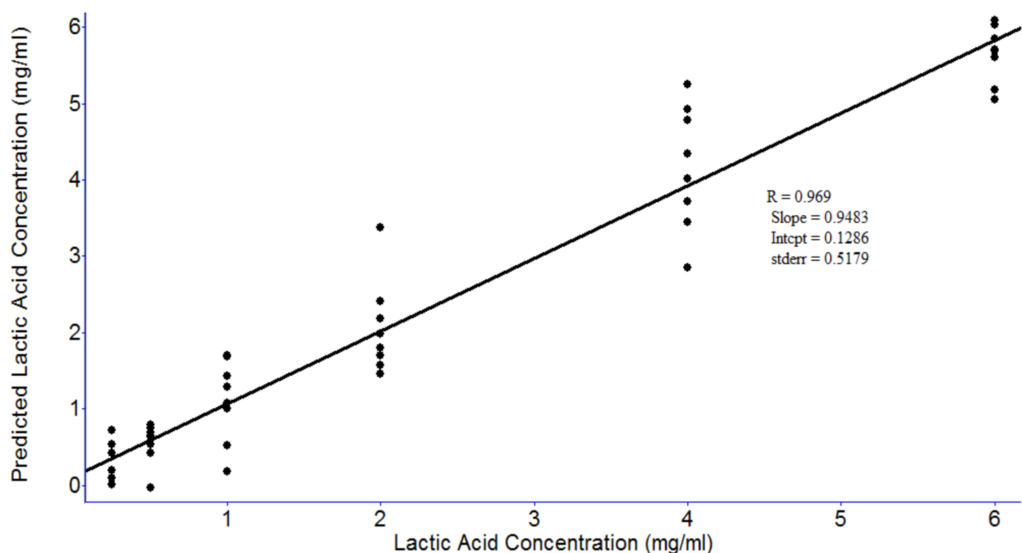

Figure 5-12. Predicted Lactic Acid Concentration (mg/ml) Versus Lactic Acid Concentration (mg/ml). Using the PLS Model; Smoothing 4 cm^{-1}, Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 6 Latent Variables; Spectral Region 4000-525 Cm^{-1}; Spectral Number N=48, Lactic Acid Concentrations 0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/ml

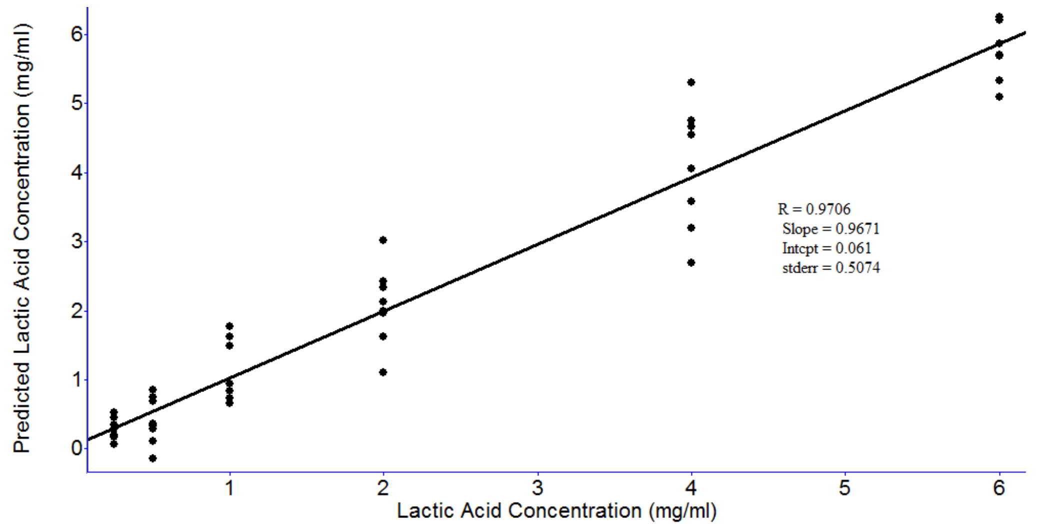

Figure 5-13. Predicted Lactic Acid Concentration (mg/ml) Versus Lactic Acid Concentration (mg/ml) using the PLS Model; Smoothing 4 cm^{-1}, Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 5 Latent Variables; Spectral Region 2000-500 cm^{-1}; Spectral Number n=48, Lactic Acid Concentrations 0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/ml

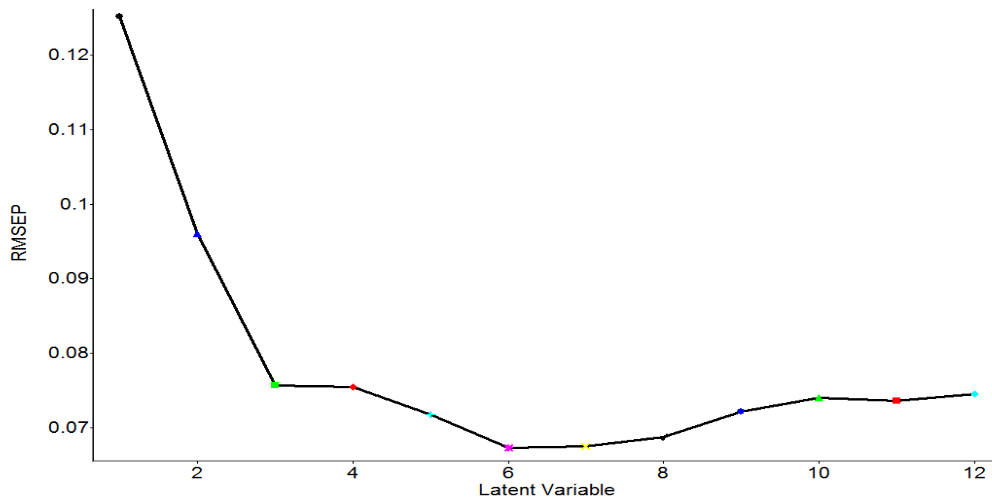

Figure 5-14. Root Mean Square Error of Prediction (RMSEP) Values Obtained from the Partial Least Squares (PLS) Models with Different Latent Variables for Spiked Samples

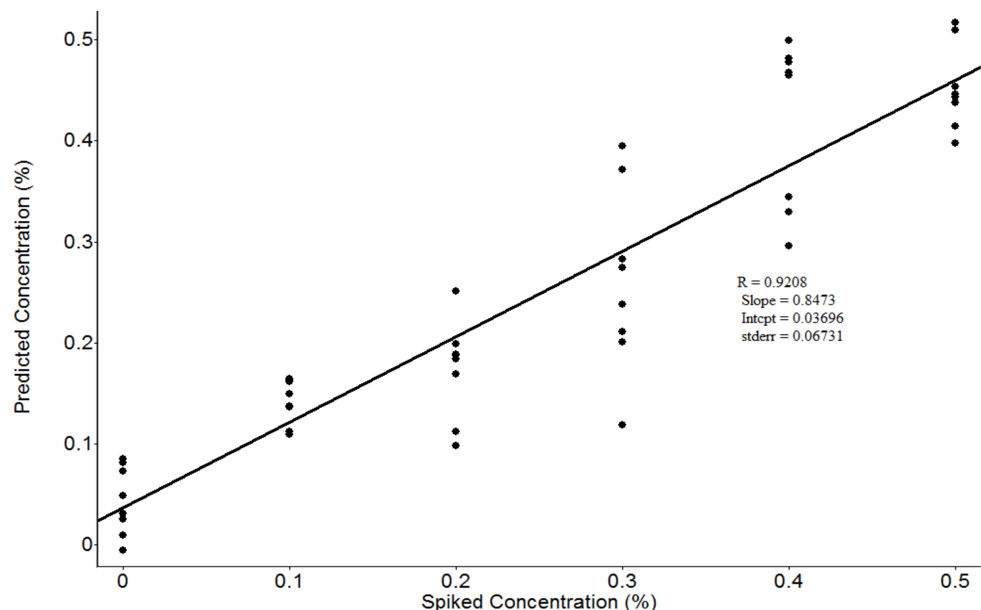

Figure 5-15. Predicted Lactic Acid Concentration (%) Versus Spiked Lactic Acid Concentration (%) using the PLS Model; Smoothing 4 cm^{-1}, Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 6 Latent Variables; Spectral Region 4000-525 cm^{-1}; Spectral Number n=48

Figure 5-14 and Figure 5-15 showed the latent values and spiked predict lactic acid. The prediction results were achieved with R=0.9208 and RMSEP=0.067. Figure 5-14 shows the PLS prediction results with spiked concentration of lactic acid with 1.0 % to 5.0 %. Therefore, in theory, the final solution concentrations become 0.1% to 0.5%. These results indicate that satisfactory quantitative results for lactic acid could be measured by FTIR techniques. However, current methods cannot be used if the lactic acid was added in very low amounts. From all these results, it can be concluded that more improvements are needed. The standard deviation should be as low as possible to improve the reproducibility of spectra. In addition, water content in testing samples may interfere with other food components and increase the difficulty of measurements. Experiments should be performed to reduce the interference from other food components and improve PLS prediction models.

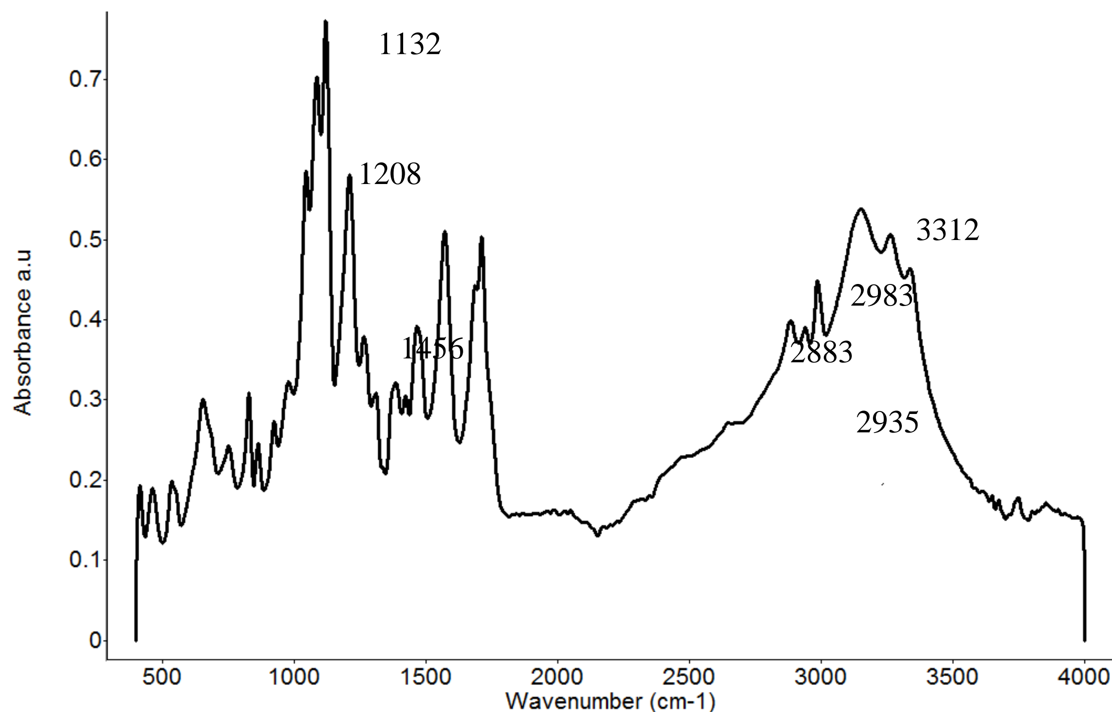

Figure 5-16. FTIR Fingerprint of Powder L-lactic Acid

Figure 5-16 shows the FTIR fingerprint of powder L-lactic acid. FT-IR intensities around the wavenumber of 3312 cm^{-1} are associated with O-H stretching. It may relate to water content in the tested lactic acid powder. C-H stretching bands are located at 2983 cm^{-1}, 2935 cm^{-1} and 2883 cm^{-1}. CH_3 is responsible for the appearance of the band at 1456 cm^{-1}. The bands at approximately 2997 cm^{-1} and 2945 cm^{-1} signal come from the CH_3 asymmetric and symmetric stretch. The bands at approximately 2854 and 2926 cm^{-1} come from the organic modifiers and are not present in pure lactic acid solution or powder. The band at 1454 cm^{-1} attributed to the asymmetric deformation mode of CH3, is split into two bands at 1458 and 1442 cm^{-1}. The region of 1300 to 1000 cm^{-1} is related to the C-O-C stretching vibrations, it is also related to band splitting, and peak shifts. The peak at 1200 cm^{-1} is due to the C-O-C asymmetric vibrations linked with asymmetric CH_3 rocking vibrations, and it shifts to higher wavenumbers with stronger intensity. This

1200 band is split into two peaks at around 1210 and 1180 cm^{-1} (AOAC 2000). Vodnar and others (2010) summarized the peak assignment of L-lactic acid, and addressed that the peak intensities at 1760 cm^{-1} and 873 cm^{-1} are its characteristic peaks. Table 5-4 is adapted from Krikorian and Pochan (2005). They summarized the peak assignments of poly L-lactic acid.

Table 5-4. Peak Assignment of PLLA (Poly L-lactic Acid)

Peak Assignment	Wave number (cm^{-1})
νCH_3 as	2998
νCH, νCH_3 S	29,542,900
$\nu C=O$	1762
δCH_3 as	1452
δCH_3 s, δCH	1385, 1365, 1292
γCH	1215, 1180
γCH_3	1129, 1091
$\nu C\alpha$-$C\beta$	1042
νC_{Ester}-$C\alpha$, νC-$C\alpha$	920,872
$\gamma C=O$	735, 707
ţC-$O_{E,}$ $\delta O=CO_E$	510, 408, 394, 340
$\delta CC=O$, γCCC	305, 237

5.5 CONCLUSION

The extraction method used in this study is a simple and an accurate analytical method for determining lactic acid or encapsulated lactic acid content in meat samples. There were no steps for fat removal and protein precipitation. It saves time and chemical reagents. The procedures that used cheesecloth to remove residual meat and centrifugation for 20 minutes can help slurry pass through the filter. In this way, it did not clog the HPLC column. In this study, two types of lactic acid application were investigated, including restructured meat containing sodium alginate and encapsulated lactic acid and spraying with lactic acid, which are two common applications applied into

meat industries. These extraction methods and analytical tools, such as HPLC and FTIR, provide references for food manufacturers if they are interested in understanding the amount of lactic acid or other types of organic acids affecting meat quality. It also provides references for food manufacturers if they use lactic acid bacteria as a food additive. Overall, this research showed that both HPLC and FTIR could be used to analyze the content of various types of lactic acid.

In the FTIR study, sample preparations included centrifugation and filtration. The same samples were used for HPLC analysis. The results showed that FTIR has the potential to quantitate the amount of lactic acid in meat samples. However, compared with HPLC methods, it requires more technical handling and analysis to obtain specific results. Compared with HPLC , FTIR cannot provide extract values and can only predict the possible amount. It still has potential to be used in the meat industry; however, more work is needed to identify more parameters and meat quality changes during storage or other types of food processing.

CHAPTER 6
PERFORMANCES OF COLD-SET BINDERS, FOOD HYDROCOLLOIDS, AND COMMERCIAL MEAT BINDER ON THE PHYSICAL AND CHEMICAL CHARACTERISTICS OF TILAPIA FISH BALLS

6.1 ABSTRACT

The overall objective of this study is to add different types of hydrocolloid additives into reformed fish products and to compare the performances of different binders by testing chemical and physical properties of restructured meat samples. In total, nine treatments were included in this study. Eight types of meat binders were formulated into fish meatballs to determine differences in physical and chemical characteristics differences. The eight types of meat binders include cornstarch, commercial meat-binder, carrageenan, methylcellulose, Activa® RM, plasma powder FG+, plasma powder FG and encapsulated lactic acid with sodium alginate and calcium sources. The results showed that Activa® RM and FG+ and FG could provide satisfactory binding properties in fish balls.

There was no significant difference among all samples for cooked meat moisture ($p<0.05$). Raw meat had slightly higher moisture than other cooked meat treatments. Samples treated with Activa® RM had the highest WHC for cooked samples; while methylcellulose treated samples had the lowest WHC. Samples treated with sodium alginate had the lowest pH values for both cooked and raw meat samples. There were no significantly differences detected for water activity for both raw and cooked meat samples. Samples treated with methylcellulose had the lowest cooking yield, and all other meat binder treatments samples had higher cooking yield than that of the control samples. Samples treated with Activa® RM, FG+ and FG showed higher puncture values than that of control samples. Except for samples treated with sodium alginate, methylcellulose, and

meatbinder treatments, all other binder treated samples had lower puncture values for cooked samples. Samples treated with Activa® RM, FG+ and FG treated samples had the best texture, hardness, springiness. In summary, Activa® RM, FG+ and FG treatments performed well for all parameters, and sodium alginate, methylcellulose, and meatbinder treatment did not show advantages when compared with the control.

6.2 INTRODUCTION

The functions of binder and stabilizer in meat products are to form various cuts of meat into affordable and acceptable innovative muscle food productions. Based on the United States Department of Agriculture (USDA) definition, binder is used to thicken or to improve texture consistency and sensory scores of meat. Stabilizer is a food additive. It contributes toward providing an optimal finished meat system product and provides value-added qualities to meat system applications. It also can improve finished product stability, provide consistent texture and viscosity, and make food products firmer (Suzuki and others 2014). Currently, there are many products available in the supermarket, which are made by binding comminuted meat products along with spices, seasonings, and stabilizer into one cohesive product. Various binders are available to meat processors. Some binders are proteins, such as soy protein isolate, pea protein, wheat protein, milk caseinate, gelatin, and egg protein. Some binders are derived from enzymes, such as transglutaminase and beef fibrin. Some binders contain little or no protein, such as fibers, flours, and starches. There are some other popular binders in meat-based products such as oatmeal and breadcrumbs. Hydrocolloids are another type binder that is widely used into meat products. Phosphate has a similar function to the most effective water-binding agents in processed meat. Phosphate and salt, with the aid of the mechanical force, can extract meat myofibrillar proteins that can hold large amount of water.

Hydrocolloids have been employed by the meat industry to function as gelling agents, stabilizers, or thickening agents. Due to their high molecular weights, they are also called macromolecules. These macromolecules can be dissolved or dispersed into aqueous solution, which can increase viscosity or gel formation (Suzuki and others 2014). Most of these hydrocolloids come from either plant (polysaccharides) or animal sources (protein). The majority polysaccharides originate from plants (Coultate and others 2002). Polysaccharides play a less important role in animal physiology. Two polysaccharides related to animals are glycogen and chitin, however, both animal polysaccharides have specialized roles in connective tissue, which contains a large amount of protein.

Polysaccharides are comprised of three sub-groups, including non-ionic, anionic and cationic. Non-ionic polysaccharides are neutral, for example hydroxyethyl cellulose and dextran; anionic polysaccharides include, but are not limit to, xanthan gum, carrageenan, guar gum, alginate and carboxymethylcellulose (CMC); cationic polysaccharides are a group of hydrocolloids with overall positive charges, including arginine hydrochloride and chitosan (Msagati 2012; Coultate and others 2002).

Many reports have addressed the applications of polysaccharides in the meat industry as meat binder. The main function of meat binder is to glue the various cuts of meat into affordable and innovative meat protein based food. The comminuted meats hold spices or seasonings together and develop into one cohesive body. In the current market, 25% of the carcass is used for steaks and kebabs and a large amount of the carcass is processed into lower value ground products such as hamburger and sausages. Restructured meat includes chorizo, kielbasa, salami, chicken nuggets, and different types of hams. For beef, pork, mutton, and lamb products, the restructuring technology has

been widely used to produce restructured roast and hams. Current trends show increasing consumer demand for ready to cook or ready to eat (RTE) meat products.

Restructuring technology is a method for transforming lower value cuts of meat and quality trimming into products with higher values. Restructured meat should not be considered as a replacement of high quality meat but as an innovative way to convert muscle meat into high value protein food. There are types of restructured meat products; products that resemble meat made from trimmings and products that resemble to whole muscle. Both finished products can show uniform product shape, color and other attractive properties. Different binders work in different ways as the interaction between the types of meat muscles and ingredients differs. Anionic polysaccharides are widely used as stabilizers in dairy products. The mechanism of milk applications involves the interaction of anionic polysaccharides with the positive charges on the surface of foodstuffs, which could stabilize the casein network and lower gel separation (Coultate and others 2002). The following hydrocolloids are recognized as common stabilizers used in the meat industry; xanthan gum, pectin, alginate, carrageenan, guar gums, and gelatin. The restructured meat products may be produced by the formation of heat set gels or cold set gels.

Restructured meat products are traditionally made by adding salt and phosphate. By the aid of mechanical action, the myofibrillar protein is extracted. Upon cooking, the protein matrix forms on the surface of meat pieces and coagulates the protein; this binds the meat pieces together. The meat pieces are cooked to a final internal temperature ranging from 57°C to 68°C to obtain the binding (Msagati 2012). If the meat pieces are not bound together efficiently, or if the protein matrix is not heated to the desired internal

temperatures, the individual meat pieces may fall apart due to the shrinkage of meat protein. The product binder is not very strong in the raw state and the product should be sold and labelled as precooked (Msagati 2012).

Cold-set binders apply chemically induced gel instead of thermal set gels into meat pieces and make products more acceptable to consumers. These products can be sold as raw chilled and refrigerated. In general, cold-set restructured meat processing contains six basic steps. First, raw material selections and preparation, such as trimmings and ground meat; Second, adding the cold-set binders into meat mixture; Third, molding or shaping new restructured meat; Fourth, cold setting process; Fifth, dividing into small portions; and Sixth, packaging and storing. The first, third and fifth steps are similar with the steps in the hot-set restructure process (Toldra 2010). The major differences between cold-set and hot-set are that cold-set binding process does not require thermal process to set the gel, and cold set binding systems can be sold in a refrigerated or frozen state. Once the cold-set binder is mixed with pieces of meat and shaped, the molded mixture should be held at a specific temperature for various amounts of time depending on the type of binder.

Several cold-set techniques have been developed in order to meet the consumer demand for various restructured meats. The techniques include using polysaccharides (Carballo and others 2006; Toldra 2010; Clarke and others 1988), pearl E and pearl F (Nielsen and others 1996), blood plasma fractions (Esguerra 1994), and microbial transglutaminase enzyme preparations (Esguerra 1994).

The first cold-set binding system is a sodium alginate system. The three most common ingredients in alginate binding or gelling systems are alginate salt, a calcium

source, and acidulant or sequestrant, such as encapsulated lactic acid or Glucono Delta Lactone (GDL) (Lennon and others 2010). The calcium source in an alginate binding system should be added at the last stage of the process to avoid pre-gelation during processing. The interaction between calcium and monomer units would develop the polysaccharide-meat protein into a composite gel, which is a thermo-irreversible gel (Suklim and others 2004). The function of acidulate or sequestrant in this system is to modify the reaction rate and to control the hydration rate and gel setting time or to accelerate the release of calcium (Suklim and others 2004; FMC-Biopolymer 2016; Shand 2000). The setting temperature and time for this system is usually 0-4 °C overnight. Once the gel system is formed, the gel interacts with myofibrillar protein. These are mainly electrostatic interactions between the anionic group on alginate and positively charged group on protein. No report has been made about whether the functionality of myofibrillar protein could be improved by conjugation with alginate. Therefore, the grade of alginate, calcium sources, and sequestering agents and their ratios must be used appropriately in order to develop the overall desired texture for different food products.

The second cold-set binding system is blood plasma fraction- Fibrimex blood plasma. This type of binding agent relies on the physiological clot forming action of the plasma proteins fibrinogen and thrombin. The available commercial binder is Fibrimex®, which is produced by the Dutch Company Sonac BV. Its binding action is based on the transformation of fibrinogen into fibrin by the action of thrombin. The fibrin interacts with collagen to bind the meat pieces and develops restructured meat products (Boles and Shand 1998). When Fibrimex® is mixed with water, it forms the binder solution. This solution can then be applied to the surface of meat pieces; the thrombin enzyme converts

fibrinogen into fibrin. Fibrin molecules develop cross-linked gel by the function of transglutaminase enzyme in the fibrinogen. Transglutaminase enzyme can connect and develop cross-linking between fibrin and collagen in meat. Therefore, this cold-set system has a big advantage if used in the muscle meat containing higher collagen, such as beef forequarter (Boles and Shand 1999).

The third cold-set binding system is pearl meat cold-set binder. Pearl F is white power with carbohydrate, protein, and bone ash mix. It is used to bind seam-boned muscle and large meat pieces. Pearl E is a protein active meat binder that can be used in binding small size pieces of raw meat. Pearl E is developed by Earlee Products Qld, Australia. Pearl F is developed by Chiba Flour Milling Co. Ltd, Japan (Ofori and Hsieh 2012).

The fourth cold-set binding system is microbial transglutaminase enzyme (protein-glutamine γ-glutamyltransferase). Transglutaminase is an enzyme that catalyzes the covalent cross-link gel formation with different types of proteins. MTGase catalyzes covalent bonds between the ε-amino group of lysyl residues and the γ-carboxamide group of glutaminyl residues of adjacent proteins (Lennon and others 2010). The role of MTGase in catalyzing the cross-linking of myosin heavy chains has been investigated, but no clear reaction mechanism has been summarized. It has a wide active pH range from 4.0 to 9.0 and the active temperature is 0-70 °C with the optimal activity at 55 °C. When applying MTGase into cold water fish muscle, the optimal temperature is in the range of 25-30 °C (Toldra 2010). Fish muscle contains an endogenous transglutaminase (TGase) of its own. Sufficient calcium ions in fish muscle promote the endogenous TGase to be activated and can develop gel at low temperature. Activa is a product that

contains microbial transglutaminase (MTGase) and sodium caseinate (Moreno and others 2008). The function of sodium caseinate is as a substrate to increase cross-linking in the meat product. The MTGase catalyzes the acyls to form covalent cross-linking in protein and peptides, most of time this occurs between glutamine and lysine residues. This helps the protein aggregation and gelation to occur. Transglutaminase has been used in pork, beef, and chicken. Some reports points out that MTGase applications are influenced by meat species (Moreno and others 2008). MTGase interacts with muscle protein to produce thermo-stable gels at temperatures below 30 °C.

Many researchers have investigated the cold set binder in meat products. Carballo and others (2006) concluded that the use of alginate and calcium system binder could improve quality of restructured beef texture and reduce formulation costs. Carballo and others (2006) addressed that alginate and MTGase were very suitable as binding ingredients for fish, Alginate has been extensively studied in restructured meat products, but there is limited research on fish products. MTGase has been widely used in pork, beef, chicken, and several studies were reported on the application in lamb, fish, and seafood products. Ensor and others (1990) used sodium alginate and microbial transglutaminase to homogenize and bind small fish muscle pieces into restructured fish products for frozen storage. Moreno and others (2008) investigated the cold set binding agents including Texor, Fibrimex, alginate, and Activa EB to reform steaks. Moreno and others (2010) used transglutaminase and sodium caseinate as binding agent to bind different amounts of walnuts with meat to form restructured steaks. Lennon and others (2010) performed research on cold set binder plasma on dry ham. The gelling capacity of fish proteins in comminuted fish products is one of their most important functional properties.

The myofibrillar protein of fish can form a firm gel, and the main gel-forming protein in fish is myosin. Myosin plays an important function for the development of the elasticity properties of gels. The gelling properties of protein in surimi products have been commercially utilized to produce imitation shellfish meat (Serrano and others 2004). However, there are very few studies on comparing the performances of fish products in the presence of different types of binders. The aim of this study was to investigate the behavior of different restructured fish products with different hydrocolloids and cold set binders during chilled storage. These parameters compared include cooked meat color and raw meat color at different storage period, water holding capacity, water activity, raw and cooked meat pH, puncture test and TPA test.

6.3 MATERIALS AND METHODS

6.3.1 Prepare Samples

Swai fillets (Beaver Street Fisheries Inc., FL, USA) were purchased from a local grocery store. The vacuum-packed fillets were sold as frozen products. The fish were farm raised, and produced in Vietnam. There were a total of nine treatments. There was not any binder added to the control treatment; treatment 2: minced fish with 5.0% corn starch; treatment 3: minced fish with 2.0% Meatbinder (GRINDSTED® meatbinder 2-555, Danisco, KS); treatment 4: minced fish with 1.5% carrageenan (GRINDSTED® Carrageenan, Danisco, KS); treatment 5: minced fish with 1.5% methylcellulose (Dow, Michigan); treatment 6: minced fish with 1.0% Activa® RM; treatment 7: minced fish with 0.7% plasma powder FG+; treatment 8: minced fish with 0.7% plasma powder FG; and treatment 9: minced fish with 0.4% (w/w) encapsulated lactic acid (IFP Incorporated, MN), 0.3% (w/w) calcium carbonate (Microwhite Codex 50, IMERYS, GA) and 0.8% (w/w) sodium alginate (FD155, Danisco, KS).

Fish samples were thawed at refrigeration temperature overnight. The semi-thawed fillets were cut into small pieces, and transferred to a food processor (Cuisinart® Prep 9™ 9-Cup Food Processor, Model DLC-2009CHBM), and blended for 2 minutes. Meat pieces around the food processor walls were scraped off with a rubber spatula to ensure even blending. Meat binders were manually sprinkled into the paste, then the binder was covered with paste before further mixing in the food processor, and blending was continued for another 3 minutes. Except for the sodium alginate system, in which limited chopping processes were used after adding the encapsulated lactic acid.

Cold water was prepared in advance. Wax paper was used to line the bottom of Styrofoam trays. The fish balls were scooped out with a tablespoon and shaped between the palms of the hands. To avoid sticking, both hands were moistened with cold water. There were about 12-15 fish balls made for each tray. The trays were covered with stretch wrap film to avoid moisture loss. Trays were placed into a refrigerator overnight to set the gel. Between each treatment, the food processor was cleaned thoroughly, and dried with paper towels prior to next treatment preparation.

The pH, a_w, and moisture of raw fish balls were measured on the first day. After overnight storage, the fish balls were cooked in a 70 °C water bath. The pH, a_w, WHC, and moisture of both cooked and raw fish balls were measured on the second day. The cooked both puncture and TPA tests were evaluated after cooling the fish balls to room temperature. The raw puncture and TPA tests were evaluated on the same day as cooked texture measurements.

6.3.2 pH Analysis

Raw meat samples (10 g each) were placed in stomach bags with 90 ml water and homogenized with the stomacher. The pH of the slurry was measured by using a Fisher

Accumet Model 230A pH/ion meter (Fisher Scientific Inc., Salt Lake City, UT). The cooked meat samples were blended in a blender for 15 seconds. The pH measurements of both raw and cooked samples were determined. The pH meter was calibrated using pH buffers 4.00 (SB 101-500, Fisher Scientific, Fair Lawn, NJ) and 7.00 (SB 107-500, Fisher Scientific, Fair Lawn, NJ). The probe was placed into the sample homogenate and allowed to equilibrate for one minute before the pH reading was recorded. All pH readings were performed in triplicate.

6.3.3 Water Activity Analysis

Water activity (a_w) measures the energy status of the water in the system. In other words, a_w is an important factor in related to microbial growth, chemical reactivity, and product stability. Aw is considered as a criterion for quality and safety measurements. Therefore, it is defined as the current volume and availability of water for microbial growth; it should not be directly compared with water content. The water activity is given as a_w value and ranges from 0 and 1. Zero represents absolute dryness and one represents condensed humidity. Homogenized raw and cooked fish samples water activities were measured with AquaLab. The sample was filled slightly less than half of the water activity container. The AquaLab was warmed up 15 minutes before use. The temperature is set at room temperature. All a_w cooked and raw readings were performed in triplicate.

6.3.4 Water Holding Capacity Analysis

The water-holding capacity method was based on previous studies with slight modification (Se Avila and others 2014). The cooked fish balls were minced with a food processor. Ten grams minced cooked samples were placed into 40 ml tubes containing 20 ml of 0.6 M sodium chloride solution, and the tube was vortexed (Votex Geniz 2 TM Cat. No.12-812 Model G 250, Fisher Scientific, McGaw, IL) for 1 minute to ensure even

distribution. The tubes were placed into a 4°C refrigerator for 15 minutes prior to centrifugation. The centrifuge (Sorvall RC-5B, Beverly, MA) was turned on 30 minutes prior to use. The tubes were centrifuged at 7000 rpm at 4°C for 15 minutes. After centrifugation, the liquid lost during centrifugation was collected. The sample was allowed to stand for 1 minute so the liquid could drain. Only the liquid was decanted and solid meat particles were kept in the tube. The WHC of cooked sample was calculated as the ratio of the water remaining after centrifugation to the initial content of meat sample, using the following formula. The WHC (%) was determined by using the following equation:

$$WHC\ \% = 100 * \left(\frac{W1 - W2}{W3}\right)$$

Where W1 represents solution added into the sample, g

W2 represents solution removed after, g

W3 represents the meat samples mass, g

6.3.5 Cooking Yield Analysis

Ten fish balls of each treatment were placed in clear reclosable zipper bags for cooking. During cooking, the bags were zipped to avoid moisture loss. The samples were heated for about 90 minutes in a water bath (70°C ±1°C) until internal temperature reached 70°C, which was monitored with copper-constantan thermocouples. The copper-constantan thermocouples were inserted into the center of the fish ball before cooking. The water bath was preheated approximately 30 minutes until the water bath temperature reached 70°C. After reach the desired internal temperature was reached, the cooked fish balls were placed on paper towels and cooled for 15 minutes at room temperature. The weight (Mettler Toledo Scales, Model: MS 3001S 103, Switzerland) of the fish balls

were recorded before and after cooking, and cooking yield was calculated using the following equation:

$$Cooking\ Yield\ \% = 100 * \frac{W2}{W1}$$

W1 represents the weight before cooking, g

W2 represents the weight after cooking, g

6.3.6 TPA Analysis

The TPA was carried out using a texture profile analyzer (TA-XT Express, Stable Micro Systems Ltd.). After the fish balls were cooked, their weights were recorded for the cooking yield. The fish balls were cooled to room temperature before performing texture profile analysis (TPA). A 5 kg load cell was applied at a crosshead speed of 1 mm/s. A double compression cycle test was performed with up to 50% compression of the original portion height with an aluminum cylinder probe 5-cm diameter. A gap of 5 seconds was allowed to elapse between the two compression cycles. Once tests were finished, the following parameters would be recorded, including hardness, springiness, adhesiveness, cohesiveness, chewiness, resilience, and gumminess. Hardness (N) is the maximum force required to compress the sample. Springiness (m) is the ability of the sample to recover its original form after deforming force was removed; Adhesiveness (N*s) is the area under the abscissa after the first compression. Cohesiveness is the extent to which the sample could be deformed prior to rupture. Chewiness (J) is the work required to masticate the sample before swallowing, which is defined as the product of hardness, springiness, and cohesiveness. Resilience is to measure how well a product fights to bounce back its original shape and size. Resilience has some similarities to springiness. Most of the time, the products spring back (recover their height) different

with the energy they exert to recover their shape. Gumminess applies only to semi-solid products and is calculated as hardness*cohesiveness. Chewiness applies only to solid products and is calculated as gumminess*springiness.

6.3.7 Puncture Test Analysis

After the fish balls were cooled to room temperature, the puncture test was performed. A Stevens-LFRA Texture analyzer was used to penetrate the approximate 2-cm diameter fish patty disks. The diameter of the spherical probe was 0.635 cm. The penetrating speed was 2.00 mm/sec. The highest value throughout puncturing was recorded. Six samples per treatment were measured. Samples were removed from refrigerated conditions and centrally placed underneath the probe. Tests were performed at ambient environment.

6.3.8 Moisture Analysis

Moisture content determination applied the method from Zayas (1997) with modifications. About 3.0 g of raw paste and cooked fish sample was placed in an aluminum tray and placed in a vacuum oven at 80 °C for 24 hours under 23kPa pressure, and cooled to room temperature in a desiccator prior to taking final weights. Three samples per treatment were measured. Moisture (%) was calculated using the following equation:

$$Moisture\ \% = 100*(1-\frac{W2}{W1})$$

W1 represents the weight before drying, g

W2 represents the weight after drying, g

6.3.9 Objective Color Measurement

The raw meat and cooked fish ball was minced with a food processor. The minced samples were transferred in to a petri dish, and color measurements were taken from the bottom dish. The raw color was measured with Hunter MiniScan XE plus colorimeter (Hunter Associated Laboratory, Inc, Reston, VA) in the same manner as cooked minced samples. The colorimeter was calibrated with a standard black tile and white tile as recommended by the manufacturer. The samples were measured at three locations for L*, a* and b* values. The instrumental color of L*, a* and b* color spectrum were recorded. Where L* represents the total light reflected on a scale ranging from 0 = black to 100 = white, while a* represents the amount of red (positive values) and green (negative values), and b* values represents the amount of yellow (positive values) and blue (negative values).

6.3.10 Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS institute, 2002) by generating an analysis of variance (ANOVA). The model includes the main effects of binder treatments and storage days for both raw and cooked fish balls. For other parameters, the main effects of treatments were analyzed. Comparisons among means were performed using SAS Tukey of SAS 9.4. P values less than 0.05 ($P < 0.05$) were considered statistically significant. The MEANS procedure was employed to analyze data and treatment.

6.4 RESULTS AND DISCUSSION

6.4.1 pH Analysis

The data demonstrated that fish balls treated with 1.5% methylcellulose and 1.5% carrageenan had the highest pH values among these treatments (Table 6-1). Fish balls

treated with sodium alginate and encapsulated lactic acid had significantly lower pH values when compared with samples treated with carrageenan and methylcellulose. The analyzed results stated that there was no statistically significant difference between samples treated with sodium alginate and control; however, both raw and cooked samples treated with sodium alginate had the lowest pH values among all treatments. The raw fish balls were set overnight before cooking. The encapsulated lactic acid are small beads of acid surrounded by a lipid coating, and the acid was gently blended into the fish mixing in order to avoid disrupting the lipid coat. When calcium ions were introduced into the alginate system, the encapsulated lactic acid helped to slowly release calcium and control the gel development rate and setting time. After 24 hours setting, it formed the thermo-irreversible gel system. The raw pH values were measured after 24 hours setting, which can explain the lower pH values of sodium alginate samples compared with other treatments.

6.4.2 Water Activity Analysis

No significant differences ($P > 0.05$) were observed among treatments regarding water activities (a_w) (Table 6-1). The water activity of the raw meat sample lies in the range of 0.962 to 0.980. After formulated with binders, due to their high moisture contents, the water activity of the product did not show significantly drop. Values still ranged from 0.975 to 0.98. Except for samples treated with carrageenan and cornstarch, other treated fish ball samples decreased in a_w after cooking. The accuracy of a_w equipment is +/- 0.003 and repeatability is +/- 0.002. In general, the a_w of fresh meat and fish has the highest a_w at 0.99, the a_w of cooked meat is around 0.91-0.98. A_w value is used to free or available water in food systems. Dissolved substances could reduce values

of water activity. In this study, all binders are used to binder water in product and bind meats together. The water activity was not affected by the binders.

Table 6-1. The pH and A_w Measurements for Raw and Cooked Fish Balls Treated with Meat Binders and Stored at 4 °C

	Attributes			
Treatment	Cooked pH	Raw pH	Cooked a_w	Raw a_w
Control	9.26^{ab}	8.96^{a}	0.977^{a}	0.972^{a}
5.0% Cornstarch	9.20^{ab}	9.09^{a}	0.978^{a}	0.980^{a}
1.2% Meatbinder	9.03^{ab}	8.71^{a}	0.975^{a}	0.962^{a}
1.5% Carrageenan	9.49^{a}	9.35^{a}	0.975^{a}	0.975^{a}
1.5% Methylcellulose	9.45^{a}	9.27^{a}	0.975^{a}	0.969^{a}
1.0% Activa® RM	9.31^{ab}	9.17^{a}	0.980^{a}	0.970^{a}
0.7% FG+	9.36^{ab}	9.22^{a}	0.976^{a}	0.962^{a}
0.7% FG	9.26^{ab}	9.10^{a}	0.980^{a}	0.976^{a}
0.8% Sodium Alginate	8.74^{b}	8.56^{a}	0.975^{a}	0.967^{a}

$^{a-b}$ Means in same column with different superscripts are significantly different ($p<0.05$)

6.4.3 Moisture Analysis

The results for both raw and cooked moisture measurements are shown in Table 6-2. There was no significant difference ($P > 0.05$) in cooked moisture among most samples with different treatments. Except for the cornstarch treatment, the moistures for binder-treated samples were consistently lower ($P > 0.05$) than control samples. Cooked samples treated with cornstarch had the lowest moisture among treatments and were significant lower ($P < 0.05$) than control samples.

6.4.4 Cooked Water Holding Capacity Analysis

The WHC percentages among different treatments showed significantly different results ($P < 0.05$) (Table 6-2). The samples treated with Activa® RM had much higher ($P < 0.05$) WHC values than control samples. Samples treated with methylcellulose had significantly lower ($P < 0.05$) WHC values when compared with the control treatment.

The cooked samples treated with meatbinder, FG+, FG and sodium alginate had similar WHC ($P > 0.05$). The cooked samples treated with carrageenan and cornstarch showed similar WHC values ($P > 0.05$) as the control samples. The samples treated with Activa® RM showed significantly higher WHC values ($P < 0.05$) than the control treatment, and methylcellulose treatments had significantly lower WHC ($P <0.05$) when compared to the control.

WHC determination is used to test the meat system's ability to hold water. The water could be added liquid or intrinsic to the meat. There are three basic principles to measure WHC, including applying no force, applying mechanical force and applying thermal force. Applying no force methods include weight loss, drip loss, or evaporation loss, and they are very sensitive and time consuming; Applying external mechanical force method uses instant centrifugation; Applying thermal force methods include cooking yield and cooking loss. WHC of cooked meat is the main interest for meat processors and consumers. In this experiment, the centrifugation method was used to determine WHC. Preliminary experimental results (data not shown) demonstrated that the centrifugation method for raw fish paste is not appropriate due to development of fish gelation. The fish muscle is broken down with different degrees of integrity during chopping and blending processing, which extracts myofibrillar protein from fish muscle. The comminuted fish incorporated with sodium chloride solution in cold environmental developed properties similar to surimi. The fish proteins were separated from centrifugation processing, and retained its gel forming ability, so the centrifugation method cannot be used for raw minced fish WHC measurements.

Table 6-2 results showed the ability of binder to uptake added water in meat when combined with sodium chloride. This method attempts to mimic the practical industry processing when salt is added as an ingredient. It is concluded that samples treated with methylcellulose had the lowest WHC while samples treated with Activa® RM had the highest WHC ability compared with control samples.

6.4.5 Cooking Yield Analysis

In general, there was a positive relationship between cooking yield and water holding capacity. The samples treated with methylcellulose showed the lowest cooking yield percentage. There was no statistical difference between control and methylcellulose treatments ($P > 0.05$), however, the control treatment showed 3.5% cooking percentage higher than that of methylcellulose treatment. Samples treated with methylcellulose had lower cooking yield than control samples. From a meat processor's point of view, this is undesirable. All other treatments showed consistently higher than control treatment. The cornstarch, meatbinder, and carrageenan treatments showed significantly higher cooked yield than the control treatment. Activa® RM, FG+, FG and sodium alginate showed similar cooking yields ($P > 0.05$) as the control. However, there was a 1-4% increase in cooking yield, which would still be important to meat processors. The higher cooking yield from binder treatments may be due to the water binding capacity of all meat binders except for methylcellulose.

Table 6-2. Cooked WHC and Cooking Yield, and Moisture for Both Raw and Cooked Fish Balls, Treated with Meat Binders and Stored at 4 °C

Treatment	Attributes			
	Cooked Moisture (%)	Raw Moisture (%)	Cooked WHC (%)	Cooking Yield (%)
Control	83.68^{a}	85.49^{a}	85.17^{bc}	88.44^{bc}
5.0% Cornstarch	80.52^{a}	80.96^{b}	82.30^{cd}	93.36^{a}
1.2% Meatbinder	84.79^{a}	84.29^{ab}	91.72^{ab}	93.14^{a}
1.5% Carrageenan	82.96^{a}	83.60^{ab}	87.00^{abc}	94.17^{a}
1.5% Methylcellulose	83.23^{a}	84.56^{ab}	76.41^{d}	84.91^{c}
1.0% Activa® RM	83.08^{a}	84.29^{ab}	95.03^{a}	90.42^{ab}
0.7% FG+	84.03^{a}	84.33^{ab}	91.87^{ab}	91.83^{ab}
0.7% FG	83.61^{a}	84.21^{ab}	92.82^{ab}	92.45^{ab}
0.8% Sodium Alginate	82.21^{a}	84.36^{ab}	92.34^{ab}	92.67^{ab}

$^{a-d}$ Means in same row with different superscripts differ significantly ($P < 0.05$)

6.4.6 Cooked and Raw Meat Samples Puncture Test Analysis

For puncture or penetration tests, the forces of deformation are used to test muscle binding or sample hardness. Small cylinders, balls, needles, and cones are used to penetrate sample to imitate month bite. Five types of puncture tests were reported in Figure 6-1. Two parameters were displayed as the results of the test: peak load and final load in units of grams. The peak load is the highest load value recorded during the test. The final load is the last load recorded prior to the probe returning to its original position. In this experiment, only peak loads were recorded. It provides the hardness of meat samples.

In this study, a 6.35 mm spherical ball probe was used. A ball probe is typically used in samples that are not consistent or are not completely flat. Since it is difficult to make exactly the same size and same shape fish balls, the ball probe was selected in this study.

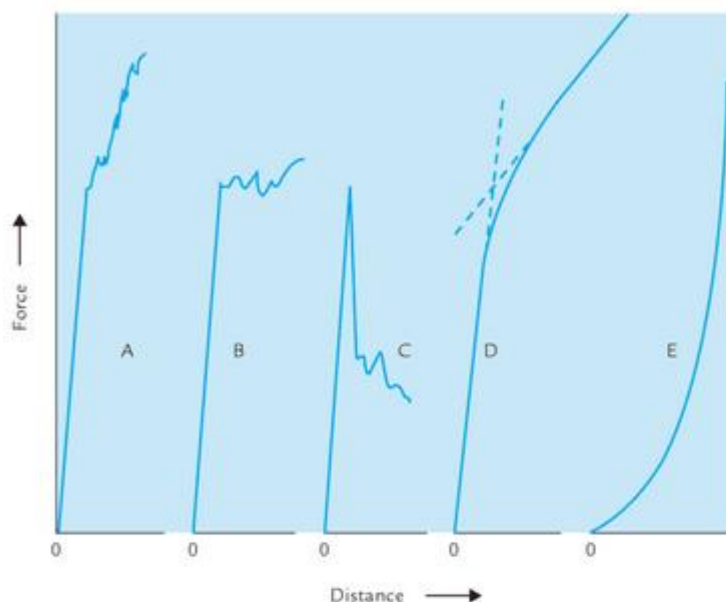

Figure 6-1. Schematic Representation of the Five Different Types of Force-Distance Curves that are Obtained in Puncture Test. (Adapted from Huang (2010); (AOAC 2000), Original from Bourne 1979, copyright Academic Press Inc. London)

The puncture value results for both raw and cooked fish balls are shown in Table 6-3. Samples treated with Activa® RM showed significantly higher puncture values ($P < 0.05$) than the rest of treatments. Both raw and cooked samples treated with meatbinder showed lower values than other binder treatments, including control samples. Some binder-treated samples had significantly higher value ($P <0.05$) than samples treated with meatbinder and some samples had slightly higher values ($P > 0.05$) than samples treated with meatbinder. The control treatments showed the second lowest hardness among treatments.

Cooked samples treated with Activa® RM and carrageenan showed significantly higher puncture values than the rest of samples ($P < 0.05$), Activa® RM showed slightly higher hardness than samples treated with carrageenan ($P > 0.05$) (Table 6-3). Control, methylcellulose, and sodium alginate treated samples had similar hardness. These three

samples had higher hardness than samples treated with meatbinder, but less hardness than samples treated with 0.7% FG+ and 0.7% FG.

Table 6-3. Puncture Tests for Both Raw and Cooked Fish Balls Treated with Different Meat Binders

Puncture Test	Attributes	
Treatment	Raw Meat	Cooked Meat
Control	22.50^{bc}	168.6^{cd}
5.0% Cornstarch	26.59^{b}	217.9^{bc}
1.2% Meatbinder	18.50^{c}	134.7^{d}
1.5% Carrageenan	22.33^{bc}	337.5^{a}
1.5% Methylcellulose	29.17^{b}	162.6^{cd}
1.0% Activa® RM	37.67^{a}	340.2^{a}
0.7% FG+	28.67^{b}	263.0^{b}
0.7% FG	28.58^{b}	257.7^{b}
0.8% Sodium Alginate	25.08^{bc}	160.5^{cd}

$^{a\text{-}d}$ Means in same column with different superscripts differ significantly (p<0.05)

6.4.7 Cooked and Raw Objective Meat Color Measurement

On the initial day, control treatments and samples treated with carrageenan were darker (P < 0.05) for raw meat samples, when compared to samples treated with meatbinder, methylcellulose, sodium alginate, and similar with samples treated with Activa® RM. Except the lightness of methylcellulose treatment did not significantly change (P>0.05), all other samples became darker as storage time increased (Table 6-4). Cornstarch and methylcellulose treatments showed the highest lightness from day 0 to day 5. This might be due to the fact that adding cornstarch into samples increases the lightness. That is also true for methylcellulose. Methylcellulose is a white powder, which can increase meat lightness when added into minced fish. In contrast, carrageenan powder is a yellowish color. It reduces lightness after being formulated into fish samples.

Sodium alginate, methylcellulose, cornstarch and meatbinder treated samples had more lightness than control samples (P< 0.05).

Table 6-4. Objective Raw Meat Color L* Values for Fish Balls Treated with Different Meat Binders and Stored at 4 °C for 5 Days

		Storage Time			
Attribute	Treatment	Day 0	Day 1	Day 3	Day 5
Raw L*	Control	$75.87^{a, y}$	$71.68^{ab,y}$	$68.68^{b,y}$	$67.69^{b,z}$
	5.0% Cornstarch	$81.51^{a, w}$	$80.4^{ab,w}$	$79.57^{bc,w}$	$78.64^{c,w}$
	1.2% Meatbinder	$80.41^{a, wx}$	$76.88^{b,wx}$	$77.06^{b,w}$	$75.88^{b,wx}$
	1.5% Carrageenan	$75.47^{a, y}$	$71.36^{b,y}$	$70.32^{bc,y}$	$68.51^{c,z}$
	1.5% Methylcellulose	$80.74^{a, wx}$	$76.68^{a,wx}$	$75.99^{a,wx}$	$76.16^{a,wx}$
	1.0% Activa® RM	$77.34^{a, xy}$	$73.19^{b,xy}$	$71.74^{bc,y}$	$70.63^{c,yz}$
	0.7% FG+	$77.59^{a, wxy}$	$73.59^{b,xy}$	$72.49^{bc, xy}$	$69.97^{c,yz}$
	0.7% FG	$76.09^{a,y}$	$71.96^{b,y}$	$71.65^{bc,y}$	$69.6^{c,xy}$
	0.8% Sodium Alginate	$80.83^{a, wx}$	$77.12^{ab,wx}$	$77.51^{ab,w}$	$73.16^{b,xy}$

[a-d] Means in same row with different superscripts differ significantly (P < 0.05);
[w-z] Means in same column with different superscripts differ significantly (P < 0.05);

Table 6-5. Objective Raw Meat Color a* Values for Fish Balls Treated with Different Meat Binders and Stored at 4 °C for 5 Days

		Storage Time			
Attribute	Treatment	Day 0	Day 1	Day 3	Day 5
Raw a*	Control	$3.59^{a,x}$	$2.45^{a,xy}$	$2.03^{a,xyz}$	$1.69^{a,x}$
	5.0% Cornstarch	$2.66^{a,xy}$	$2.68^{a,xy}$	$2.69^{a,xy}$	$2.77^{a,x}$
	1.2% Meatbinder	$2.06^{a,y}$	$1.44^{a,y}$	$1.32^{a,z}$	$1.76^{a,x}$
	1.5% Carrageenan	$2.09^{a,y}$	$1.78^{a,xy}$	$1.8^{a,xyz}$	$1.76^{a,x}$
	1.5% Methylcellulose	$1.99^{a,y}$	$1.75^{a,y}$	$1.72^{a,yz}$	$1.79^{a,x}$
	1.0% Activa® RM	$2.58^{a,xy}$	$1.94^{ab,xy}$	$1.66^{b,yz}$	$2.03^{ab,x}$
	0.7% FG+	$2.51^{a,xy}$	$1.98^{a,xy}$	$1.74^{a,xyz}$	$2.13^{a,x}$
	0.7% FG	$3.02^{a,xy}$	$2.65^{a,xy}$	$2.53^{a,xyz}$	$2.78^{a,x}$
	0.8% Sodium Alginate	$3.43^{a,xy}$	$3.06^{a,x}$	$2.96^{a,x}$	$2.78^{a,x}$

[a-d] Means in same row with different superscripts differ significantly (P < 0.05)
[w-z] Means in same column with different superscripts differ significantly (P < 0.05)

Except for Activa® RM treatment, there was no significant difference in redness (a*) detected during storage period from day 0 to day 5 for raw fish samples (Table 6-5). However, when only comparing day 0 and day 5, except for cornstarch treatments, the fish balls from all treatments insignificantly decreased the redness. For the cornstarch treatment, the redness slightly changed during 5 days of storage. Control samples decreased redness more sharply than other treatments. It started with the highest initial redness, and ended with the lowest redness. Therefore, compared with control treatments, all binders could efficiently protect the redness of meat samples.

Table 6-6. Objective Raw Meat Color b* Values for Fish Balls Treated with Different Meat Binders and Stored at 4 °C for 5 Days

		Storage Time			
Attribute	Treatment	Day 0	Day 1	Day 3	Day 5
Raw b*	Control	$8.53^{a,wxyz}$	$7.33^{b,y}$	$6.35^{c,y}$	$5.97^{c,x}$
	5.0% Cornstarch	$9.06^{a,vwx}$	$9.32^{a,v}$	$9.25^{a,v}$	$9.39^{a,uv}$
	1.2% Meatbinder	$7.90^{a,z}$	$7.02^{ab,y}$	$6.85^{b,y}$	$7.34^{ab,wx}$
	1.5% Carrageenan	$10.95^{a,u}$	$10.6^{ab,u}$	$10.51^{ab,u}$	$9.98^{b,u}$
	1.5% Methylcellulose	$8.18^{a,yz}$	$8.53^{a,vw}$	$8.15^{a,w}$	$8.19^{a,vw}$
	1.0% Activa® RM	$8.31^{a,xyz}$	$7.50^{ab,xy}$	$6.94^{b,xy}$	$7.06^{b,wx}$
	0.7% FG+	$9.21^{a,vw}$	$8.28^{ab,wx}$	$7.91^{b,wx}$	$8.02^{b,vw}$
	0.7% FG	$9.51^{a,v}$	$8.80^{ab,vw}$	$8.52^{ab,vw}$	$8.41^{b,uvw}$
	0.8% Sodium Alginate	$8.71^{a,wxy}$	$8.71^{a,vw}$	$8.56^{a,vw}$	$7.95^{a,vw}$

$^{a-d}$ Means in same row with different superscripts differ significantly ($P < 0.05$)
$^{w-z}$ Means in same column with different superscripts differ significantly ($P < 0.05$)

There was no significant b* value (yellowness) reduction for cornstarch, methylcellulose, and sodium alginate samples as storage period increased from day 0 to day 5. For the rest of the treatments, the b* value decreased as storage time increased (Table 6-6). Among these treatments, meatbinder samples showed the lowest yellowness values compared with all other treatments for all storage days. The yellowness was

significantly lower (P < 0.05) in some days and little bit lower in other days. Carrageenan samples had higher yellowness than other treatments, this is due to the yellow color of carrageenan itself, which can increase the yellowness of samples.

6.4.8 Cooked Meat L*, a* and b*

Table 6-7. Objective Cooked Meat Color L* Values for Fish Balls with Different Binders and Stored at 4 °C for 5 Days

		Storage Time	
Attribute	Treatment	Day 1	Day 5
Cooked L*	Control	$76.15^{a,yz}$	$76.23^{a,x}$
	5.0% Cornstarch	$79.59^{a,uw}$	$78.48^{b,vw}$
	1.2% Meatbinder	$81.35^{a,u}$	$80.37^{b,x}$
	1.5% Carrageenan	$74.88^{a,z}$	$74.01^{b,y}$
	1.5% Methylcellulose	$78.98^{a,wx}$	$77.62^{a,wx}$
	1.0% Activa® RM	$76.62^{a,y}$	$76.29^{a,x}$
	0.7% FG+	$77.43^{a,y}$	$76.53^{b,x}$
	0.7% FG	$77.61^{a,xy}$	$76.43^{b,x}$
	0.8% Sodium Alginate	$80.83^{a,uv}$	$79.96^{b,uv}$

$^{a-d}$ Means in same row with different superscripts differ significantly (P < 0.05)
$^{w-z}$ Means in same column with different superscripts differ significantly (P < 0.05)

After meat samples were cooked in a water bath for 30 minutes, cooking yield, texture, pH, moisture, and WHC were evaluated. A blender was used to grind fish balls for 15 seconds and the ground fish samples were placed into petri dishes, covered with lids. The cooked meat color was measured using colorimeter through a petri dish lid. The grinder was clean thoroughly before preparing the next treatment. When compared, all cooked meat color changed from day 0 to day 5. All meat sample treatments decreased in lightness during the storage period. There were no significantly differences in lightness detected for control samples or those treated with methylcellulose or Activa® RM (P >

0.05). For other treatments, the cooked lightness values changed significantly (P < 0.05). However, overall, the lightness of samples decreased as storage time increased.

Table 6-8. Objective Cooked Meat Color a* Values for Fish Balls with Different Binders and Stored at 4 °C for 5 Days

		Storage Time	
Attribute	Treatment	Day 1	Day 5
Cooked a*	Control	-7.69^{a,z}	-0.89^{a,xyz}
	5.0% Cornstarch	-5.34$^{a, xyz}$	-0.64^{a,x}
	1.2% Meatbinder	-0.62^{a,xyz}	-0.67^{a,xy}
	1.5% Carrageenan	-0.38^{a,wx}	-0.55^{b,x}
	1.5% Methylcellulose	-0.49^{a,xyz}	-0.62^{b,x}
	1.0% Activa® RM	-0.70^{a,yz}	-1.06^{b,z}
	0.7% FG+	-0.77^{a,z}	-1.01^{b,yz}
	0.7% FG	-0.48^{a,wxy}	-0.61^{b,x}
	0.8% Sodium Alginate	-1.69^{a,w}	-0.20^{a,w}

$^{a\text{-}d}$ Means in same row with different superscripts differ significantly (P < 0.05);
$^{w\text{-}z}$ Means in same column with different superscripts differ significantly (P < 0.05);

Table 6-9. Objective Cooked Meat Color b* Values for Fish Balls with Different Binders and Stored at 4 °C for 5 Days

		Storage Time	
Attribute	Treatment	Day 1	Day 5
Cooked b*	Control	9.37$^{a, yz}$	9.41^{a,xy}
	5.0% Cornstarch	9.69^{a,xyz}	9.56^{a,xy}
	1.2% Meatbinder	9.22^{b,z}	9.40^{a,y}
	1.5% Carrageenan	10.49^{a,x}	10.32^{b,w}
	1.5% Methylcellulose	9.62^{a,xyz}	9.60^{a,xy}
	1.0% Activa® RM	9.38^{a,yz}	9.16^{a,y}
	0.7% FG+	9.88^{a,xy}	9.67^{a,wxy}
	0.7% FG	10.11^{a,wx}	10.09^{a,wx}
	0.8% Sodium Alginate	9.54^{b,yz}	9.71^{a,wxy}

$^{a\text{-}d}$ Means in same row with different superscripts differ significantly (P < 0.05);
$^{w\text{-}z}$ Means in same column with different superscripts differ significantly (P < 0.05);

The same trends were observed with raw carrageenan treatments when compared with cooked b* values (yellowness) (Table 6-9). The cooked samples treated with carrageenan showed the highest yellowness value among treatments, and had significantly higher yellowness than samples treated with control, meatbinder, Activa® RM and sodium alginate. Meatbinder samples had the lowest yellowness values among all treatments. When compared with yellowness on day 0 and day 5, except for meatbinder and sodium alginate treatments, there were no yellowness changes among samples (P >0.05).

6.4.9 Cooked and Raw TPA Analysis

Carrageenan samples had the highest hardness, adhesiveness, gumminess, and chewiness parameters, and had the lowest springiness and cohesiveness parameters (Table 6-10). Samples treated with 1.0% Activa® RM, 0.7% FG+ and 0.7% FG had similar hardness (P > 0.05). Compared with samples treated with control and cornstarch, there was no advantages found (P > 0.05) when adding cornstarch as a binder. However, there were slightly harder than control samples. Samples treated with methylcellulose had the softest texture among treatments. Except for samples treated with methylcellulose, all other binder treatments had a firmer texture than the control treatment. Therefore, except for methylcellulose, all these binders can be applied into fish balls to improve the hardness texture. The 1.0 % Activa® RM, 0.7% FG+ and 0.7% FG treatments had less stickiness, more cohesiveness, more gumminess, more chewiness, and more resilience than control treatments.

Compared with cooked fish ball samples, the raw TPA data demonstrated that 1.0% Activa® RM (2182.7 g) had the firmest texture among all treatments (Table 6-11). It was 7 times the hardness of the control samples (319.0 g). Next, samples treated with 0.7%

FG+ and 0.7% FG treated samples had firmer textures than control samples. When compared with control samples, sodium alginate system, cornstarch, and meatbinder did not show any advantage. Samples treated with 1.0% Activa® RM had the lowest adhesiveness, followed by samples treated with 0.7% FG+ and 0.7% FG. Samples treated with 1.0% Activa® RM showed the highest gumminess and chewiness among treatment samples. For sodium alginate treated samples, all parameter values were higher than control samples. Control treated samples had the softest texture among all treatments.

Table 6-10. Cooked TPA for Fish Balls with Different Binder Treatments

Cooked TPA	Attribute						
Treatment	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Control	1588.3^{ef}	-15.17 ^{ab}	0.956^{a}	0.510^{bcd}	810.9^{c}	773.2^{c}	0.411^{b}
5.0% Cornstarch	1858.7^{de}	-24.10^{b}	0.952^{ab}	0.504^{cd}	937.6^{c}	894.3^{c}	0.384^{bc}
1.2% Meatbinder	2125.8^{cd}	-24.04^{b}	0.904^{bc}	0.497^{d}	1057.2^{bc}	955.1^{bc}	0.364^{c}
1.5% Carrageenan	3226.1^{a}	-2.01^{a}	0.833^{d}	0.456^{e}	1477.8^{a}	1239.3^{a}	0.320^{d}
1.5% Methylcellulose	1188.1^{f}	-1.58^{a}	0.880^{cd}	0.438^{e}	521.8^{d}	458.8^{d}	0.320^{d}
1.0% Activa® RM	2775.8^{ab}	-4.78^{a}	0.963^{a}	0.531^{ab}	1477.3^{a}	1421.6^{a}	0.492^{a}
0.7% FG+	2579.6^{bc}	-5.99^{a}	0.938^{ab}	0.526^{abc}	1360.4^{a}	1277.6^{a}	0.458^{a}
0.7% FG	2473.8^{bc}	-3.67^{a}	0.922^{abc}	0.515^{bcd}	1274.1^{ab}	1175.6^{ab}	0.466^{a}
0.8% Sodium Alginate	1791.1^{de}	-26.53^{b}	0.945^{ab}	0.454^{a}	976.3^{c}	921.6^{bc}	0.387^{bc}

Table 6-11. The TPA Values for Raw Fish Balls with Different Binder Treatments

Raw TPA	Attribute						
Treatment	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Control	319.0^{e}	-222.3^{b}	0.541^{c}	0.311^{d}	99.3^{d}	55.3^{e}	0.072^{e}
5.0% Corn Starch	333.6^{e}	-517.22^{d}	0.800^{ab}	0.383^{c}	127.7^{d}	102.2^{de}	0.074^{e}
1.2% Meatbinder	327.4^{e}	-338.2^{c}	0.734^{b}	0.326^{d}	106.7^{d}	78.7^{de}	0.069^{e}
1.5% Carrageenan	546.6^{d}	-112.4^{ab}	0.475^{c}	0.283^{d}	154.8^{cd}	73.4^{de}	0.107^{d}
1.5% Methylcellulose	396.1^{e}	-425.7^{cd}	0.860^{a}	0.486^{a}	194.1^{c}	169.2^{c}	0.150^{c}
1.0% Activa® RM	2182.7^{a}	-8.67^{a}	0.762^{ab}	0.416^{bc}	907.7^{a}	691.7^{a}	0.320^{a}
0.7% FG+	693.9^{c}	-48.7^{a}	0.529^{c}	0.301^{d}	207.1^{c}	110.4^{d}	0.141^{c}
0.7% FG	914.9^{b}	-12.2^{a}	0.729^{b}	0.399^{c}	366.3^{b}	267.6^{b}	0.255^{b}
0.8% Sodium Alginate	396.1^{e}	-112.4^{ab}	0.854^{a}	0.443^{ab}	134.6^{d}	115.1^{d}	0.078^{c}

Table 6-12. Relationship among Cooked TPA Parameters

Pearson Correlation Coefficients, N=81

Prob > |r| under H0: Rho=0

	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Hardness	1.000	0.116	-0.160	0.178	0.973	0.924	0.275
Adhesiveness	0.116	1.000	-0.355	-0.337	0.043	-0.014	0.135
Springiness	-0.160	-0.355	1.000	0.687	0.009	0.184	0.604
Cohesiveness	0.178	-0.337	0.687	1.000	0.384	0.499	0.753
Gumminess	0.973	0.043	0.009	0.384	1.000	0.983	0.445
Chewiness	0.924	-0.014	0.184	0.499	0.983	1.000	0.556
Resilience	0.275	0.135	0.604	0.753	0.445	0.556	1.000

Table 6-13. Relationship among Raw TPA Parameters

Pearson Correlation Coefficients, N=81

Prob > |r| under H0: Rho=0

	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
Hardness	1.000	0.542	0.039	0.154	0.989	0.963	0.879
Adhesiveness	0.542	1.000	-0.594	-0.467	0.454	0.361	0.610
Springiness	0.039	-0.594	1.000	0.852	0.162	0.280	0.122
Cohesiveness	0.154	-0.467	0.852	1.000	0.290	0.391	0.324
Gumminess	0.989	0.454	0.162	0.290	1.000	0.992	0.891
Chewiness	0.963	0.361	0.280	0.391	0.992	1.000	0.876
Resilience	0.879	0.610	0.122	0.324	0.891	0.876	1.000

Table 6-14. Relationship among Raw and Cooked TPA Hardness and Puncture Tests

Pearson Correlation Coefficients, N=81

Prob > |r| under H0: Rho=0

	Raw-Puncture Test	Cooked-Puncture Test	Cooked Hardness	Raw Hardness
Raw-Puncture Test	1.000	0.309	0.180	0.199
Cooked-Puncture Test	0.309	1.000	0.367	0.101
Cooked Hardness	0.180	0.367	1.000	0.695
Raw Hardness	0.199	0.101	0.695	1.000

Hardness is the force required to break food samples into pieces during first bite or the maximum force of first compression. 1.0% Activa® RM, 0.7% FG+ and 0.7% FG of raw samples had the top three highest hardness values among nine treatments. For cooked samples, the top four highest hardness values were achieved with carrageenan, 1.0% Activa® RM, 0.7% FG+ and 0.7% FG treatments. There is positive correlation between hardness of cooked samples and hardness of raw samples (Table 6-14). The correlation coefficient is 0.695. The value of a correlation coefficient ranges between -1 and 1. The greater the absolute value of a correlation coefficient, the stronger the linear relationship. While the correlation coefficient between hardness and punctures values of cooked samples was 0.367 (Table 6-14).

Adhesion is to measure stickiness of food products. A higher value means the food is sticker. For raw samples, the top three low adhesive products were samples treated with 1.0% Activa® RM, 0.7% FG+ and 0.7% FG. For cooked samples, the top three low adhesive products were samples treated with carrageenan and methylcellulose and 1.0% Activa® RM samples.

Springiness is interchangeable with the term “elasticity”. A higher value means the food is stickier. It describes the remaining structural integrity to spring back. It also describes how well the food samples spring back after they are deformed during the first compression. In general, the more the product is destroyed, the lower the springiness value. Control samples and samples treated with Activa® RM had the highest springiness among cooked samples. Samples treated with sodium alginate and methylcellulose had the highest springiness for all raw samples.

Gumminess applies only to semi-solid products and chewiness applies only to solid products. In this experiment, only chewiness values were considered and investigated. Chewiness is the energy required to break down the solid food products. It has highly positive correlation with hardness. The correlation coefficients between gumminess and chewiness with hardness are 0.973 and 0.924 respectively (Table 6-12). Activa® RM has the largest chewiness among nine treatments for both cooked and raw meat samples.

Cohesiveness was calculated as the ratio between that the area of work during the second compression and the area of work during the first compression (Area 2/Area 1). It is defined as how well the product can withstand the second deformation relative to its resistance under the first deformation. In food systems, the cohesiveness is the energy or the number of times the food to can be broken down until it can be swallowed. The correlation coefficient between hardness and cohesiveness is 0.154 for raw fish balls (Table 6-13). It was concluded that the hardness and springiness of foods were uniformly distributed on an evaluation scale. The results may be opposite for cohesiveness.

6.5 CONCLUSIONS

This study showed that samples treated with Activa® RM and FG+ and FG produced satisfactory binding in fish balls. These three binders can result in higher cooking yield, hardness texture, and maintain both cooked and raw fish ball lightness during storage period. Considering overall parameters evaluated in this study, it is concluded that Activa® RM binder showed the best functionality or performance, following with FG+ and FG treatments. Samples treated with sodium alginate performed at medium level. Moreover, studies showed that salt could inhibit alginate from forming a

gel with meat protein. A sodium alginate system is not suitable for products with salt. Samples treated with meatbinder and methylcellulose showed the worst performance.

In conclusion, as storage period increased, the lightness, redness and yellowness of all samples decreased. Except for carrageenan, all other binders showed more lightness than control samples. All samples showed more redness and yellowness than control samples. These binders could be efficiently applied into meat samples to protect lightness, redness, and yellowness of meat samples. As storage times increased, the lightness of cooked samples decreased, the meat samples became darker. There was no significantly difference detected among all samples for cooked meat moisture. Raw meat has slightly higher moisture than other cooked meat treatments. Activa® RM had the highest WHC compared with all cooked samples. Samples treated with methylcellulose had the lowest WHC. Sodium alginate had the lowest pH values for both cooked and raw meat samples. There were no significantly differences detected for water activity for both raw and cooked meat samples. The samples treated with methylcellulose had the lowest cooking yield. All other samples treated meat binders had the higher cooking yields than control samples. Samples treated with Activa® RM, FG+ and FG showed higher values for the puncture tests than control samples. Except for samples treated with sodium alginate, methylcellulose, and meatbinder, all other binders-treated samples had lower puncture values for cooked samples. Activa® RM, FG+ and FG treated samples had the best texture, hardness, and springiness. Overall, samples treated with Activa® RM, FG+ and FG demonstrated the best performance with regard to all parameters. Samples treated with sodium alginate, methylcellulose and meatbinder did not have advantage over

control samples. More research is needed to compare commercial products when adding salt as ingredient into fish balls.

CHAPTER 7

QUANTITATIVE AND QUALITATIVE ANALYSIS OF TOTAL SODIUM ALGINATE IN RESTRUCTURED MEAT PRODUCTS BY FTIR

7.1 ABSTRACT

In this study, a method was developed to quantify sodium alginate, which was added into tilapia paste at specific concentrations (0, 0.5, 1.0, 2.0, and 5.0%). There were two pre-preparation methods. The first method is called direct oven drying sample preparation, which removes moisture with an oven; and the second method is called indirect sample preparation or an extraction method that includes several steps. These exactions step are as following fat removal by acetone extraction, enzymatic protein degradation, ethanol precipitation of the polysaccharides, centrifugation, and air-drying for 30 minutes. FTIR was used to determine the amount of sodium alginate or calcium alginate. When sodium alginate and calcium carbonate are formulated into restructured meat, they react to form calcium alginate. The FTIR analysis was performed at room temperature. The objective of this research is to develop useful alternative methods for directly quantifying total content of sodium alginate in foods and screening hydrocolloid type by using FTIR. The results showed that FTIR could potentially be a good technique for rapid measurement of the amount of sodium alginate added to meat. PLS and PCA models can be useful tools to develop quantitative and qualitative models for hydrocolloid analysis.

7.2 INTRODUCTION

“Low Fat Meat Product” is a magic labeling strategy to attract consumers. It has been a fashionable advertisement in meat industry for decades. The function of fat is to develop unique flavor and contribute (to) the texture of meat products. Therefore,

reducing fat content in the meat product can result in a firmer, more rubbery, less juicy product with dark color and a higher cost (Bourne 2002). Removing fat from meat requires a fat replacement to supplement the lost flavor, texture, and mouth feel to mimic traditional meat samples. Alginate is one of the most popular hydrocolloid fat substitutes due to its higher binding water capacity. Using alginate as fat replacer can supplement the juice loss from fat removal and can provide creamy meat texture (Bourne 2002).

Sodium alginate is the sodium salt of alginic acid, a natural polyuronided constituent of certain brown algae. Alginates and alginic acid are widely used in the food industry, biotechnology, and medicine because of their gel-forming capacity. Alginates are polysaccharides obtained from marine brown algae. Alginic acid is a linear, 1, 4-linked copolymer of β-D-mannuronic acid (M unit) and its C5 epimer, α-l-guluronic acid (G unit), as seen in Figure7-1 (Mallika and others 2009; Rourke 1992; Pignolet and others 1998). The G and M units are joined together in homopolymeric and heteropolymeric sequentially alternating blocks that affect the strength of the gel formed in restructured food products. The carboxyl groups present at C-6 of the G and M uronic acid units stabilize the glyosidic bonds from acid hydrolysis (Oztekin and others 2007).

Alginate system restructuring has been developed to meet the demand for restructured meats. The process of making this structured meat with alginate gels was patented by researchers at Colorado State University (Jani and Salamone 2005). The patent described the process of development of restructured meat products by using sodium alginate, calcium carbonate, and glucono-delta-lactone (GDL). Once adding sodium alginate into meat blocks, sodium alginate is hydrated by the moisture of meat. Calcium carbonate has low solubility, which can be used to control the rate of gel

formation, and allow more time for meat processors to stuff the meat mixture into casing. Continuous exposure of alginate to calcium solution can increase the firmness of the gel due to the calcium ion binding with G block within the alginate structure (Rourke 1992). The function of GDL is to change the pH value of meat, which causes the increase of solubility of calcium carbonate. This leads to release of more calcium to the meat system, and causes gel formation. The alginate gel is able to glue the meat particles into larger meat blocks. Once the gel has set, the product is sliced into steaks or chops since the calcium alginate gel is heat stable, and thus the product will not fall apart during cooking. This technique can eliminate the disadvantages of the traditional methods, which use salt and phosphate with mechanical action. The additional salt can alter meat color and cause lipid oxidation, which also can increase sodium content.

There are two functions of alginate application in the meat industry, including fat replacement and gelling in restructured meats. The two applications significantly increase the demands for this chemical. In the current market, only 15% to 20% of a carcass is used as steaks and chops, the remaining carcass is fabricated into ground meat and sausages. There is huge profit by adding alginate, in addition, the USDA limitation of usage and more marketing on restructured meat demands and consumer desire for meat products. The expected increase of alginate in processed meat requires a quantitative method for detection of the sodium alginate in meat products. Therefore, the validated methods should be able to monitor the chemical efficiently. Based on the current USDA regulations, a mixture of sodium alginate (not to exceed 1% of product formulation), calcium carbonate (not to exceed 0.2%), and calcium lactate/lactic acid (not to exceed 0.3%) is permitted for use in restructured meat food products to bind meat pieces. The

entire mixture is not to exceed 1.5% of product at formulation and it must be added dry. For ground formed raw and cooked poultry pieces, sodium alginate cannot exceed 0.8%, calcium carbonate cannot exceed 0.15%, and calcium lactate/lactic acid cannot exceed 0.6%. The entire mixture cannot exceed 1.55% of product formulation and it must be added dry. A mixture containing water, sodium alginate, calcium chloride, carboxymethyl cellulose (CMC), and corn syrup solids may not exceed 1.5% of hot carcass weight when applied and chilled weigh cannot exceed hot carcass weight (no added water). USDA also allows for the application of an alginate film on freshly dressed meat carcasses to reduce cooler shrinkage and help protect surfaces (Means and Schmidt 1986).

General methods used for quantification of alginate have depended on the colorimetric or chromatographic analysis of uronic acids released from enzymatic depolymerization or chemical hydrolysis of alginate (Tarte 2009; USDA-FSIS 2016a). The disadvantage of these methods is the indirect quantification of alginate content via determination of uronic acids. Colorimetric methods are not specific to the various forms of uronic acids. Chromatographic methods are based on an estimation of polysaccharide content from the mean uronic acid concentrations (Awad and Aboul-Enein 2013). Oztekin and others (2007) reported using a cation exchange HPLC to analyze alginate content added into pork product, and this methodology was based on the indirect quantification of analysis of uronic acids of alginate copolymer.

Figure 7-1. The Chemical Structure of Alginate; Chemical Structure of G and M Blocks; Calcium Alginate and Alginate Polymer at Sodium Chloride (No Crosslinking) and Calcium Chloride Solutions (Crosslinking) (Oztekin and others 2007; Rourke and others 1993; Pignolet and others 1998).

Other methods may be useful for direct measurement of the total content of alginate without hydrolysis. Oztekin and others (2007) directly determined the alginate content in brown algae by near infrared (NIR) spectroscopy. Jani and Salamone (2005) developed and validated the method for the quantitative determination of alginic acid, which involved a preliminary separation of the alginic acid from an antacid formulation, and followed by using capillary electrophoresis wherein alginic acid was measured by a UV detector. Horn and others (1999) concluded that the micellar electrokinetic

chromatography (MEKC) method was a promising method to be applicable to the quality control of alginate content in antacid formulations. The solution preparation was fast without purification except for centrifugation and the sample matrix did not interfere with the analysis. Moore and others (2004) also determined the alginic acid content of both solid and liquid antacid formulations treated with alginate lyase followed by capillary electrophoresis and UV detection. Oztekin and others (2007) studied a direct method using HPLC to quantify directly the total alginate content without sample pretreatment in a medicine formulation. In 2011, British Pharmacopeia and U.S. Pharmacopeia recommended development of a method for the assay of alginic acid and alginate as raw material for pharmaceutical applications. Because this calls for development representing an application for a pure condition, this method is unlikely to be applicable to a finished meat matrix.

Currently, there is an increasing demand for seaweed polysaccharides by the food industry. Quick and reliable non-destructive methods to assess the application of polysaccharides are required. Based on the literature review, FTIR or Raman could be used as preliminary methods to identify the main polysaccharides in an unknown seaweed sample. These methods are used to measure the sodium/calcium film to determine the mannuronate/guluronate (M/G) ratios when sodium alginate is immersed in calcium chloride solution. Peak shift, peaks shape and new peak appearance was observed by FTIR techniques (Volpi 2008). Awad and Aboul-Enein (2013) combined two spectroscopies techniques, FTIR-ATR and FT-Raman, to identify the principal different types of carrageenan colloids in ground seaweed powder, and perform analysis based on spectra peaks. Sartori and others (1997) proposed that the FTIR-ATR

spectroscopy could be a useful tool for the food, pharmaceutical and cosmetics industry to check the hydrocolloids types and differentiate sodium alginate and carrageenan by a quick and non-destructive method. Pereira and others (2011) summarized the FTIR-ATR and FT-Raman spectroscopy peaks in main seaweed polysaccharides spectra with attributed bonds (Table 7-1). The seaweed polysaccharides include alginate, fucoidan, laminaran, agars, kappa-, iota- and lambda-carrageenan. Gomez-Ordonez and Ruperez (2011) introduced the method to determine the M and G ratio by FTIR. Various approaches have been reported for the alginate determination in the pharmaceutical field, but there is no general procedure for their analysis in food systems, not to mention more complicated meat systems. Therefore, the objective of this study was therefore to develop a valid method that can directly quantitate the total alginate content in restructured meat by using FTIR technique coupled with PLS and PCA analysis methods.

7.3 MATERIALS AND METHODS

7.3.1 Product Preparation

Before use, fish samples were thawed at refrigerated conditions overnight, the semi-thawed fillets were cut into small pieces, and then were transferred to a food processor (Cuisinart® Prep 9™ 9-Cup Food Processor, Model DLC-2009CHBM), and blended for 2 minutes. The food processer was paused and different amounts of sodium alginate were sprinkled manually into the paste, while a rubber spatula was used to cover the binder to avoid sticking around the inside of the processor's internal wall. The food processor was turned on again for another three minutes. Encapsulated acids release acid more slowly and prevent texture breakdown. After adding encapsulated acid, limited mixing was made to avoid damage to the encapsulation coating. The packages were placed in the refrigerator overnight to set the gel.

Polysaccharide extraction methods include five steps, including fat extraction, protein degradation, and precipitation of polysaccharide, centrifugation, and air drying. Three samples per treatment were randomly collected from each package. Fat was removed by adding 100 mL of acetone into 5 grams samples with or without sodium alginate. Mixture was stirred and extracted for one hour. After the extraction, the mixture was filtered through Whatman® Grade 1 Qualitative Filter Paper. The residue was saved in the original 500 ml polyethylene beaker. Next, About 50 ml distilled deionized water and 1 mL 1N NaOH were added to the meat protein with agitation for 10 minutes. The mixture was incubated at 100°C in a water bath for 10 minutes to degrade the protein. The mixture was cooled to room temperature (24°C) and pH value was adjusted to 7.5 with 1N HCl. Protease was added to the mixture to digest the protein for 16 hours in a 37 °C water bath. The enzymatic reaction was stopped and the remaining protein was coagulated by heating the mixture at 70°C for 20 minutes. After fat extraction and protein degradation, the next step was polysaccharide precipitation. Polysaccharides were precipitated from previously treated samples by adding 350 mL 90% ethanol at room temperature and mixing for 2-3 hours. Excess ethanol was removed and the samples were centrifuged at 10,000 rpm for 15 minutes. The polysaccharides were carefully removed with small spatula and air-dried for 30 minutes. The samples were dried by applying tissue paper until no more water showed. The samples had to be dry before testing by FTIR analysis.

7.3.2 FTIR Spectroscopy Measurements

FTIR analysis was carried out using a Thermo Nicolet 380 FTIR spectrometer (Thermo Electron Corporation, Madison, Wis.). The FTIR spectra using Attenuated Transmission and an internal reflection accessory made of Composite Zinc Selenide

(ZnSe) and Diamond crystals. Each spectrum was scanned from 4000 to 400 cm-1. The FTIR spectra were acquired for each treatment at room temperature. Each spectrum is composed of an average of 32 separate scans. The spectra were measured by summing 32 scans at a resolution of 4 cm-1. The software Delight Version 3.2.1 (D-Squired Development Inc., LaGramde, OR, USA) was used in data analysis. FTIR spectra data were converted to trt format. Data pre-processing algorithms including polynomial substrate and Gausssian smoothing was used to subtract the baseline shift and eliminate high frequency noises from the instrument. The partial least square (PLS) model, as a multivariate statistical regression model, was used to predict analyst concentrations in tested samples. The number of PLS latent variables was optimized based on the lowest root mean square error of prediction (RMSEP) values to avoid overfitting of spectral data.

7.4 RESULTS AND DISCUSSION

The FTIR spectroscopy of commercial sodium alginate and pure alginic acid are shown in Figure 7-2. The most remarkable difference between commercial sodium alginate and pure alginic acid standard in Figure 7-3 was in the wavenumber range of 1550 to 1750 cm^{-1}, which is a carboxylic ester band. This carbonyl group showed carboxylic acid ester form in alginic acid at C=O at 1730 cm^{-1}, and-carboxylate anion form COO- form at 1600 cm^{-1} in sodium alginate samples, which was in agreement with Pereira and Neto (2014).

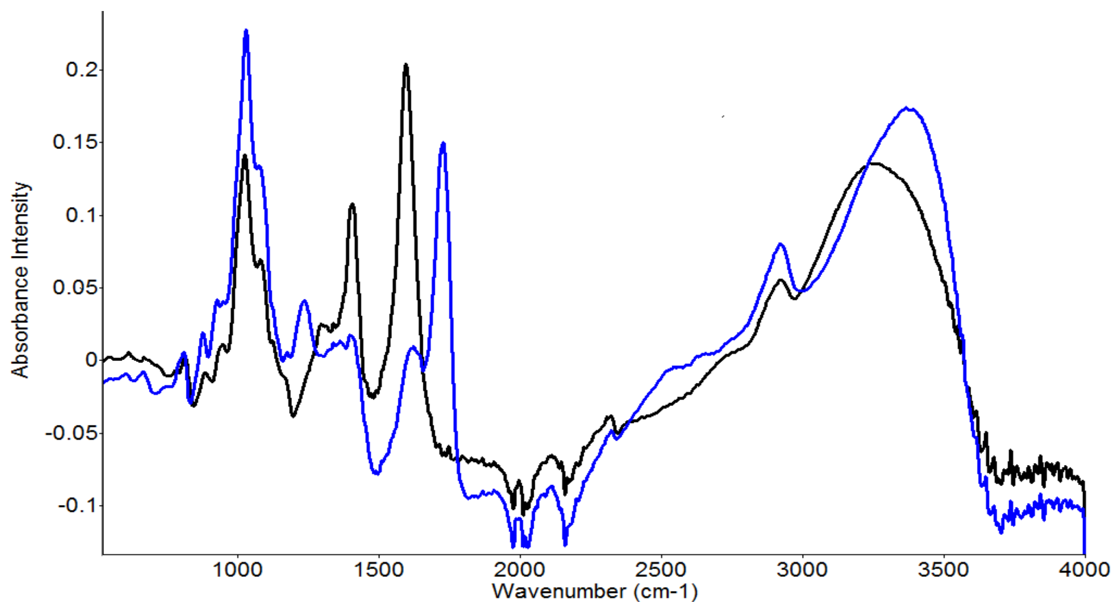

Figure 7-2. Spectra of Commercial Sodium Alginate (blue) and Pure Alginic Acid (black)

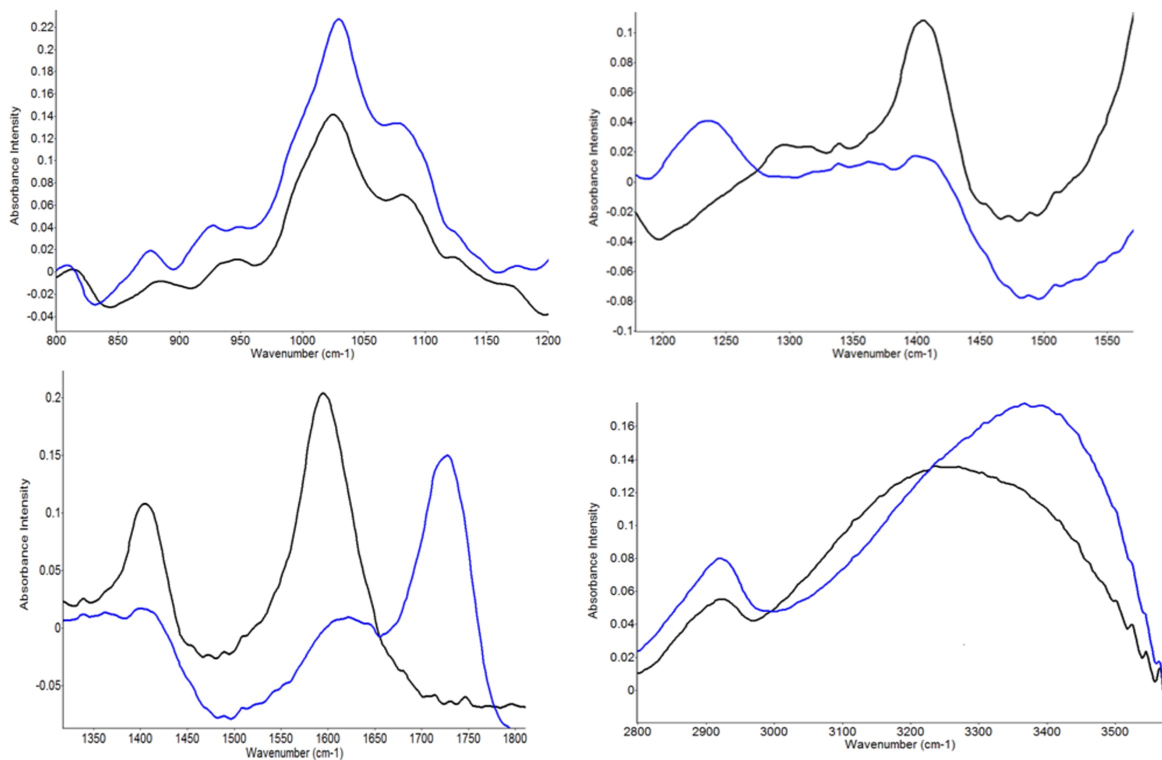

Figure 7-3. The Spectra of Commercial Sodium Alginate (blue) and Pure Alginic Acid (black) at Bands Approximately 817, 1030, 1417, 1617, 2920 and 3426

The band 817 cm^{-1} is characteristic of mannuronic acid residues which showed at both sodium alginate and alginic acid samples. The band 878 cm^{-1} is assigned to the C1-H deformation vibration of β-mannuronic acid residues. The band 948 cm^{-1} is assigned to C-O stretching vibration of uronic acid residues. Sakugawa and others (2004) concluded the same results that both alginate and alginic acid samples in the anomeric region of fingerprint from 750-950 cm^{-1} showed these three characteristic absorption bands. The approximately 1083 cm^{-1} and 1024 cm^{-1} bands were assigned to C-O and C-C stretching vibrations of pyranose ring and C-O-C glyosidic bonds. Gomez-Ordonez and Ruperez (2011) also stated that a peak at wavenumber of approximately 1030 cm^{-1} peak appears due to vibration of C-O, C-C-C and vibrational asymmetry of pyranose ring. Gomez-Ordonez and Ruperez (2011) agreed that the band at 1030 cm^{-1} may be due to C-C stretching vibrations of pyranose ring. The band at 1406 cm-1 was assigned to C-OH deformation vibration with O-C-O symmetric stretching vibration of carboxylate group. The weak signal is approximately 2926 cm^{-1} due to C-H stretching vibrations. The broad band centered at approximately 3260 cm^{-1} was assigned to hydrogen bonded O-H stretching vibrations (Mohamed and others 2011; Praveena and others 2014). The strong peak was located at 3446 cm^{-1} in pure alginic acid due to O-H stretching, which indicated the peak was at 3249 cm-1 in commercial sodium alginate spectra in this study.

Gomez-Ordonez and Ruperez (2011) also demonstrated the FTIR spectra of pure alginate. They addressed that the bands around 1030 cm^{-1} (C-O-C stretching) is due to the saccharide structure of sodium alginate. The bands at 1617 and 1417 cm^{-1} were assigned to asymmetric and symmetric stretching peaks of carboxylate salt groups. Praveena and others (2014) pointed out that the molecule chain of sodium alginate contains both –OH

and –COO- groups. The O-H stretching vibration showed approximately at 3388 cm^{-1}. The peaks at 1604 cm^{-1} and 1411 cm^{-1} bands were assigned for the asymmetric –COO-stretching vibration and symmetric –COO-stretching vibration, respectively. Table 7-1 summarized the FTIR spectral band assignments of commercial sodium alginate, pure sodium alginate, and pure alginic acid. Table 7-2 summarized FTIR spectral band assignments of sodium alginate. Researchers concluded slightly different bands and wavenumbers at the same function groups.

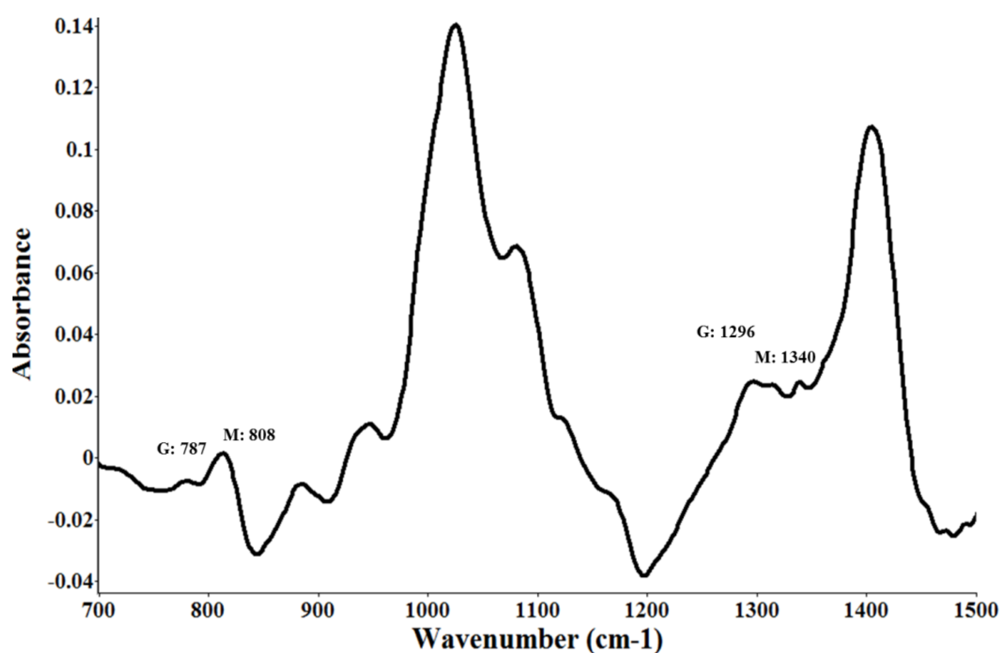

Figure 7-4. Sodium Alginate Spectra at 525-4000 cm^{-1}

Malesu and others (2011) summarized that different types of alginate showed different G block and M block ratios and patterns. These differences can cause differences in the physical properties of sodium alginate. The guluronic units can be identified from a band around 1025 cm^{-1}. The mannuronic units can be identified from a band around 1100 cm^{-1}. The ratio of guluronic and mannuronic concentration ratio of alginate samples can be determined from the relative intensity ratio of 1025 and

1100 cm^{-1} bands. Swamy and others (2008) recommended that determination of M block to G block values in alginate can be measured with the ratio of absorption band intensities at 808 (M) and 787 (G) in the IR spectra. The author also pointed out that comparison of band intensities at 1320 (M) and 1290 (G) is another good way to determine the ratio. However, in my study, the ratio seemed opposite. Pereira and others (2003) summarized that sodium alginate showed two characteristic bands in IT spectra. The band at 808 cm^{-1} was assigned to M units, and 787 cm^{-1} was assigned to G units. However, some researchers assigned both 808 and 787 cm^{-1} bands to G units. Some studies showed the M/G ratios could be estimated from the ratio of absorbance of the bands at 1320 and 1290 cm^{-1} in FTIR spectra. Usov (1999) addressed that polymannuronate does not have a strong interaction with divalent cations like the egg-box structure in polyglucuronate. The peaks of divalent polyglucuronate were sharper than the peaks of polyglucuronicacid. In accordance with the authors, the absorbance at 1030 cm^{-1} is reflected by the change of mannuronate concentration of calcium alginate and 1025 cm^{-1} is attributed to the OH bending of guluronate. Alginate also can be estimated from the intensities of 1030 to 1080 cm^{-1} in infrared spectra. The author suggested that samples with high content in guluronic acid showed an intense broad band centered at approximately 1025 cm^{-1} .

Pereira and Neto (2014) pointed out that the most prominent Raman shift bands at 950 cm-1, while in FTIR the intensity at 950 cm^{-1} is very weak. Both FTIR and Raman showed strong bands at 1400 cm^{-1}, which is due to the deformation of the CH2 group. The C-O-C and C-OH stretching modes showed in the spectral regions of 1250-1290 and 1000-1025 cm^{-1}, respectively.

Table 7-1. Peak Found in Seaweed FTIR Spectra with Their Attributed Bonds (Summarized and Adapted from Sakugawa and others (2004))

Wave numbers (cm-^{1})	Bonds/Assignments
3500	O-H
2960	CH_2
2900-2920	C-H (good reference for total sugar content)
2845	O-CH_3 (shoulder on the band at 2920 in highly methylated agars)
1725	COOH
1690-1695	Amide I from proteins
1640-1650	H_2O and proteins CO-NH/amide II from proteins
1605	Carboxylate anion of pyruvate
1450	Ester-sulfate
1420	Amide III from proteins
1370-1320	Ester-sulfate
1210<1240<1260	S=O of ester-sulfate (good indicator for total sulfate content)
1180	P-O-C (alkyl substituent's suggesting organic phosphates)
1150	Ester-sulfate
1040-1080	Skeleton of galactans
1070	C-O of 3,6-anhydrogalactose (shoulder)
1065	Gelling type carrageenan
1040	C-O of ester-sulfate and hydroxyl
1037-1071	Symmetric C-O vibration associated with a C-O-SO, of heterofucans
1020	Non-gelling type carrageenan
1000-1200	Sulfates and floridean starch
970-975	Galactose: peak with alkali modified iota carrageenan, small peak with unmodified iota, and also present in agars
930-940	Vibrations of the C-O-C of 3,6-anhydrogalactose
905	C-O-SO_4 on C_2 of 3,6-anhydrogalactose (shoulder)
890-900	Unsulfated β-D-galactose (or with 6-0-methylgalactose or with pyruvate); agar specific band
867	C-O-SO_4 on C_6 of 3,6-anhydrogalactose (shoulder)
845-850	C-O-SO_4 on C_4 of 3,6-anhydrogalactose (shoulder)
825-830	C-O-SO_4 on C_2 of 3,6-anhydrogalactose (shoulder)
820	Galactose 6-sulfate
815-820	C-O-SO_4 on C_6 of galactose
805	C-O-SO_4 on C_2 of 3,6-anhydrogalactose
790	Characteristic of agar-type in second derivative spectra
730-750	C-S/C-O-C bending mode in glycosidic linkages of agars
717	Characteristic of agar-type in second derivative spectra/ C-O-C bending mode in glycosidic linkages of agars
705	C-O-SO4 on C-4 of galactose
580	S-O in sulfated galactans

Table 7-2. Summarization of FTIR Spectral Band Assignments of Sodium Alginate

Sodium Alginate	Band Assignments
3388/3450/3426	O-H band stretching (Intermolecular hydrogen bonded)
2924/2926	C-H stretching
1604/1614/1617	O-C=O asymmetric stretching
1411/1420/1417/1400	O-C=O asymmetric stretching
1035/1030/1025	C-O-C stretching

*Summerized based on (Pereira and Neto 2014; Praveena and others 2014; Mohamed and others 2011)

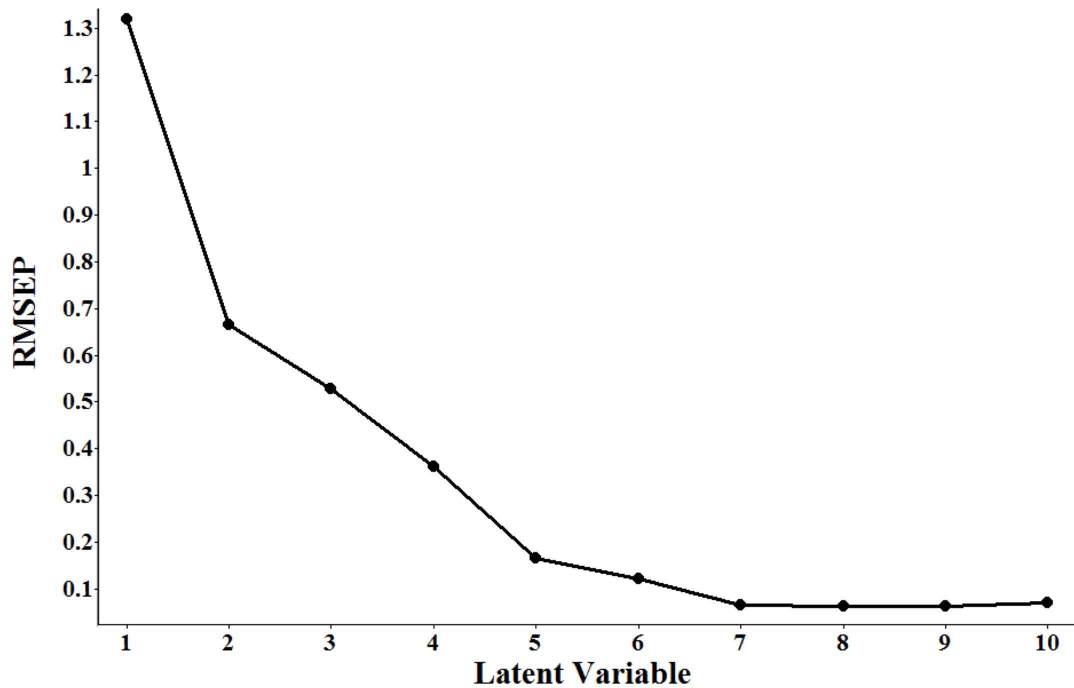

Figure 7-5. Root Mean Square Error of Prediction (RMSEP) Values Obtained from the Partial Least Square (PLS) Models with Different Latent Variables by Directly Drying Method

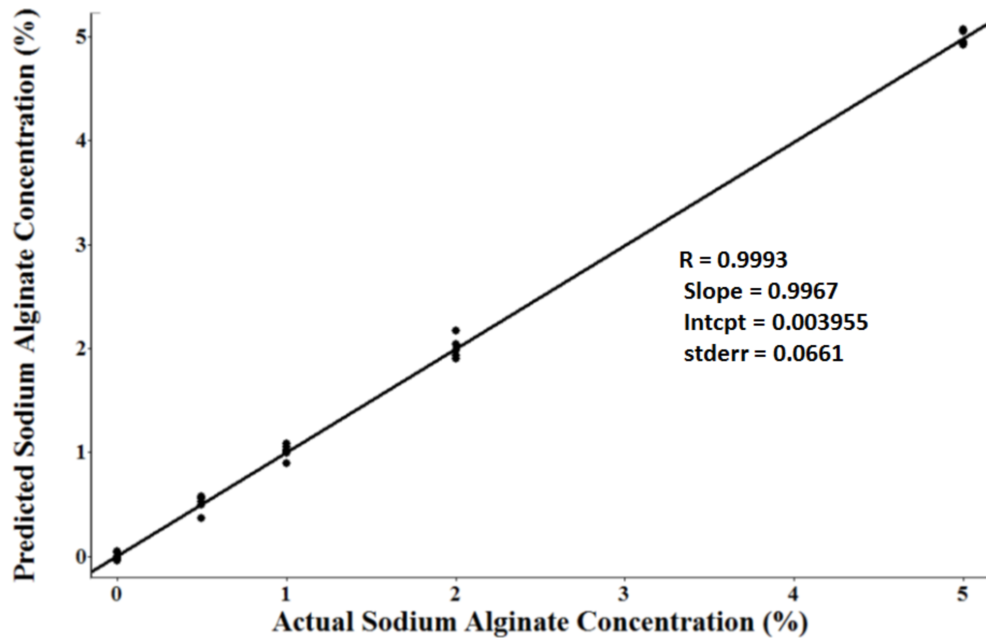

Figure 7-6. Actual Sodium Alginate Concentration (%) Added into Meat Samples Versus Predicted Sodium Alginate Concentration using the PLS Model by Directly Drying Method; Smoothing 4 cm-1, Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 7 Latent Variables; Spectral Region 800-2000 cm^{-1}, Spectra Number n=30.

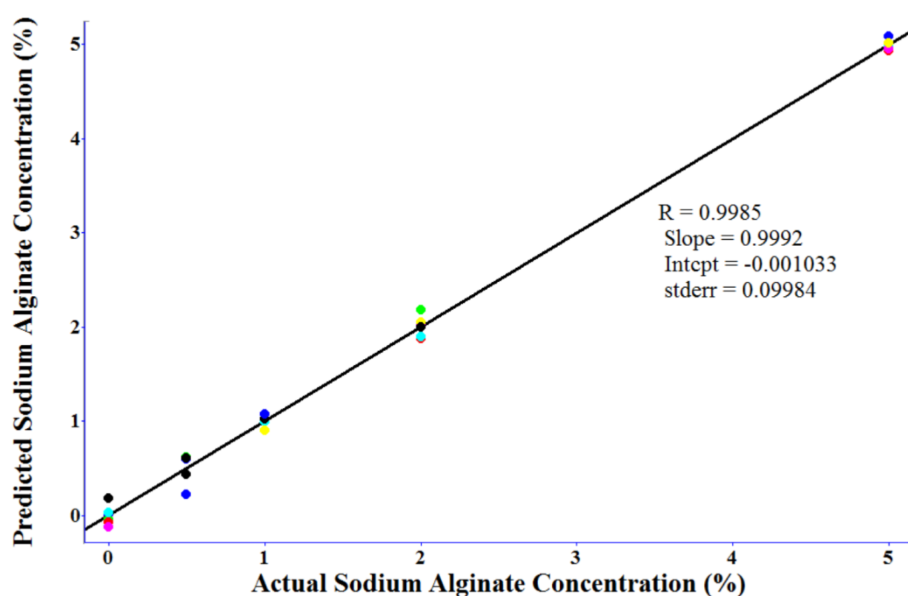

Figure 7-7. Actual Sodium Alginate Concentration (%) Added into Meat Samples Versus Predicted Sodium Alginate Concentration using the PLS Model by Directly Drying Method; Smoothing 4 cm-1; Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 7 Latent Variables; Spectral Region 800-1200 cm^{-1} , Spectra Number n=30.

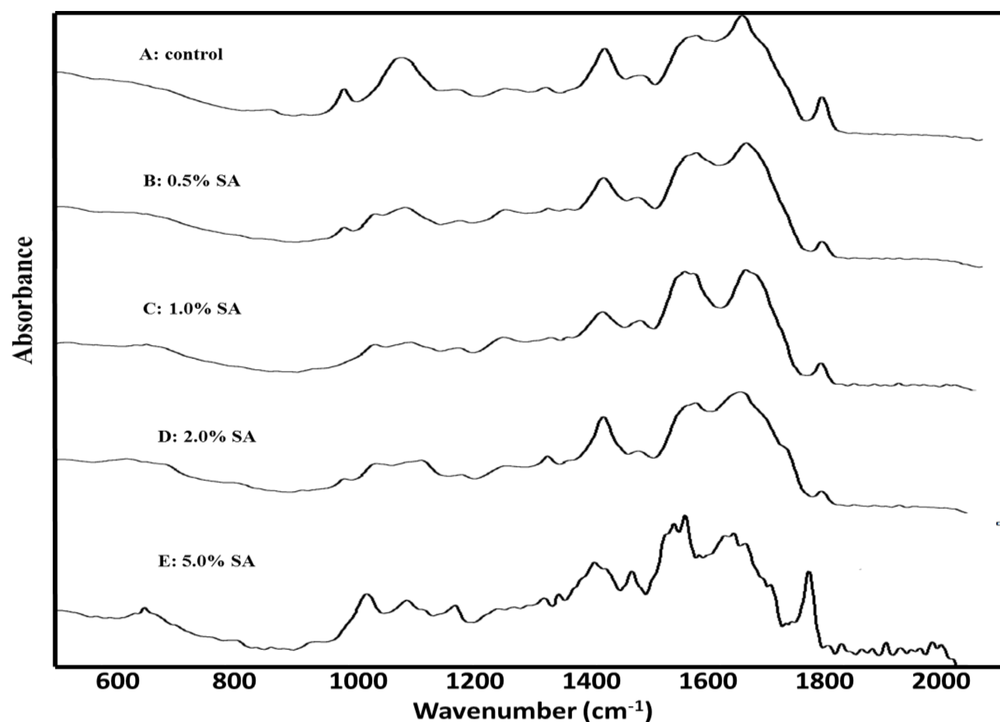

Figure 7-8. Average FT-IR Spectra (n=6) Acquired From Dry Fish Meatball Samples Containing Different Concentration of Sodium Alginate by Directly Drying Method. A: Fish Ball without Sodium Alginate; B: 0.5% Sodium Alginate; C: 1.0% Sodium Alginate; D: 2.0% Sodium Alginate; E: 5.0% Sodium Alginate; the Ratio among Sodium Alginate: CaCO3: Encapsulated Lactic Acid= 6:1:1.5 Spectra were Presents with Smoothing at 5 cm^{-1} and Baseline Adjustment by Subtracting a 2nd Order Polynomial Function. Analysis was Conducted from 525-2000 cm^{-1}.

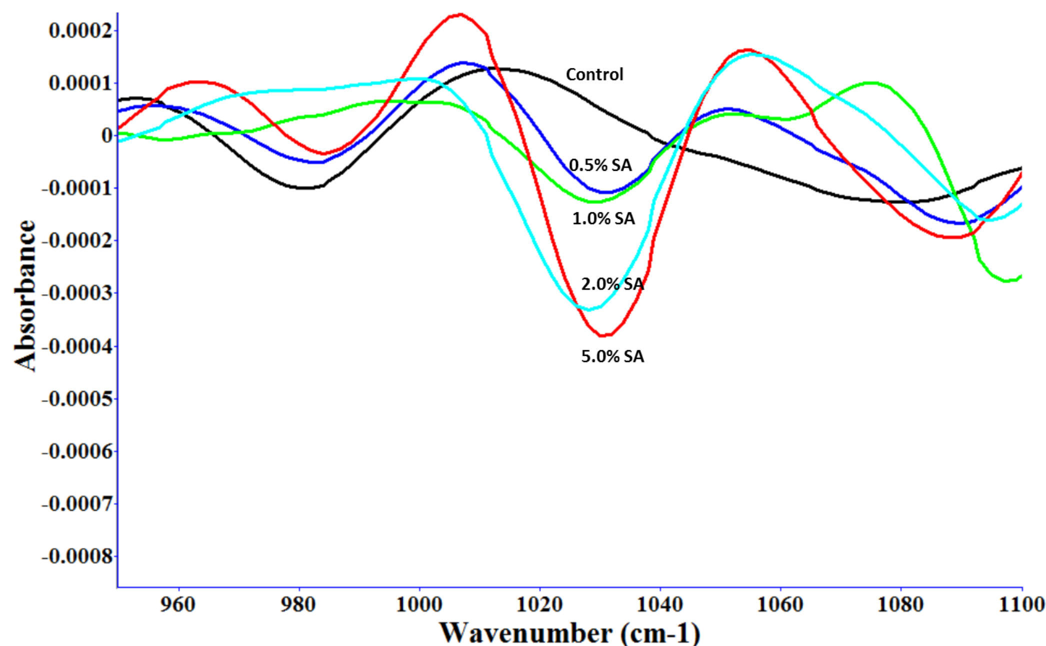

Figure 7-9. Part of Second Derivative Transformation of Average FTIR Spectra (n=6) Acquired from Different Concentration of Sodium Alginate by Directly Drying Method

Due to the fact that the medium to strong IR absorption bands at 1200-970 cm^{-1} were mainly due to C-C and C-O stretching in pyranoid ring and to C-O-C stretching of glyosidic bonds (Pereira and Neto 2014), this range is commonly used to distinguish all polysaccharides. In this study, 1200-800 cm^{-1} were chosen. The 2000-800 cm^{-1} spectra range was also used for comparison. The second-derivatives of FTIR spectra were used to determine weak absorption bands or to improve resolution of overlapped bands to distinguish different concentrations of samples.

In this study, treatments of dry meat containing different concentrations of sodium alginate and calcium sources were analyzed by FTIR. Average IR spectra (n=6) of dry meat samples are shown in Figure 7-8. It showed that the most prominent peaks of sodium alginate and calcium alginate were at around 1030 cm^{-1} , which was present in the sodium alginate treated meat samples, but was absent in the control dry meat sample (Figure 7-8). In addition, second derivative transformation can separate the over-lapped

peaks, eliminate baseline effect, and increase the spectral resolution ability. This has been applied to this study for analyzing spectra. Figure 7-8 depicts clearly that there were different spectra at wavenumber at 1030 cm^{-1}. Therefore, FTIR techniques with proper data analysis can be used to detect the different amount of sodium alginate usage in meat samples.

RMSEP values obtained from the PLS models with different latent variables are shown in Figure 7-6 and Figure 7-7. The spectra data were pre-treated with smoothing at 4cm-1 and second order polynomial subtraction in the whole spectra region. The lowest RMSEP values were achieved. The latent variable was five (Figure 7-5), which is the optimal value of latent variable to build a PLS model in this study. Figure 7-6 shows the PLS prediction results (n=6) by plotting actual sodium alginate concentration against predicted sodium alginate concentration. The prediction result was achieved with R=0.9993 and RMSEP= 0.066% at wavenumbers in the range of 2000-800 cm^{-1} (Figure 7-6)' and R= 0.9985 and RMSEP=0.09984 at wavenumber range of 1200-800 cm^{-1} (Figure 7-7). The results indicate that the PLS model in this study could provide satisfactory quantitative results for sodium alginate added into restructured meat. Based on USDA regulations, sodium alginate added to restructured meat product cannot exceed 1 percent of the product formulation. With the PLS model, very accurate quantitative results are hard to achieve if the concentration of sodium alginate is this low.

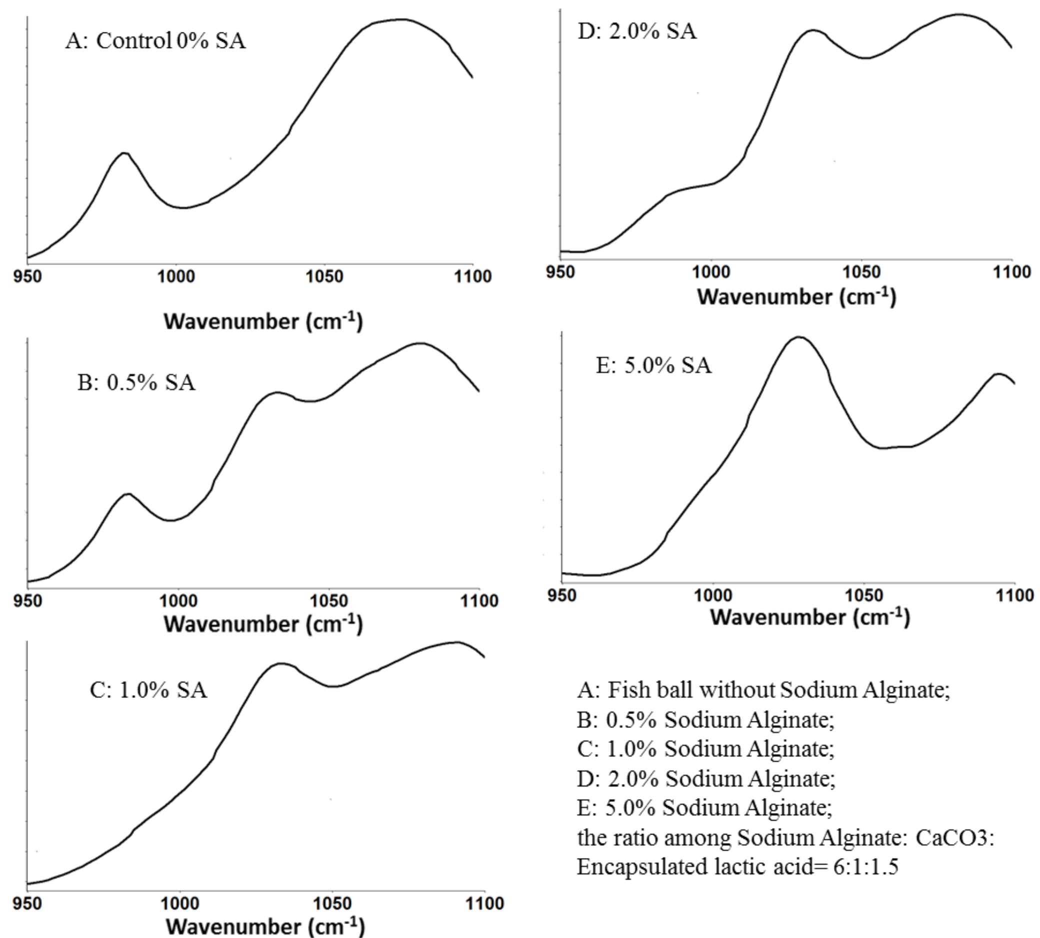

Figure 7-10. Average FT-IR Spectra (n=6) Acquired from Dry Fish Meatball Samples Containing Different Concentration of Sodium Alginate by Directly Drying Method. Spectra were Presents with Smoothing at 5 cm^{-1} and Baseline Adjustment by Subtracting a 2nd Order Polynomial Function. Analysis was Conducted from 950-1100 cm^{-1}

Figure 7-10 clearly depicts a different spectrum at wavenumber at 1030 cm^{-1}. When compared among five different treatments, and especially control and 0.5% sodium alginate treatments, there is a peak around 950-1000 cm^{-1} region, which is associated with C-O, C-C stretching and C-O-H and C-O-C deformation of carbohydrate (Pereira and others 2003).

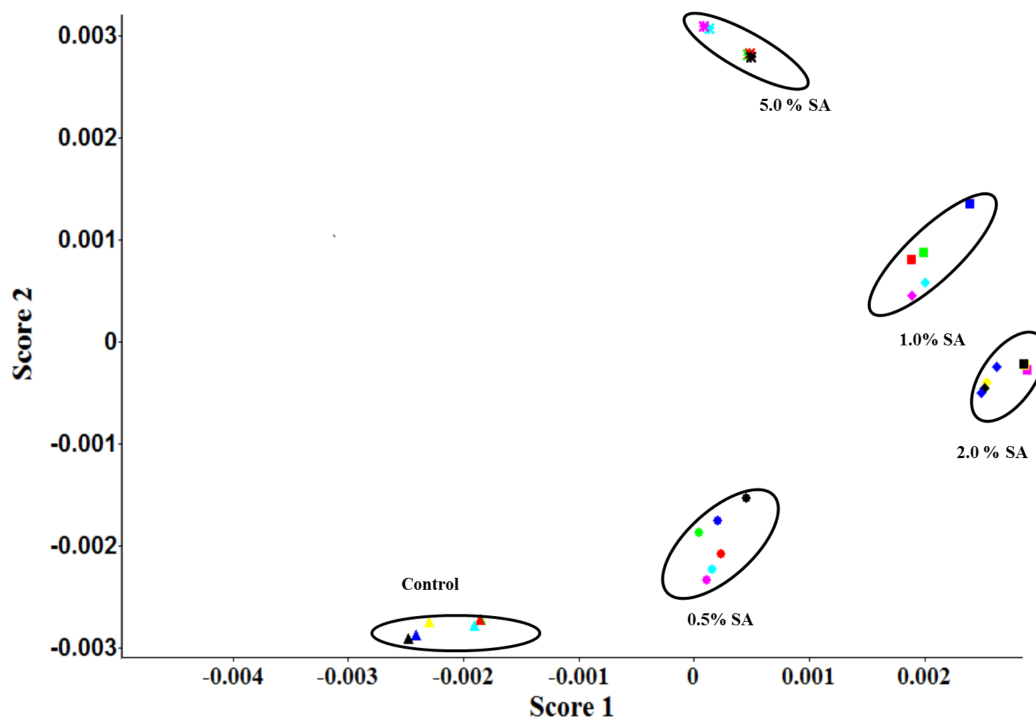

Figure 7-11. Classification of Sodium Alginate Treatments with Control Group using First Two Principal Components (PCs) at Wavenumber 800-2000 cm^{-1} by Directly Drying Method

The PCA score plot is a latent variable. Samples with the same PC scores are similar in terms of chemical composition. The samples used in this study were labeled as control, 0.5%SA, 1.0% SA, 2.0% SA and 5.0% SA. From Figure 7-11, it could be concluded that the control treatment and sodium alginate treated samples failed to classify the same group. There was no overlap between the control and 0.5% SA treated samples. This may be due to the fact that even low concentrations of sodium alginate show obvious gelling and change the meat composition structure. These results indicated that adding sodium alginate into meat samples with different concentrations could be distinguished by FTIR techniques.

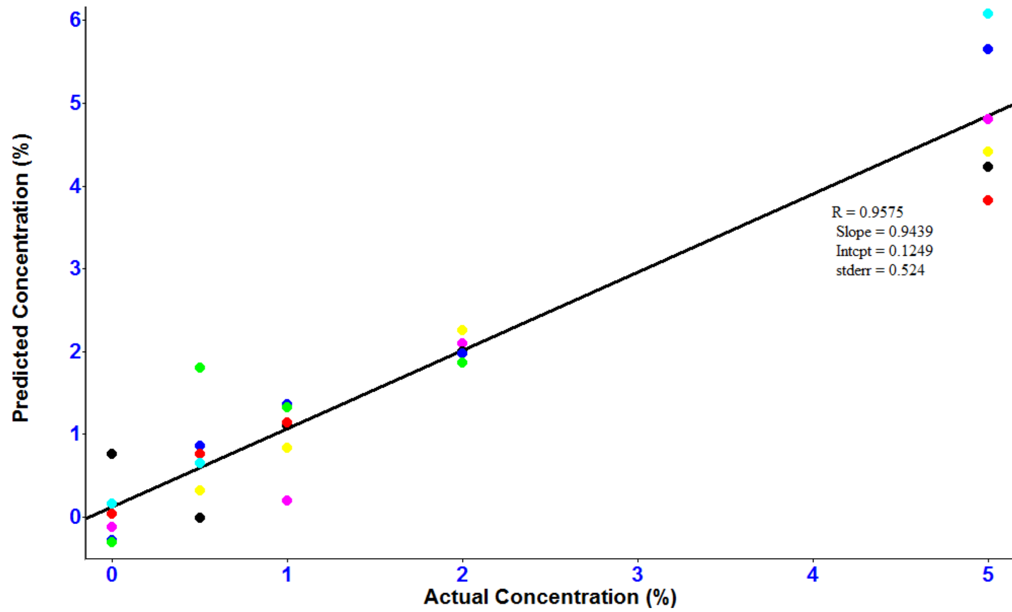

Figure 7-12. Extraction Method: Actual Sodium Alginate Concentration (%) Added into Meat Samples Versus Predicted Sodium Alginate Concentration using the PLS Model; Smoothing 4 cm^{-1}, Baseline Adjustment by Subtracting a 2nd Order Polynomial Function; 5 Latent Variables; Spectral Region 525-4000 cm^{-1}, Spectra Number n=30

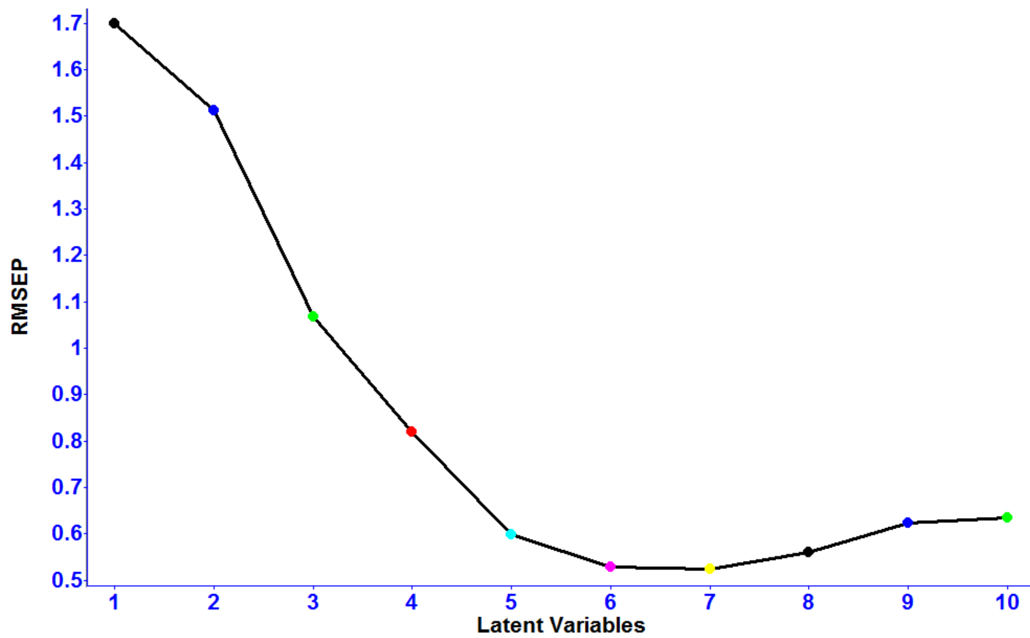

Figure 7-13. Extraction Method: Root Mean Square Error of Prediction (RMSEP) Values Obtained from the Partial Least Square (PLS) Models with Different Latent Variables

The extraction method involved fat removal and a protein degradation process. However, the residual may still contain carbohydrate, residual fat and amino acids, and minerals and vitamins. When centrifuged and air-dried for 30 minutes, the total residue is the mixture of these compounds. This explains why a fat peak was showed 1744 (C-O) cm^{-1} on Figure 7-14. This extraction method causes the protein degradation. There was no peak around 1548 cm-1 (Gomez-Ordonez and Ruperez 2011) which indicates the IR absorption band of protein. However, the spectral of the extract method for all treatments contain 1520 cm^{-1} , which is Amide II band of proteins.

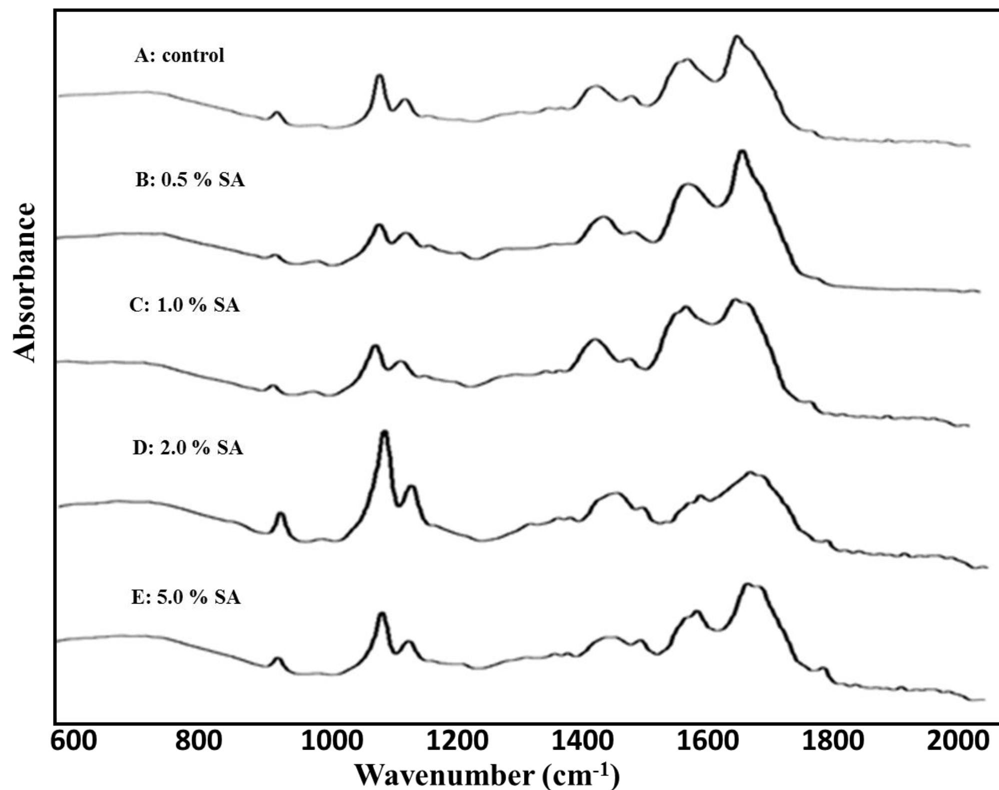

Figure 7-14. Average FT-IR Spectra (N=6) Acquired from Dry Fish Meatball Samples Containing Different Concentration of Sodium Alginate by Extraction Method. A: Fish Ball without Sodium Alginate; B: 0.5% Sodium Alginate; C: 1.0% Sodium Alginate; D: 2.0% Sodium Alginate; E: 5.0% Sodium Alginate; the Ratio among Sodium Alginate: $CaCO_3$: Encapsulated Lactic Acid= 6:1:1.5. Spectra were Presents with Smoothing at 5 cm^{-1} and Baseline Adjustment by Subtracting a 2nd Order Polynomial Function. Analysis were Conducted from 525-2000 cm^{-1}

When comparing Figure 7-14 with Figure 7-8, there was a peak at 877 cm^{-1} showing for all treatments, which may be due to an epoxy ring from epoxy fatty acid in fish products. The prominent peak was shift to 1043 cm^{-1} instead of 1030. The 1085 cm^{-1} peak might be due to P=O symmetric stretching in DNA, RNA and phospholipids (Rossell 2013). Wavenumber at1400 cm^{-1} peak is due to C=O symmetric stretching of COO- group in amino acids and fatty acids. Bands at 1540 cm^{-1} peak is due to Amide II band of proteins. 1620 cm^{-1} band at control and 0.5% SA showed sharper compared with other SA treatments. The treatments containing higher SA showed broader bands. This may be due to the chemical reaction between sodium alginate and calcium carbonate, and development of calcium alginate compounds.

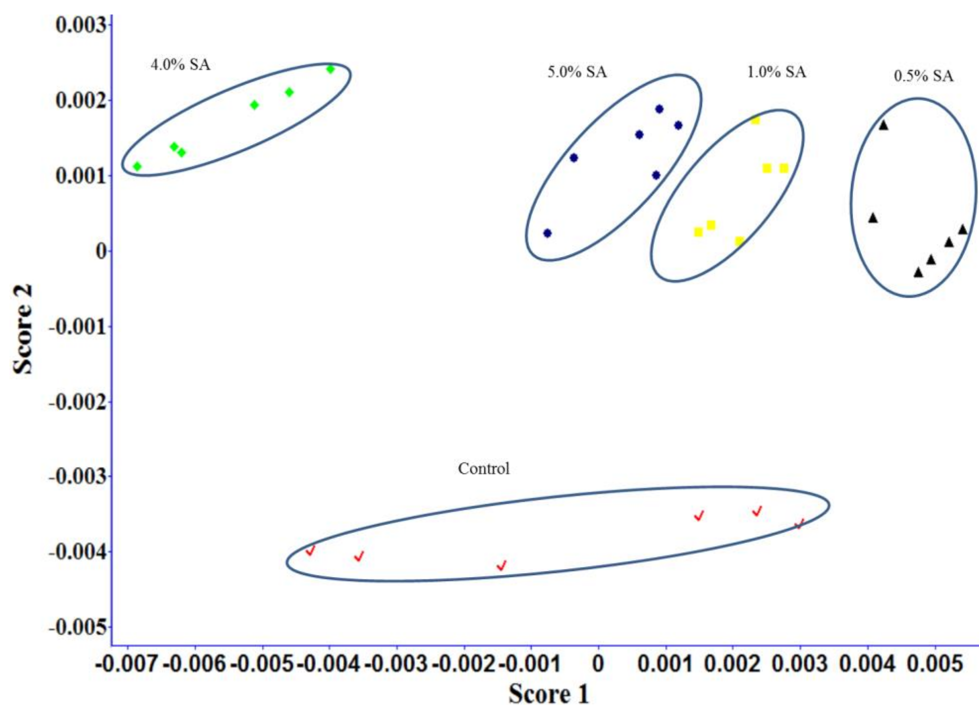

Figure 7-15. Classification of Sodium Alginate Treatments with Control Group Using First Two Principal Components (PCs) at Wavenumber 800-2000 cm^{-1} by Extraction Method

PCA was conducted based on spectra acquired from all five treatments to check whether PCA can distinguish different treatments. The results of using the first two PCs

to classify treatments are shown in Figure 7-15. The results indicated that PCA is useful to discriminate between the five treatments.

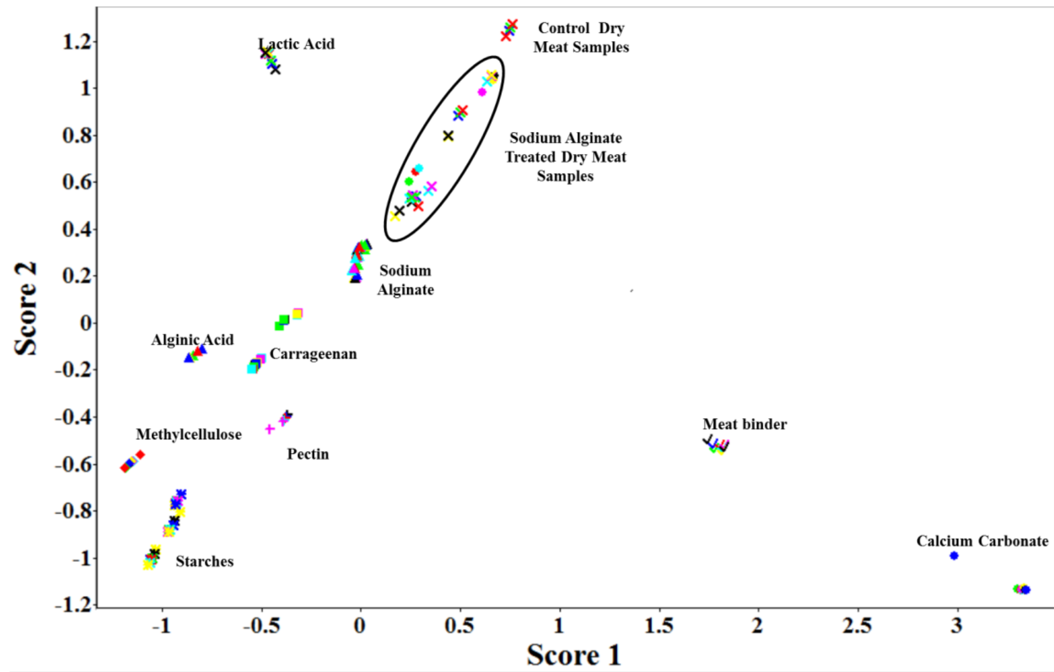

Figure 7-16. Dry Powder and Dry Meat Samples from 800-2000 cm^{-1}

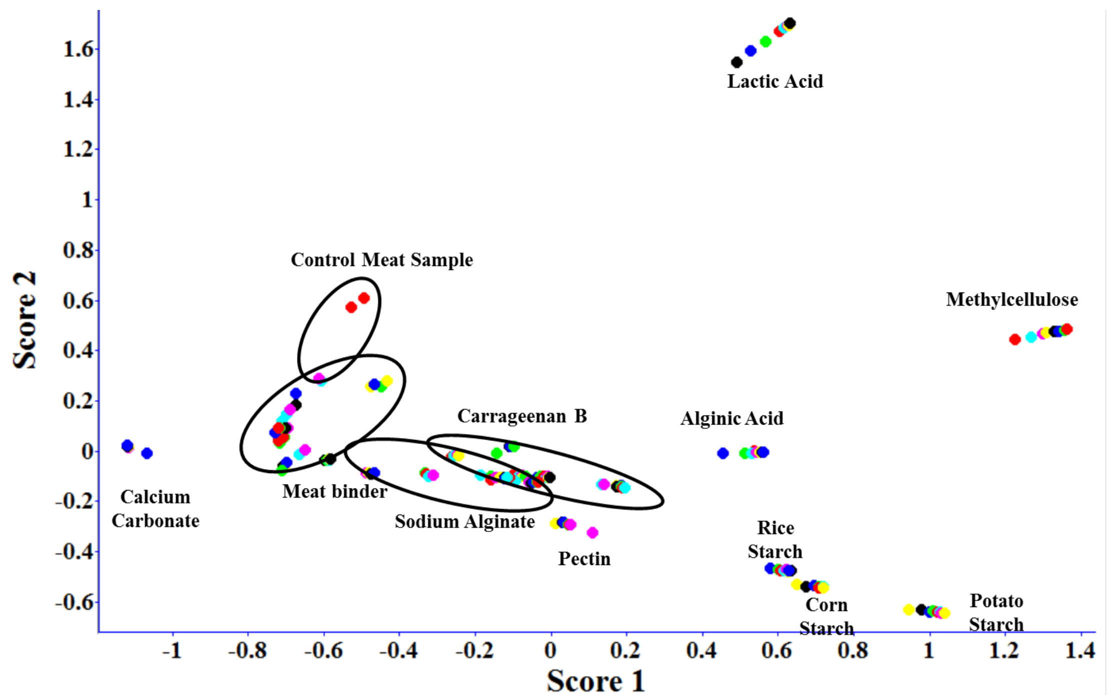

Figure 7-17. Dry Powder and Dry Meat Samples from 800-1200 cm^{-1}

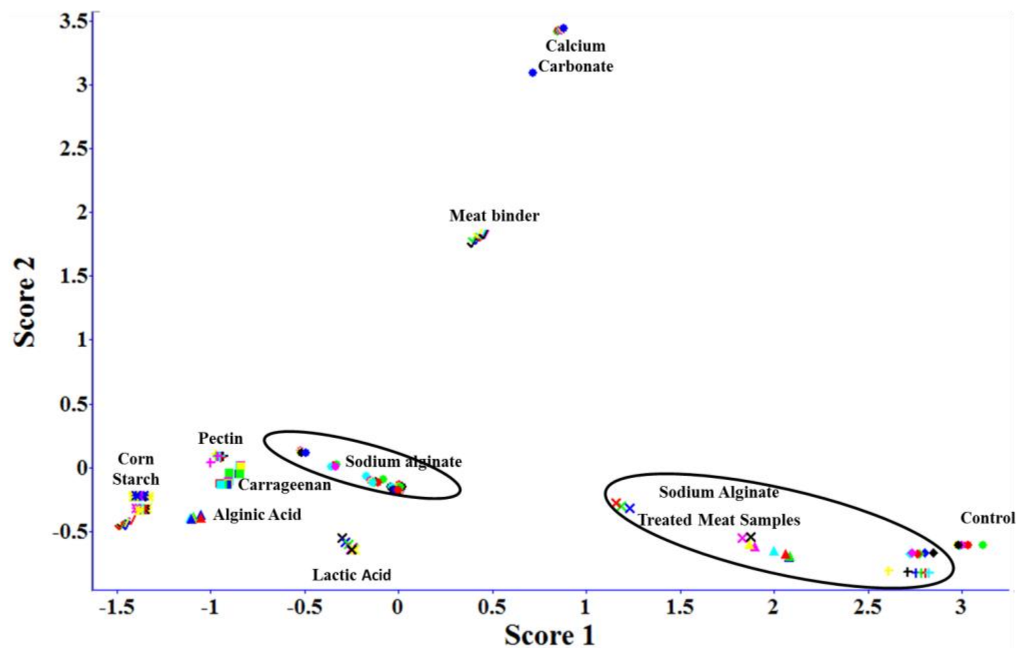

Figure 7-18. Dry Powder and Extracted Meat Samples from 800-2000 cm^{-1}

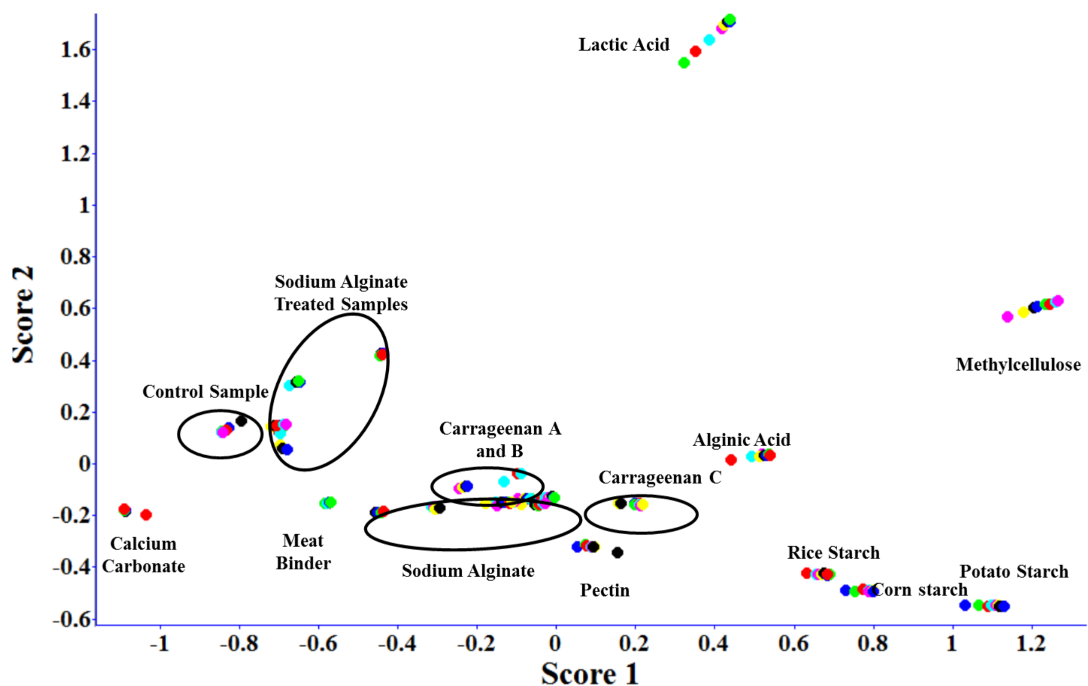

Figure 7-19. Dry Powder and Extracted Meat Samples from 800-1200 cm^{-1}

The second derivative of spectra is calculated by resolving the over-lapping bands in the original spectrum. The second derivative spectra show much more details. FTIR spectroscopy can be used for food authentication of polysaccharide-based additives and can be used as screening tests for polysaccharide presence in food products. Due to the limitation of strong absorption of water in the IR spectral region, it cannot be widely used in the meat industry. The meatball formulas containing no SA, 0.5% SA, 1.0% SA, 2.0% SA and 5.0% SA were classified using the PCA. The PCA is used for classification of different sample treatments.

Avan de Voort (1994) summarized several hydrocolloid characteristic band maxima. Such as galactose showed the strongest IR band at 1078 cm^{-1} , mannose at 1070 cm^{-1} , and glucose at 1035 cm^{-1}. The author concluded that FTIR spectra at the range of 800-1200 cm^{-1} could be useful for identification of polysaccharides with different structures and compositions. In this range, it is dominated by stretching vibrations of C-O, C-C, ring structures, and deformation vibration of CH_2 groups.

Different types of hydrocolloids and meat binders were used to build a FTIR model based on their absorbance in the region from 800 to 1200 cm^{-1}. The binders include sodium alginate from different suppliers, carrageenan, pectin, meat binder, methylcellulose, starches (potato starches and corn starches), and other ingredients that are related to restructured meat, such as lactic acid, calcium carbonate and alginic acid. Davis and Mauer (2010) pointed out that 900-1200 range is C-O-C, C-O dominated by ring vibrations in various polysaccharides.

Figure 7-15 and Figure 7-16 data results were used to compare oven dried fish samples and various types of hydrocolloids at different wavenumber ranges, such as 800-

2000 cm^{-1} and 800-1200 cm^{-1} . The results showed that at 800-2000 cm^{-1} range, the PCA technique could distinguish hydrocolloids, including pectin, methylcellulose, carrageenan, various starches, and sodium alginate. It also can separate the treated meat samples from hydrocolloids or other chemical compounds, such as alginic acid, lactic acid, and calcium carbonate. As we know, the average moisture is around 80%. After drying, the meat samples contained approximately 2.5%, 5.0%, 10%, and 25% calcium alginate. Meat binder (GrindSTED MEATBINDER MB 2555) is a food grade blend in powder form from Danisco. The composition of this stabilizer includes sodium alginate, calcium carbonate, Glucono delta lactone, and sodium phosphate. This explains why meatbinder was located between sodium alginate commercial powder and sodium alginate treated meat samples when using PCA method to distinguish the hydrocolloids,.

Figure 7-17 and Figure 7-18 show the sodium alginate treated samples can be separated from control meat sample, which also can be confirmed in Figure 7-15 and Figure 7-16. The reason that meat binder was not located at the region with other hydrocolloids may be due to sodium phosphate in this stabilizer. When analyzed at range between 800 and 1200 cm^{-1}, there was clearly segregation between hydrocolloids, restructured compounds, and meat treated samples. A small portion of sodium alginate and carrageenan overlapped each other. FTIR reflects the chemical structure of chemical compounds. Carrageenan is a linear polysaccharide comprising of repeating galactose units and 3,6-anhydrogalactose (3,6 AG) joined together by alternating α-(1,3) and β-(1,4) glycosidic links. Sodium alginate composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues linked by (1→4) glycosidic bonds in homopolymer blocks. FTIR spectra exhibited more differences at higher concentrations compared with the

same analyte at lower concentrations. Both alginate and carrageenan were produced from different types of seaweeds. Red algae (Rhodophyta) produce galactans, such as carrageenan and agars. The brown algae (Phaeophyceae) produce urinates, such as alginate. Both of them can be used in the food industry for different functions and both are generally recognized as safe (GRAS) (Kacurakova and others 2000).

When comparing the two methods-direct oven drying without any pretreatment and extraction method, there was no advantage in the extraction method. Based on spectra, there are no different peaks found when compared with control and sodium alginate treated samples. For the direct oven drying method, there was a peak at range 950-100 cm^{-1} for the control and 0.5% sodium alginate treatment samples. When the sodium alginate concentration increased, the peak disappeared. Since sodium alginate reacts with calcium carbonate, the possible explanation is that the peak shifted from sodium alginate to calcium alginate. The spectra clearly showed the different peaks at 1030 cm^{-1} when compared with control and sodium alginate treated samples. The PLS model obtained higher R-value and lower standard error at both 800-2000 cm^{-1} and 800-1200 cm^{-1} ranges when compared with the extraction method. The PCA model can be a good tool to distinguish sodium alginate treated samples.

7.5 CONCLUSION

The FTIR spectroscopy combined with PLS and PCA methods at wavenumber of 800 cm^{-1} can be used for the quantitative analysis of control and different concentrations of sodium alginate. The R^2 and RMSEC values obtained for quantification were 0.998 and 2.00%, respectively. The PCA was successfully used for the classification of lower and higher concentration of sodium alginate.

Our study showed that using FTIR with PLS and PCA techniques could be used to quantitatively measure, distinguish, and characterize the samples. The only sample preparation is removing the moisture from fish samples. Further research is needed to test many different types of hydrocolloid treated meat samples and to compare the spectral characteristics to determine the hydrocolloid type and usage amount. This method can be used to test hydrocolloids in the meat samples.

CHAPTER 8
CONCLUSION

In this study, the applications of lactic acid have been investigated. In the first part of the study, lactic acid functions as a permeabilizer of gram-negative bacterial outer membrane due to the low pH. The results showed that when lactic acid solution is applied at 55°C, it significantly reduced the microbial counts to below detection limit regardless of the length of contact time with STEC-8 bacteria. When 2.0 % lactic acid was applied to pre-chilled beef at 55°C, it caused 1.8-log reduction. There was only a 1.2 log reduction when spraying carcasses with 25°C at 2.0 % lactic acid. These data indicate that the lactic acid might be applied for STEC-8 pathogen reduction in the beef carcass pre-chilling process.

In the second part of the study, instead of conventional spray systems, ESS (Electrostatic Spraying Systems) system was adapted to spraying lactic acid onto warmed carcasses. The results showed that the fatty and lean sides of brisket had similar reactions toward lactic acid treatment at different temperatures. After being treated with lactic acid, the pH values of the fatty and lean sides decreased significantly ($P<0.05$). The log reductions of the fatty and lean sides after being treated with lactic acid with electrostatic sprayer showed no significant differences at different temperatures and spray times. Further research should be designed to increase the environmental temperature when ESS with lactic acid solution is used.

In addition to the application of antimicrobial characteristics of lactic acid, the third part of this study, HPLC and FTIR methods had been developed to determine lactic acid in meat samples. The results implied that FTIR could be potentially used to quantitate the lactic acid amount in meat samples. FTIR cannot provide exact values, but only predicts

the estimated amounts. It can be applied potentially in the meat industry to identify more parameters and meat quality changes during storage. It could also be used in other types of food processing. However, HPLC can provide the exact value of lactic acid amounts. In this study, HPLC was used to compare amounts of lactic acid in various meat samples.

In the fourth part of the study, encapsulated lactic acid, rather than lactic acid solution, was added into restructured meat. It was used to release free calcium ions from the calcium carbonate and hence develop alginate gelation. Sodium alginate treated samples were compared with other treatments with or without meat binders. The results showed that samples treated with sodium alginate and encapsulated lactic acid system did not show significant advantages over with control samples and other meat binders, but had the lowest pH values for both raw and cooked samples. The samples treated with Activa® RM and FG+ and FG provided the satisfactory binding capacity in fish balls. Further research should be performed to add salt and other spices or ingredients to investigate the interaction with binders.

In the final part of the study, the data analysis demonstrated that the FTIR spectroscopy combined with PLS and PCA method at wavenumber of 800 cm^{-1} can be used for the quantitative analysis of control and different concentrations of sodium alginate. The PCA was successfully used for the classification of low and high concentrations of sodium alginate. Further research is needed to test many different types of hydrocolloid treated meat samples, compare the spectral characteristics to determine the hydrocolloid type and usage amount. This method can be used to screen hydrocolloids in meat samples.

In conclusion, the applications of lactic acid and its derivatives in meat samples have been investigated. The analysis methods with HPLC and FTIR have been discussed. Overall, these results can be used as reference data for meat industry and provide useful data for the microbiological, chemical and physical characteristics of meat samples. They provide useful data with added-value meat samples to optimize meat quality.

APPENDIX

SAS Code Example

```
data huang;
input treatment$ rep$ day$ rawL rawa rawb@@;
cards;

1       1       0       79.42 1.75  7.90
1       1       0       79.82 1.66  7.78
1       1       0       79.86 1.81  7.91
2       1       0       79.30 2.39  8.57
2       1       0       83.08 2.48  8.99
2       1       0       81.74 2.52  8.92
3       1       0       80.80 2.25  7.80
3       1       0       81.35 2.42  7.94
3       1       0       82.57 2.36  8.03
4       1       0       76.09 1.96  11.07
4       1       0       75.65 2.00  11.14
4       1       0       75.45 2.09  10.87
5       1       0       83.11 1.98  7.75
5       1       0       84.11 1.89  7.87
5       1       0       83.79 1.93  8.06
6       1       0       78.15 1.90  7.60
6       1       0       78.57 1.95  7.85
6       1       0       79.76 1.77  7.87
7       1       0       75.37 2.45  9.02
7       1       0       75.86 2.34  8.96
7       1       0       76.46 2.65  9.41
8       1       0       75.02 3.42  9.41
8       1       0       75.51 3.37  9.30
8       1       0       75.13 3.16  9.34
9       1       0       81.68 3.08  8.78
9       1       0       81.65 3.00  8.65
9       1       0       82.44 3.04  8.82
1       1       1       75.9  0.92  7.2
1       1       1       75.45 1.08  6.96
1       1       1       76.71 1.13  7.39
2       1       1       80.47 2.43  9.17
2       1       1       80.62 2.59  9.29
2       1       1       82.05 2.60  9.28
3       1       1       76.61 1.62  7.16
3       1       1       79.44 2.09  7.45
3       1       1       78.18 2.17  7.47
4       1       1       69.89 2.01  10.73
4       1       1       69.99 1.94  10.55
4       1       1       71.15 1.82  10.61
5       1       1       78.88 2.01  10.73
5       1       1       69.99 1.94  8.12
5       1       1       78.95 2.04  8.17
6       1       1       72.01 1.56  7.08
6       1       1       74.28 1.71  7.76
6       1       1       72.47 1.43  7.00
7       1       1       71.12 1.83  7.96
7       1       1       72.95 2.60  8.71
7       1       1       72.79 2.62  8.83
```

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8	1	1	72.79	3.36	9.18
8	1	1	72.98	3.37	8.86
9	1	1	78.3	3.05	8.72
9	1	1	78.6	3.26	8.99
9	1	1	79.66	3.37	9.27
1	1	3	71.52	1.00	6.21
1	1	3	71.18	0.36	5.35
1	1	3	71.88	0.86	6.27
2	1	3	79.17	2.53	9.15
2	1	3	80.59	2.52	9.54
2	1	3	79.53	2.36	9.2
3	1	3	78.15	1.86	7.24
3	1	3	78.34	1.41	6.76
3	1	3	77.97	1.86	7.44
4	1	3	68.44	2.11	10.61
4	1	3	68.48	1.99	10.29
4	1	3	68.71	1.85	10.47
5	1	3	69.07	2.13	8.52
5	1	3	77.45	1.98	8.30
5	1	3	79.00	2.08	8.53
6	1	3	70.48	0.97	6.11
6	1	3	71.44	1.31	6.75
6	1	3	71.7	1.22	6.54
7	1	3	69.64	1.66	7.43
7	1	3	70.82	2.25	8.21
7	1	3	71.54	2.02	7.97
8	1	3	72.01	3.07	8.95
8	1	3	71.94	3.18	8.87
8	1	3	73.46	3.42	9.11
9	1	3	77.45	3.12	8.69
9	1	3	78.56	3.41	9.38
9	1	3	79.19	3.45	9.62
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1	1	5	70.86	0.1	4.87
1	1	5	72.38	0.83	6.27
2	1	5	78.27	2.68	9.47
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2	1	5	78.85	2.84	9.57
3	1	5	76.1	2.67	8.11
3	1	5	77.34	2.59	8.18
3	1	5	77.3	2.61	8.14
4	1	5	68.59	2.44	10.77
4	1	5	67.38	2.44	10.39
4	1	5	67.83	2.37	10.41
5	1	5	76.46	2.24	8.69
5	1	5	77.31	2.24	8.92
5	1	5	77.5	2.24	8.75
6	1	5	71.1	2.28	7.71
6	1	5	70.96	2.36	7.62
6	1	5	71.98	2.24	7.48
7	1	5	68.95	2.96	8.93
7	1	5	68.89	3.2	8.97
7	1	5	68.68	3.19	8.85
8	1	5	71.04	3.96	9.39
8	1	5	70.7	3.96	9.26
8	1	5	71.01	3.95	9.10

9	1	5	76.25	3.6	9.08
9	1	5	76.42	3.63	9.23
9	1	5	77.61	3.61	9.37
1	2	0	72.06	5.37	9.11
1	2	0	72.42	5.87	9.45
1	2	0	72.21	5.06	9.03
2	2	0	80.77	2.77	9.27
2	2	0	81.27	2.95	9.05
2	2	0	82.92	2.86	9.57
3	2	0	78.3	1.64	7.69
3	2	0	80.2	2.12	8.20
3	2	0	79.22	1.55	7.73
4	2	0	74.34	2.31	11.3
4	2	0	75.99	2.09	11.00
4	2	0	75.32	2.1	10.34
5	2	0	77.12	2.01	8.34
5	2	0	78.15	1.93	8.69
5	2	0	78.14	2.21	8.35
6	2	0	74.06	3.3	8.72
6	2	0	76.47	3.13	8.74
6	2	0	77.03	3.45	9.09
7	2	0	77.62	2.59	9.10
7	2	0	79.49	2.36	9.14
7	2	0	80.76	2.67	9.63
8	2	0	75.96	2.74	9.43
8	2	0	77.2	2.71	9.56
8	2	0	77.72	2.71	10.00
9	2	0	78.76	3.55	8.41
9	2	0	79.76	3.82	8.72
9	2	0	80.68	4.06	8.87
1	2	1	65.39	3.54	7.14
1	2	1	67.62	3.86	7.55
1	2	1	69.03	4.16	7.72
2	2	1	79.02	2.89	9.12
2	2	1	79.49	2.88	9.29
2	2	1	80.72	2.70	9.74
3	2	1	75.01	0.83	6.54
3	2	1	76.00	1.03	6.81
3	2	1	76.02	0.87	6.68
4	2	1	71.5	1.69	10.41
4	2	1	72.98	1.62	10.63
4	2	1	72.64	1.61	10.64
5	2	1	77.01	1.84	8.09
5	2	1	77.4	1.22	8.06
5	2	1	77.83	1.47	7.98
6	2	1	72.73	2.03	7.58
6	2	1	72.42	2.08	7.33
6	2	1	75.2	2.84	8.27
7	2	1	74.56	1.40	7.80
7	2	1	74.56	1.82	8.15
7	2	1	75.56	1.58	8.23
8	2	1	70.47	1.74	8.29
8	2	1	70.95	1.82	8.15
8	2	1	72.33	2.33	9.03
9	2	1	76.61	3.34	8.35
9	2	1	70.95	2.04	8.25
9	2	1	78.61	3.32	8.65

```
1       2       3       65.29 3.20  6.49
1       2       3       65.48 3.22  6.82
1       2       3       66.7  3.55  6.95
2       2       3       78.8  2.94  9.05
2       2       3       79.71 2.81  9.31
2       2       3       79.62 2.97  9.25
3       2       3       74.06 0.52  5.71
3       2       3       76.68 1.21  6.98
3       2       3       77.15 1.08  6.94
4       2       3       71.69 1.64  10.54
4       2       3       71.67 1.40  10.1
4       2       3       72.9  1.79  11.06
5       2       3       76.08 1.51  7.66
5       2       3       77.65 1.22  8.15
5       2       3       76.72 1.41  7.71
6       2       3       71.41 2.00  7.02
6       2       3       72.83 2.34  7.75
6       2       3       72.55 2.12  7.46
7       2       3       74.25 1.52  8.01
7       2       3       74.77 1.70  8.17
7       2       3       73.92 1.27  7.67
8       2       3       69.81 1.67  7.64
8       2       3       71.68 2.19  8.53
8       2       3       71.01 1.65  8.00
9       2       3       76.19 2.63  7.74
9       2       3       76.82 2.73  8.14
9       2       3       76.87 2.40  7.76
1       2       5       63.45 2.87  6.16
1       2       5       63.78 2.69  6.19
1       2       5       65.28 3.14  6.64
2       2       5       77.71 2.68  9.22
2       2       5       78.42 2.81  8.95
2       2       5       79.31 2.82  9.35
3       2       5       73.89 0.72  6.15
3       2       5       75.18 0.92  6.63
3       2       5       75.52 1.03  6.85
4       2       5       68.29 1.15  9.46
4       2       5       69.95 1.10  9.50
4       2       5       69.03 1.05  9.33
5       2       5       74.55 1.36  7.47
5       2       5       74.7  1.08  7.39
5       2       5       76.41 1.59  7.93
6       2       5       70.78 1.77  6.63
6       2       5       68.6  1.53  6.15
6       2       5       70.36 2.00  6.74
7       2       5       71.53 1.48  7.51
7       2       5       70.2  0.91  6.75
7       2       5       71.56 1.04  7.12
8       2       5       67.46 1.50  7.28
8       2       5       67.4  1.39  7.32
8       2       5       70.00 1.91  8.10
9       2       5       70.43 2.06  6.63
9       2       5       68.91 2.42  7.52
9       2       5       69.35 1.35  5.89;
proc sort data=huang;
by day treatment;
run;
```

```
proc means data=huang min max range maxdec=2;
var rawL rawa rawb;
by day treatment;
run;
proc GLM data=huang ;
   class day treatment;
   model rawL rawa rawb = treatment day treatment*day;
   means treatment day / tukey alpha=0.05;
run; quit;
```

Cooked Meat color L* , a* and b*

```
data huang;
input treatment$ rep$ day$ cookedL cookeda cookedb@@;
cards;
1      1      0      78.33 -0.91 9
1      1      0      78.12 -0.87 8.85
1      1      0      77.29 -1.13 8.83
1      1      0      77.30 -1.13 8.83
1      1      0      77.91 -0.97 8.89
2      1      0      79.52 -0.61 9.06
2      1      0      79.29 -0.57 9.69
2      1      0      79.51 -0.58 9.68
2      1      0      79.64 -0.67 9.73
2      1      0      79.79 -0.69 9.70
3      1      0      81.54 -0.61 9.32
3      1      0      81.32 -0.62 9.29
3      1      0      81.59 -0.63 9.19
3      1      0      81.55 -0.58 8.91
3      1      0      81.52 -0.57 8.98
4      1      0      74.76 -0.36 10.41
4      1      0      74.64 -0.38 10.40
4      1      0      74.66 -0.34 10.36
4      1      0      75.71 -0.49 10.47
4      1      0      75.78 -0.53 10.47
5      1      0      78.29 -0.43 9.43
5      1      0      78.22 -0.43 9.41
5      1      0      78.29 -0.39 9.63
5      1      0      77.47 -0.54 9.84
5      1      0      77.31 -0.54 9.74
6      1      0      76.61 -0.88 9.08
6      1      0      76.68 -1.01 8.88
6      1      0      76.8  -0.97 8.85
6      1      0      76.61 -0.80 9.01
6      1      0      76.82 -0.80 9.10
7      1      0      77.11 -0.74 9.96
7      1      0      77.36 -0.81 9.93
7      1      0      77.4  -0.77 9.93
7      1      0      77.33 -0.91 9.90
7      1      0      77.17 -0.84 10.00
8      1      0      77.4  -0.5  10.26
8      1      0      77.56 -0.47 10.29
8      1      0      77.53 -0.53 10.26
8      1      0      78.36 -0.47 10.24
8      1      0      78.44 -0.49 10.17
9      1      0      81.22 -0.34 9.80
```

9	1	0	81.11	-0.36	9.79
9	1	0	81.18	-0.36	9.77
9	1	0	80.97	-0.38	9.53
9	1	0	80.21	-0.39	9.60
1	1	5	77.27	-1.26	8.73
1	1	5	77.2	-1.33	8.74
1	1	5	77.29	-1.27	8.76
1	1	5	77.18	-1.27	8.79
1	1	5	77.23	-1.28	8.77
2	1	5	78.68	-0.63	9.66
2	1	5	78.48	-0.59	9.71
2	1	5	78.4	-0.63	9.66
2	1	5	78.66	-0.64	9.63
2	1	5	78.84	-0.62	9.69
3	1	5	80.67	-0.61	9.33
3	1	5	80.68	-0.62	9.38
3	1	5	80.58	-0.62	9.44
3	1	5	80.51	-0.62	9.44
3	1	5	80.61	-0.62	9.40
4	1	5	73.59	-0.54	10.31
4	1	5	73.47	-0.53	10.24
4	1	5	73.63	-0.53	10.26
4	1	5	73.55	-0.53	10.33
4	1	5	73.64	-0.5	10.24
5	1	5	75.84	-0.59	9.46
5	1	5	75.79	-0.58	9.46
5	1	5	75.66	-0.54	9.58
5	1	5	75.87	-0.53	9.56
5	1	5	75.72	-0.6	9.46
6	1	5	75.72	-1.33	8.64
6	1	5	75.61	-1.41	8.45
6	1	5	75.55	-1.25	8.67
6	1	5	75.54	-1.16	8.79
6	1	5	75.65	-1.25	8.74
7	1	5	76.07	-0.87	9.85
7	1	5	76.44	-0.91	9.88
7	1	5	76.12	-0.89	9.86
7	1	5	76.63	-0.9	9.93
7	1	5	75.82	-0.88	9.79
8	1	5	76.76	-0.52	10.29
8	1	5	76.73	-0.54	10.22
8	1	5	76.82	-0.54	10.21
8	1	5	76.58	-0.55	10.31
8	1	5	76.75	-0.55	10.29
9	1	5	79.98	-0.28	10.02
9	1	5	80.01	-0.25	9.96
9	1	5	80.27	-0.26	9.98
9	1	5	79.84	-0.27	9.94
9	1	5	80.09	-0.26	9.96
1	2	0	73.4	-0.38	10.18
1	2	0	73.44	-0.38	10.2
1	2	0	73.41	-0.38	10.17
2	2	0	79.63	-0.39	9.95
2	2	0	79.89	-0.38	9.86
2	2	0	79.47	-0.38	9.87
3	2	0	80.77	-0.65	9.33
3	2	0	81.61	-0.63	9.42

```
3       2       0       80.9  -0.64 9.33
4       2       0       74.52 -0.3  10.62
4       2       0       74.49 -0.33 10.54
4       2       0       74.46 -0.31 10.61
5       2       0       80.6  -0.51 9.66
5       2       0       80.93 -0.55 9.71
5       2       0       80.76 -0.55 9.55
6       2       0       76.51 -0.4  10.06
6       2       0       76.51 -0.35 10.01
6       2       0       76.43 -0.39 10.05
7       2       0       77.65 -0.68 9.75
7       2       0       77.64 -0.71 9.81
7       2       0       77.74 -0.67 9.79
8       2       0       77.3  -0.46 9.84
8       2       0       76.96 -0.44 9.92
8       2       0       77.36 -0.46 9.91
9       2       0       80.68 0.04  9.28
9       2       0       80.67 0.09  9.30
9       2       0       80.58 0.08  9.28
1       2       5       74.73 -0.22 10.47
1       2       5       74.23 -0.2  10.57
1       2       5       74.68 -0.31 10.42
2       2       5       79.24 -0.7  9.55
2       2       5       79.19 -0.78 9.54
2       2       5       76.34 -0.56 9.07
3       2       5       80.33 -0.76 9.42
3       2       5       80.62 -0.74 9.35
3       2       5       78.95 -0.74 9.40
4       2       5       74.8  -0.59 10.32
4       2       5       74.81 -0.58 10.43
4       2       5       74.55 -0.59 10.45
5       2       5       80.53 -0.73 9.50
5       2       5       80.58 -0.66 9.96
5       2       5       80.93 -0.69 9.84
6       2       5       76.74 -0.63 9.96
6       2       5       76.76 -0.59 10.02
6       2       5       78.73 -0.84 9.97
7       2       5       76.5  -1.35 9.07
7       2       5       77.39 -1.12 9.53
7       2       5       77.27 -1.18 9.44
8       2       5       74.12 -0.7  9.55
8       2       5       76.97 -0.72 9.89
8       2       5       76.7  -0.74 9.93
9       2       5       79.47 -0.06 9.25
9       2       5       80.02 0.02  9.28
9       2       5       80.03 0.01  9.31
;
Proc sort data=huang;
By day treatment;
Run;
proc print data=huang;
run;
proc means data=huang min max range maxdec=2;
var cookedL cookeda cookedb;
by day treatment;
run;
proc glm data=huang ;
```

```
   class day rep treatment;
   model cookedL cookeda cookedb = treatment day treatment*day
treatment*rep treatment*day*rep day*rep;
   means treatment day / tukey;
run; quit;
```

Results for cooking yield, moisture, pH, Aw, and WHC

```
data huang;
input treatment$ rep cookedmoisture rawmoisture cookedWHC cookedpH
rawpH cookedAw rawAw cookingyield@@;
cards;
1      1      84.65 87.54 84.60 9.24  9.29  0.991 0.950 86.83
1      1      85.22 87.59 90.37 9.24  9.33  0.992 0.979 86.92
1      1      85.20 87.41 86.10 9.27  9.21  0.990 0.986 89.21
2      1      81.65 81.78 86.06 9.44  9.64  0.989 0.978 94.39
2      1      82.99 83.08 78.26 9.41  9.68  0.995 0.968 94.80
2      1      82.55 82.82 83.42 9.43  9.56  0.984 0.975 93.34
3      1      86.28 86.67 94.50 9.35  9.09  0.989 0.949 92.58
3      1      88.08 87.00 94.77 9.34  9.21  0.983 0.918 93.49
3      1      87.29 84.97 91.32 9.33  9.38  0.980 0.970 95.04
4      1      86.53 83.68 92.68 9.95  9.99  0.980 0.975 93.93
4      1      83.83 86.46 90.29 9.92  9.94  0.993 0.966 94.77
4      1      86.39 85.86 92.77 9.98  10.01 0.988 0.980 95.46
5      1      86.36 87.10 82.36 9.85  9.82  0.987 0.968 78.08
5      1      83.83 85.43 83.61 9.87  9.78  0.980 0.958 80.81
5      1      86.75 87.79 84.97 9.86  9.79  0.982 0.950 88.16
6      1      84.40 86.22 95.74 9.57  9.76  0.986 0.967 92.97
6      1      84.43 84.81 96.61 9.61  9.62  0.979 0.950 91.44
6      1      82.95 85.59 94.62 9.61  9.60  0.996 0.954 88.89
7      1      86.32 88.60 95.87 9.72  9.79  0.985 0.949 93.36
7      1      87.30 85.29 96.99 9.71  9.83  0.984 0.947 90.87
7      1      84.52 86.45 95.70 9.75  9.67  0.986 0.958 94.09
8      1      85.27 85.67 93.64 9.60  9.65  0.996 0.977 92.28
8      1      85.77 85.96 92.68 9.59  9.62  0.986 0.962 93.18
8      1      86.05 86.94 96.36 9.65  9.48  0.985 0.977 95.65
9      1      84.17 87.68 97.04 8.68  8.62  0.979 0.962 89.08
9      1      82.75 86.08 92.52 8.65  8.81  0.988 0.955 92.20
9      1      86.45 85.84 94.38 8.67  8.72  0.978 0.960 96.13
1      2      83.23 83.84 81.43 9.77  8.79  0.959 0.971 87.99
1      2      81.09 83.33 80.72 9.06  8.57  0.962 0.974 89.84
1      2      82.69 83.25 87.80 9.00  8.54  0.965 0.972 89.86
2      2      79.01 80.09 81.78 8.96  8.52  0.972 0.987 93.89
2      2      78.21 79.05 81.10 8.99  8.53  0.963 0.990 91.96
2      2      78.69 78.95 83.14 8.99  8.62  0.962 0.979 91.77
3      2      81.41 83.22 90.62 8.75  8.18  0.960 0.975 94.14
3      2      83.19 81.88 88.64 8.72  8.18  0.969 0.980 91.65
3      2      82.48 82.01 90.47 8.69  8.20  0.971 0.978 91.93
4      2      82.64 83.53 82.50 9.03  8.66  0.967 0.979 94.93
4      2      77.22 80.90 84.99 9.05  8.75  0.963 0.973 93.38
4      2      81.12 81.15 78.78 9.04  8.77  0.960 0.976 92.57
5      2      80.38 82.13 73.02 9.05  8.76  0.966 0.981 82.31
5      2      80.36 82.40 67.41 9.03  8.76  0.968 0.973 90.32
5      2      81.67 82.48 67.11 9.01  8.73  0.967 0.985 89.76
6      2      82.78 80.90 91.43 9.01  8.71  0.982 0.990 89.42
6      2      81.52 83.74 93.04 9.02  8.65  0.967 0.979 89.74
6      2      82.42 84.45 98.75 9.02  8.65  0.967 0.981 90.04
```

```
7      2      81.49 81.95 83.06 8.99  8.67  0.964 0.976 92.82
7      2      83.69 82.33 89.59 9.00  8.67  0.970 0.971 90.38
7      2      80.87 81.36 89.98 8.99  8.67  0.966 0.973 89.43
8      2      82.11 81.71 89.07 8.91  8.62  0.976 0.971 92.66
8      2      80.70 81.26 92.81 8.90  8.66  0.970 0.989 90.71
8      2      81.77 83.72 92.34 8.89  8.57  0.966 0.980 90.20
9      2      80.74 83.03 95.09 8.81  8.48  0.970 0.974 95.00
9      2      82.84 82.35 82.90 8.78  8.39  0.969 0.979 92.07
9      2      76.31 81.16 92.08 8.87  8.31  0.966 0.974 91.54

;
Proc sort data=huang;
By treatment;
Run;
proc print data=huang;
run;
proc means data=huang min max range maxdec=2;
var cookedmoisture rawmoisture cookedWHC cookedpH rawpH cookedAw rawAw
cookingyield;
by treatment;
run;
proc anova data=huang ;
   class treatment;
   model cookedmoisture rawmoisture cookedWHC cookedpH rawpH cookedAw
rawAw cookingyield = treatment;
   means treatment / tukey alpha=0.05;
run; quit;
```

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VITA

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