12-2017

Radiofrequency processing for inactivation of Salmonella spp. and Enterococcus faecium NRRL B-2354 in whole black peppercorn and ground black pepper

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Radiofrequency processing for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 in whole black peppercorn and ground black pepper

by

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A THESIS

Presented to the Faculty of

The Graduate College at University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professors Jeyamkondan Subbiah and Jayne Stratton

Lincoln, Nebraska

December, 2017

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Black pepper has been implicated in several foodborne illness outbreaks and food product recalls due to *Salmonella* contamination. Conventional decontamination methods for black pepper are challenged by harmful residues or quality deterioration. Radiofrequency (RF) heating reduces the come-up time which allows to design a high-temperature short-time processing to inactivate *Salmonella* with minimal deterioration in product quality. The objectives of this study were to investigate RF heating for inactivation of *Salmonella* in whole black peppercorn and ground black pepper samples, evaluate *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella,* and assess quality deterioration during RF heating of whole black peppercorn and ground black pepper samples. Both samples were inoculated with a 5-strain cocktail of *Salmonella* or *E. faecium*, equilibrated to the target water activity, and RF heated for 150 s for whole black peppercorn and 130 s for ground black pepper. Microbial stability and homogeneity tests were conducted for both *Salmonella* and *E. faecium* during moisture equilibration before RF heating to evaluate the inoculation method. Piperine, total phenolic and, volatile oil, and the antioxidant activity were assessed for the quality of both whole black peppercorn and ground

black pepper samples. RF heating was demonstrated to provide 5.31 and 5.98 log CFU/g reduction of *Salmonella* for whole black peppercorn and ground black pepper, respectively. Whole black peppercorn and ground black pepper samples were dried to their optimal storage moisture after RF heating. The higher thermal resistance of *E. faecium* was observed during RF heating of both black pepper samples. The quality analysis showed that the quality of both black pepper samples did not experience a considerable change after RF heating. In this study, an applicable RF pasteurization process was developed for whole black peppercorn and ground black pepper, which provided effective inactivation of *Salmonella* while minimizing the quality deterioration. *E. faecium* was found to be a suitable surrogate for *Salmonella* during the RF heating of both whole black peppercorn and ground black pepper samples.

Keywords: dielectric heating, electromagnetic heating, process validation, quality analysis, surrogate

Acknowledgement

Foremost, I would like to express my deepest gratitude to my advisor Dr. Jeyamkondan Subbiah for the support of my Master's study and research. He has been supportive since the days I began working in the lab as an undergraduate. His guidance provided me the chance to participate in multiple research projects, which helped me come up with the thesis topic. His encouragement supported me through the rough road to finish this thesis. Thanks to Dr. Subbiah for the life advice, I started to build up the leadership and communication skills which I would benefit from in the rest of my life.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Jayne Stratton who is my co-advisor, Dr. Sibel Irmak and Dr. Andreia Bianchini. With their expertise in food microbiology, Dr. Stratton and Dr. Bianchini provided a lot of advice for my design of microbial challenge studies and the critical comments on the microbiological side. My sincere thanks to Dr. Irmak, for her time and efforts spent on my chemical analysis.

I can still remember the first day when Jiajia Chen and Soon Kiat Lau introduced me to the lab. Thanks to their help, I started to get familiar with every instrument in our lab, and got the chance to work under Dr. Subbiah. Thanks to Kiat's time for helping with my projects and reviewing my manuscripts. I would also like to thank Tushar Verma. We worked together almost all the time in the lab during the past two years. From helping with his project, I have gained so much knowledge and experience. I am also grateful to Long Chen, Sabrina Vasquez, Ryan Anderson and

Emily Bender for their support and help. I will always remember the fancy meals we had on every foodie Friday.

Finally, I am grateful to my parents, as their only child, I have been absent for four years from their life. I appreciate their support and understanding.

Declaration of Grant Information

This project is supported by funding from United States Department of Agriculture (USDA) - National Institute of Food and Agriculture (NIFA) Agriculture and Food Research Initiative (AFRI) grant 2015-68003-23415.

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Chapter I: Introduction

1.1 *Salmonella* in low moisture foods and black pepper

Defined by Blessington (2013), low moisture foods are those foods with water activity (a_w) lower than 0.7 which is of high prevalence in daily life. Typically, nuts, peanut butter, dry fruits, grain products and spices are the examples of low moisture foods. Historically, low moisture foods were not considered as foodborne pathogens carriers because the low a_w environment does not support the growth of foodborne pathogens like *Salmonella, Campylobacter, Listeria* and *Escherichia coli* (Beuchat, 1981). However, several outbreaks of *Salmonella* were reported to be linked to low moisture foods (Centers for Disease Control, 2010, 2007, 2004, 1998; Johnson et al., 2014), and thus low moisture can no longer be considered microbiologically safe (Beuchat et al., 2013).

Black pepper has also been reported to cause several outbreaks (Centers for Disease Control, 2010, 1982) and recalls (Dey et al., 2013) because of *Salmonella* contamination. Although *Salmonella* cannot reproduce themselves in black pepper, it has been reported that *Salmonella* could survive for several years even in the extremely low a_w environment of black pepper at room temperature (Keller et al., 2013). Therefore, contaminated black pepper could still sicken people after a long period of storage without effective pasteurization. Black pepper is a common ingredient in many ready-to-eat food products and a seasoning to enhance flavor. On the consumer's side, black pepper is not always subjected to further heating, and thus it requires effective decontamination from the food industry's side to ensure food

safety.

Black pepper is one of the most popular spices in the world and usually can be found in forms of ground black pepper and whole black peppercorn at retail. Several decontamination methods have been developed to control *Salmonella* in black pepper for spice manufactories to ensure the food safety. Although non-thermal treatments like ethylene oxide fumigation and gamma irradiation treatment have been shown to effectively reduce *Salmonella* population in ground black pepper or whole black peppercorn, their disadvantages regarding potential harmful residues and poor customer acceptance limit their promotions (Song et al., 2014; Toofanian, 1986). Thermal treatments like steam treatment and hot air treatment are more commonly used in commercial pasteurization of black pepper. However, since the conventional thermal treatments only rely on thermal conduction, it would take a long time for those thermal treatments to heat up the low moisture food products because of the poor thermal conductivities of low moisture food. Due to longer treatment at high temperature, the food quality has to compromise on safety, and it has been reported that significant quality deterioration of black pepper resulted from the conventional thermal treatments (Chacko et al., 1996; Schneider, 1993; Waje et al., 2008).

1.2 Radiofrequency (RF) heating

RF heating is a kind of dielectric heating which has high potential to be applied to decontamination low moisture foods. The conventional heating is based on thermal conduction or convection, the heat comes from an outside heat source to the interior by a temperature gradient and the heating rate depends on the thermal properties of

food products. Most of food products are dielectric materials with poor electrical-conduction properties, and it has been found that poor electrical-conduction properties correlated with low thermal conductivity (Barber, 1983). Thus, the conventional heating would take a long time to heat up food products, and the slow heating rate could result in severe non-uniform heating when processing a large scale of food products in the food industries. The dielectric heating like RF and microwave heating could be a novel alternative for conventional heating to heat food products because of its characteristic heating mechanism. In dielectric heating, the electromagnetic energy is directly transferred into food products, and the high-frequency alternating electric field induces the vibration of molecules which generates heat due to friction. Therefore, the dielectric heating provides a fast and volumetric heating to food products. In addition, RF and microwave heating can be turn on and off instantaneously and also provide a much higher heating efficiency than conventional heating (Mermelstein, 1997).

Compared to microwave heating, RF heating has advantages in terms of large-scale processing because of its longer wavelength which provide a greater penetration (Birla et al., 2004; Chen et al., 2017; Piyasena et al., 2003; Tang et al., 2005). In addition, RF heating is also more suitable for heating of low moisture food (Wang et al., 2003). Unlike gamma irradiation, RF heating is nonionizing radiation and cannot produce sufficient energy to change molecular structures, which indicates RF heating is a pure thermal treatment (Mitcham et al., 2004).

RF heating has been shown to effectively inactivate *Salmonella* in several low

moisture foods such as in-shell almonds (Li et al., 2017), dry milk (Michael et al., 2014), wheat flour (Liu et al., 2017; Villa-Rojas et al., 2017), red and black pepper (Song et al., 2014). Before food manufacturers could apply RF heating in their facilities, the validation should be conducted to give a scientific proof of this process for effective hazard control as it is required by Food Safety Modernization Act (Food and Drug Administration, 2013). However, it is risky to directly introduce the pathogen like *Salmonella* into the food processing facility, and the use of surrogate provides a practical way to conduct the process validation. *Enterococcus faecium* NRRL B-2354 has been demonstrated to possess a higher heat resistance than *Salmonella* in many low moisture food (Almond Board of California, 2007; Kopit et al., 2014; Liu et al., 2017), and has also been evaluated as a suitable surrogate for *Salmonella* in some thermal processing of different low moisture foods (Bianchini et al., 2014; Enache et al., 2015; Shah et al., 2017; Verma, 2017; Villa-Rojas et al., 2017).

1.3 Objectives

This research is aimed to develop an applicable RF heating pasteurization process for both ground black pepper and whole black peppercorn which could provide adequate and effective inactivation of *Salmonella* spp. while minimizing quality deterioration. The main objectives of this research are:

1. To determine RF heating process parameters for effective inactivation of *Salmonella* spp. in whole black peppercorn and ground black pepper.
2. To evaluate the use of *Enterococcus faecium* NRRL B-2354 as a suitable

surrogate for *Salmonella* spp. in the validation of RF heating.

3. To assess the quality deterioration of whole black peppercorn and ground black pepper after the RF heating.

1.4 Thesis organization

Chapter II is a literature review and provides information on *Salmonella* and its behavior in low moisture food. Background on black pepper including production, quality, application and association with *Salmonella* is provided. In this chapter, the current applications and limitations of RF heating are also reviewed.

In Chapter III, the efficacy of RF heating to inactivate *Salmonella* spp. and *E. faecium* on whole black peppercorn was assessed. A proper RF heating pattern was developed to provide an adequate inactivation for *Salmonella* spp. on the equilibrated whole black peppercorn samples, and the microbial challenge studies were conducted in terms of average enumeration and cold spot enumeration. The surrogate study was conducted and quality of whole black peppercorn was evaluated for the determined RF heating pattern.

Chapter IV describes the development of RF heating pasteurization for ground black pepper. From the results of Chapter III, it was determined that for ground black pepper only samples at the cold spot would be sufficient to validation the process. Surrogate evaluation and quality analysis were also performed for this RF heating pattern.

Chapter V is the conclusion of this thesis and summarizes that RF heating could be a feasible tool to be applied in the processing of spice to ensure the food safety of

black pepper products. In addition, suggestions for future research are also mentioned in this chapter.

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Chapter II: Literature Review

2.1 General introduction to *Salmonella*

The name of *Salmonella* was given by Daniel Elmer Salmon, who identified *S.* Choleraesuis in 1885 as a bacteriologist (Smith, 1894). *Salmonella* is gram-negative, rod-shaped bacilli which range from 0.7 to 1.5 μm in diameter and from 2 to 5 μm in length, facultatively anaerobic bacteria in the family *Enterobacteriaceae* (Issenhuth-Jeanjean et al., 2014). The optimal living temperature for *Salmonella* is 37°C while it can survive in a wide temperature range from 2 to 46°C, and *Salmonella* has a pH tolerance from 4.0 to 9.5 with an optimum growth pH from 6.5 to 7.5 (Andino and Hanning, 2015). Based on their phenotypic profile, *Salmonella* can be divided into two species, *Salmonella enterica* and *Salmonella bongori.* However, based on biochemical and genomic modifications, *Salmonella enterica* is further divided into 6 subspecies: *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica* (Brenner et al., 2000). There are currently 2,463 serotypes associated with *Salmonella*. Out of those, Typhimurium*,* Newport*,* and Javiana are the most common serotypes that cause human infection (Issenhuth-Jeanjean et al., 2014).

Based on their serotype, *Salmonella* are classified as either “typhoidal” or “nontyphoidal”. Typhoidal is the *Salmonella* that causes typhoid fever which spread mainly among human via the fecal-oral route and has no significant animal reservoirs. Nontyphoidal salmonellosis also can be spread from person to person, while its main reservoir is animals and this disease is usually foodborne (Gal-Mor et al., 2014). Most human infections with *Salmonella* caused by the ingestion of contaminated food or

water and some result from contacting with infected animals.

2.2 *Salmonella* in low-moisture food

Salmonellosis is one of the most common and worldwide distributed foodborne diseases. Annually, non-typhoidal *Salmonella* result in approximately 1.2 million illnesses and approximately 450 deaths in the United States (Johnson et al., 2014). *Salmonella* could be commonly found in raw food products like eggs, poultry, unpasteurized milk, meat, cheese, fruits and vegetables, spices, and nuts.

Low-moisture foods are those with water activity (a_w) lower than 0.70 (Blessington et al., 2013). Powdered infant formula, peanut butter, spices, dry fruits, nuts, wheat flour, puffed cereals, and cookie dough are some examples of low-moisture food (Carrasco et al., 2012; Jiao et al., 2014; Podolak et al., 2010). Historically, low-moisture foods were not considered as a carrier for *Salmonella* because low a_w is a barrier for the growth of *Salmonella* (Beuchat, 1981). Though these products do not support the growth of *Salmonella*, *Salmonella* is able to survive in low-moisture foods for long periods of time (Keller et al., 2013). Recently many low-moisture foods have been reported to be contaminated with foodborne pathogens, most noticeably and generally *Salmonella*, leading to numerous food product recalls and foodborne illness outbreaks. As reported by Centers for Disease Control (CDC), the major low-moisture foods that were implicated in foodborne illness included oats cereal (Centers for Disease Control, 1998), peanut butter (Centers for Disease Control, 2007), raw almond (Centers for Disease Control, 2004), red and black pepper (Centers for Disease Control, 2010). From 2007 to 2012, the records of low-moisture

foods on the CDC website revealed that there were a total of 119 recalls in United States which included pet food, powdered infant formula, peanut butter, spices, dry nuts, dry milk, and seeds. Therefore, low moisture food can no longer be considered microbiologically safe.

Water activity in foods is defined as the ratio of the vapor pressure of water in a food matrix compared to that of pure water at the same temperature. Water plays an important role in microbial inactivation, because a_w is related to the interaction between cells and water which reflects the ability to growth of cell (Bowman et al., 2015). The minimum a_w for growth of most bacteria is approximately 0.87 and minimum a_w for mycotoxin production by molds is 0.80 (Beuchat et al., 2013). However, low a_w could increase the heat resistance of *Salmonella* cells, and it was reported that the reduced a_w protected against the inactivation of *Salmonella* in low-moisture foods (Beuchat et al., 2013). Therefore, *Salmonella* is extremely difficult to control in a desiccated environment.

Many low-moisture food products are ready-to-eat foods like salami, nuts, spices and dry fruits which do not require further cooking at consumer's end. In addition, even consumption of only one *Salmonella* cell in a food product may be sufficient to cause illness (Little et al., 2003). Hence, the presence of *Salmonella* in low-moisture foods can pose a threat to the public health and potentially result in foodborne outbreaks (Kopit et al., 2014). Therefore, effective decontamination process must be conducted to ensure the safety of low-moisture foods.

In most of food products, *Salmonella* requires a minimal ingestion dose of 10^5 to

10^7 CFU/g depending on the strain and host to cause foodborne illness(Hara-Kudo and Takatori, 2011; Teunis et al., 2010). However, it was reported that a low infectious dose of 10 to 100 CFU/g of *Salmonella* contaminated low moisture food has resulted in many outbreaks(Gill et al., 1983; Greenwood and Hooper, 1983). It is likely that the desiccated environment or a high-fat content provide protection to *Salmonella* during invading the gastrointestinal tract and immune system, which has been shown in some studies(Aviles et al., 2013; Possemiers et al., 2010).

2.3 *Enterococcus faecium* as a surrogate of *Salmonella*

According to FDA Food Safety Modernization Act, food manufacturers are required to validate their process controls to ensure food safety (Food and Drug Administration, 2015). However, it is not feasible to validate the process using pathogenic bacteria like *Salmonella* in their facilities because *Salmonella* could pose a threat to the health of workers and the safety of food products. Therefore, it is essential to find a non-pathogenic surrogate to be inoculated into the product and subjected to the actual process to conduct validation studies in the processing plant. A proper surrogate can be defined as “a non-pathogenic organism that behaves similarly to the pathogenic organism when exposed to the same conditions or treatment” (Jeong et al., 2011). The identification of surrogate is beneficial for determining and validating the efficacy of decontamination treatments in food manufacturing facilities.

Enterococcus faecium NRRL B-2354 is a Gram-positive, spherical cell, and facultative anaerobic organism (Byappanahalli et al., 2012). *E. faecium* is commonly used as surrogate for *Salmonella* in testing of thermal processing treatments used in

the production of low-moisture foods. The genetic and phenotypic characteristics of *E. faecium* show that it is safe to be selected as a surrogate for food processing (Kopit et al., 2014). Almond Board of California first identified *E. faecium* as a suitable surrogate for *Salmonella* during the almond process (Almond Board of California, 2007). It has also been evaluated as a good surrogate for *Salmonella* in many low-moisture food matrices, such as extrusion of carbohydrate-protein meal (Bianchini et al., 2014), extrusion of oat flour (Verma, 2017), RF heating of wheat flour (Villa-Rojas et al., 2017), and thermal treatments of chicken meat powder, pet food and savoury seasoning (Rachon et al., 2016).

2.4 Black pepper

As defined by the International Standard Organization (ISO), spices are "vegetable products or mixtures thereof, free from extraneous matter, used for flavoring, seasoning, and imparting aroma in foods." Considered as "King of spice" or "Black Gold", black pepper is one of the most popular spices in the world. In the world trade of spice, black pepper accounts for 35% of the total trade, and India is the largest producer, consumer and importer of black pepper (Dhas and Korikanthimath, 2003). Black pepper is the fruit of *Piper nigrum*, and has been commonly used as flavoring and seasoning agent to enhance the flavor of foods. According to American Spice Trade Association (2011), black pepper should achieve a uniform moisture content that is certainly not higher than 12.0% (w.b.), and 10.5% (w.b.) moisture content is optimal for storage of ground black pepper. Black pepper originated from the Western Ghat in India where more than hundred known cultivated types are

reported, and it was spread to other countries subsequently (Ravindran et al., 2000). The humid environment and warm temperature are optimal for the growth of black pepper (Sivaraman et al., 1999). Black pepper is also used for the preparation of its derivatives such as essential oils and oleoresin because of its excellent aroma, flavor and pungency (Dhas and Korikanthimath, 2003).

In addition to their flavor and use in human dietaries, black pepper is also known for its preservation (Nielsen and Rios, 2000), antioxidative (Shobana and Naidu, 2000) and antimicrobial properties (Salie et al., 1996). The aroma of black pepper is contributed by the essential oil, while the pungency is due to the alkaloid piperine (Srinivasan, 2007). Piperine is a compound belongs to the alkaloid family representing the major component in the black pepper, and it was reported that the distinct biting quality of black pepper is attributed to piperine (Govindarajan and Stahl, 1977). It has been reported that piperine possesses an anti-inflammatory and an analgesic effect (Gupta et al., 2000). In addition, some studies have also shown that piperine could possess pharmacological effects such as anti-diarrhoeal (Bajad et al., 2001) and hepatoprotective (Koul and Kapil, 1993). Piperine has also been demonstrated in in vitro studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species and has also been evidenced to lower lipid peroxidation (Srinivasan, 2007). Moreover, piperine was also demonstrated to have the neurotrophic effect in hippocampus, and it could be used for the precaution of Alzheimer diseases due to its high antioxidant activity (Chonpathompikunlert et al., 2010).

Pepper essential oil plays an important role in the manufacture of perfumery and confectionery products. It has been reported that volatile oils of black pepper possess strong antioxidant and antimicrobial activities (Singh et al., 2004). It has been demonstrated that supercritical CO_2 extracted oleoresin and essential oils from black pepper were effective for reducing lipid oxidation in ground pork, and pepper can avoid meat flavor deterioration (Tipsrisukond et al., 1998).

The quality of black pepper depends on the quality of raw materials, the methods used in processing, packaging and marketing practices (Dhas and Korikanthimath, 2003). Therefore, in order to ensure quality of the product, effective inspection should be maintained from the time of pre-harvesting to post-harvesting.

Black pepper has been known to harbor bacteria, yeasts, and molds, and nature black pepper usually has a high microbial load (McKee, 1995). Usually the presence of these microorganisms is considered a product quality issue rather than a safety problem (Bowman et al., 2015).

2.5 *Salmonella* in black pepper

The commercial production of black pepper contains multiple steps including harvesting, drying, grinding, packaging and storage, during which cross-contamination with pathogenic microorganism may occur (Keller et al., 2013). It has been reported that black pepper could get contaminated with *Salmonella* during the packing process, in food manufacturing facilities and even in retail (Food and Drug Administration, 2012; Moreira et al., 2009; Sagoo et al., 2009). During the cultivation, harvesting and post-processing, black pepper could get contaminated with

Salmonella. The contamination may occur when black pepper get contact with animal waste from pests and birds, during sun drying of spice berries on the ground, and because of poor personal hygiene of laborers (Nair, 2004).

Salmonella has been reported to readily reproduce themselves at room temperature in ground black pepper when $a_w > 0.94$ (Keller et al., 2013). Although *Salmonella* can not reproduce themselves in the low a_w environment, it has been reported that it may survive for possibly years at a low a_w storage conditions (Keller et al., 2013). Black pepper is commonly used as a raw ingredient in many ready-to-eat foods, and it is also used to enhance the flavor cooked food. In both cases, black pepper are not subjected to further thermal treatment, thus *Salmonella* contaminated black pepper could potentially pose a threat to public health and may result in foodborne outbreaks (Little et al., 2003). In 2010, a multistate foodborne outbreak caused by *Salmonella* Montevideo was traced back to salami products made with contaminated imported black pepper, which sickened 272 people in 44 states (Centers for Disease Control, 2010). It has also been reported that black pepper imported from Brazil into Norway was responsible for 126 cases of *Salmonella* Oranienburg infection (Centers for Disease Control, 1982). There were a total of 76 recalls in 2010 associated with different food products contained the potential contaminated black pepper (Dey et al., 2013). Contaminated black pepper not only causes food safety issue, but also results in an enormous economic loss. Thus, effective decontamination methods need to be developed for food manufactory to ensure the food safety of black pepper.

To reduce the microbial load in black pepper, several decontamination methods have been developed such as ozone treatment, fumigation by ethylene oxide, irradiation with gamma ray and steam treatment. Although ethylene oxide has been proven to significantly reduce microbial load (Leistritz, 1997; Toofanian, 1986), it is generally considered as a carcinogen and mutagen and even banned in the European Union (Uijl, 1992). Gamma irradiation has also shown to be an effective method for black pepper decontamination. A dose of 5 kGy decreased *S.* Typhimurium populations by more than 5.2 log CFU/g in black pepper (Song et al., 2014). However, it is scarcely used in the food industry because of its poor consumer acceptance. A 10 min of ozone treatment at a ozone concentration of 6.7 mg/L has been reported reduce the microbial population of ground black pepper by 3-6 log CFU/g, while it resulted in the oxidation of certain volatile oil constituents (Zhao and Cranston, 1995). High temperature steam treatment is extensively used in the European spice industry for denomination of whole spices (Schweiggert et al., 2007). However, the commercial steam treatment was reported to cause significant color loss and quality deterioration to black pepper (Schneider, 1993; Waje et al., 2008). Therefore, innovative technologies are demanded for the spice industry to decontaminate black pepper while maintaining product quality.

2.6 Radiofrequency (RF) heating

RF heating is a novel and promising technology for food applications. The advantages of RF heating over the conventional heating are its rapid heating, uniform heat distribution and less energy consumption (Birla et al., 2004; Jiao et al., 2014;

Wang et al., 2008). Basically, conventional heating depends on temperature gradient which energy transfers from a hot medium to a cooler product. The process is not only time consuming and non-uniform, but also plenty of energy is wasted by dissipating into the environment. RF heating is based on volumetric and dielectric heating which transfers the electromagnetic energy directly into the food product and generates the heat from molecular friction. Similar to microwave heating, RF heating is a kind of dielectric heating. Food products are applied as dielectric between the two metal capacitor plates, the two electrodes are alternatively charged at a relative high frequency. The polar molecules like water molecules in food products try to continuously realign themselves according to the high frequency alternatively electromagnetic field. The result of this motion of molecules generates heat from their kinetic energy and friction caused by colliding neighboring molecules. Radio frequency (RF) heating involves utilizing electromagnetic energy at a frequency range of 1–300 MHz. However, radio frequencies lies in radar range and can interface with communication system, so according to Electromagnetic Compatibility (EMC) regulations only selected frequencies can be used for radiofrequency and microwave heating. They are 13.56, 27.12 and 40.68 MHz for radio frequency heating and 915 MHz, 2,450 MHz, 5.8 GHz for microwave heating (Davis and Showers, 1974).

RF heating has already been widely applied in food industry for post-baking of cookies (Palazoğlu et al., 2012). It has also been investigated in the research laboratories for various applications such as drying of grain product (Jumah, 2005) and pet food (McCulloch and Nelson, 1977), , pest control of walnuts (Wang et al.,

2008), grain (Nelson and Whitney, 1960) and food pasteurization (Al-Holy et al., 2005; Bengtsson et al., 1970; Houben et al., 1991).

2.7 Applications of RF on low moisture food

Conventional thermal pasteurization process for dry food products usually heats the food products by hot air or steam. Because of the low thermal conductivity of low moisture foods, using conventional oven to heat up dry food products can take several hours to a couple of days to reach the requirement of pasteurization. It has been reported that low moisture food like dry egg white powder are generally dry-heated at 60-80°C for 3-30 days for pasteurization (Handa et al., 2001). Slow and longtime heating of dry food materials usually leads to food quality deterioration in terms of texture, color, flavor, and nutritional value (Talansier et al., 2009). In order to minimize the quality loss and provide a fast and efficient thermal processing, RF heating would be a promising tool to be applied.

Due to the low a_w, low-moisture food usually has higher percentage water exists as the form of bound water rather than the free water (Rockland and Stewart, 2013). Within the radio frequency range, the bound water has its maximum dielectric loss factor at around 100 MHz, which indicates that RF heating tends to heat up low moisture food more effectively than microwave heating (Tang et al., 2005).

Several researches showed that radio frequency heating improved food product quality compared to conventional heating process and reduce energy consumption at the same time (Nijhuis et al., 1998; Zhao et al., 2000). RF heating has also been evaluated to effectively inactivate *Salmonella* in many low-moisture food products,

e.g. in-shell almonds (Li et al., 2017), red and black pepper (Kim et al., 2012), nonfat dry milk (Michael et al., 2014) and wheat flour (Liu et al., 2017).

2.8 Non-uniform heating of radio frequency

Although RF provides much higher penetration depth because of its longer wavelength than microwave, one of the major problems associated with radio frequency heating is the non-uniform temperature distribution due to the edge over-heating and thermal runaway (Tiwari et al., 2011). The heating performance of RF could be affected by a lot of factors such as settings of RF, the geometry, salt content, moisture content, thermal properties and dielectric properties of food. It was reported that non-uniform temperature distribution might cause the food safety issue or food quality deterioration in different food products (Nijhuis et al., 1998; Piyasena et al., 2003; Tiwari et al., 2011).

To improve the heating pattern of RF heating, researchers have tried to develop different models for RF heating process to compute temperature distribution during heating directly, and thus could change the heating setting more straight forward (Chen et al., 2013; Jiao et al., 2014; Tang et al., 2005).Several methods have also been developed to improve radio frequency heating uniformity. Jiao (2014) surrounded foods with material with similar dielectric constant improves RF heating uniformity. Liu (2013) reported that combining radio frequency with hot air treatment could improve the heating uniformity. Wang (2006) mixed the food sample during heating and redistribute electric field and heat within the food product. Due to the non-uniform heating, there will be cold spot and hot spot formed within the food

samples. It is important to locate the cold spot during RF heating and evaluate the worst-case scenario to ensure the food safety. Liu (2017) showed that evaluating the microbial reduction of the cold spot can potentially provide enough evidence for the validation studies of RF heating.

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Chapter III: Radiofrequency processing for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 in whole black peppercorn

Abstract

Several *Salmonella* spp. outbreaks linked to black pepper calls for effective inactivation processes, because current decontamination methods are limited by quality deterioration or potential harmful residues. Radiofrequency (RF) heating provides a fast heating rate and volumetric heating, resulting in a shorter come-up time. This allows for choosing a higher temperature and short time combination to achieve desired inactivation with minimal quality deterioration. The objectives of this study were to investigate RF heating for inactivation of a 5-strain *Salmonella* cocktail in whole black peppercorn, evaluate *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* spp. in RF heating and assess quality change of RF-treated whole black peppercorn. Whole black peppercorns were adjusted to a moisture content of 12.7% (wet basis, wb), and were then inoculated with a 5-strain cocktail of *Salmonella* spp. or *E. faecium* to attain initial population levels of 6.8 log CFU/g and 7.3 log CFU/g, respectively. Stability test was performed to quantify the microbial reduction during inoculation and storage before RF heating inactivation. During RF heating, the cold spot was determined to be at the center on the top surface of the treated sample. Therefore, in addition to the inoculated sample, an inoculated packed sample was placed at the cold spot to evaluate the worst-case scenario. The 2.5 min of RF heating provided an average of 5.31 log CFU/g reduction and 5.26 log CFU/g reduction for *Salmonella* spp. and *E. faecium,* respectively. Color parameters (L*, a*,

b*), piperine content, total phenolics, scavenging activity and most of the volatile compounds of 2.5 min of RF treated samples were not significantly different from the control samples. These data suggest that RF heating is a promising thermal inactivation treatment for *Salmonella* without significant quality deterioration, and *E. faecium* is a suitable surrogate for *Salmonella* to validate the efficacy of RF heating of whole black peppercorn.

3.1 Introduction

Salmonella related foodborne illness is a major public health problem worldwide. It has been reported that non-typhoidal *Salmonella* causes approximately 1.2 million illnesses and approximately 450 deaths in the United States annually (Johnson et al., 2014). Low-moisture foods are those with water activity (a_w) levels lower than 0.70 and are not usually considered a high-risk food product, because low a_w is barrier against *Salmonella* growth (Blessington et al., 2013). Although low-moisture foods do not support the growth of *Salmonella* and have historically been considered to be microbiologically safe (Beuchat, 1981), many outbreaks reported by CDC implicated *Salmonella* in low-moisture food products as the causative agent of those illnesses (Centers for Disease Control, 2004, 2007, 2010).

Black pepper is the fruit of *Piper nigrum*, and has been used as flavoring and seasoning agents to enhance the flavor of foods. The commercial production of black pepper contains multiple steps including harvesting, drying, grinding, packaging and storage, during which cross-contamination may occur (Keller et al., 2013). In addition, *Salmonella* has been reported to readily reproduce themselves at room

temperature in ground black pepper when $a_w > 0.94$ and may survive for possibly years at a low a_w storage conditions (Keller et al., 2013). In addition, *Salmonella* exhibits increasing thermal resistance at decreasing water activity during heat treatment (Beuchat et al., 2013). This could potentially create a public health risk, because black pepper is frequently added as an ingredient in many ready-to-eat foods which are not subjected to further thermal treatment (Little et al., 2003). In 2010, a multistate foodborne outbreak caused by *Salmonella* Montevideo was traced back to salami products made with contaminated imported black pepper, resulting in 272 sick people in 44 states (Centers for Disease Control, 2010).

To reduce the microbial load in black pepper, several decontamination methods such as ozone treatment, fumigation by ethylene oxide, irradiation with gamma ray and steam treatment have been developed. Although ethylene oxide has been proven to significantly reduce microbial load (Leistritz, 1997; Toofanian, 1986), it is generally considered as a carcinogen and mutagen and even banned in the European Union. Gamma irradiation has also shown to be an effective method for black pepper decontamination. A dose of 5 kGy decreased *S.* Typhimurium populations by more than 5.2 log CFU/g in black pepper (Song et al., 2014). However, it is scarcely used because of its poor consumer acceptance. Ozone treatment reduced the microbial population of ground black pepper by 3-6 log CFU/g, but resulted in the oxidation of certain volatile oil constituents (Zhao and Cranston, 1995). High temperature steam treatment is extensively used in the European spice industry on whole spices before grinding (Schweiggert et al., 2007). However, the commercial steam treatment has

been reported to cause significant color loss and quality deterioration to black pepper (Schneider, 1993; Waje et al., 2008). Therefore, innovative technologies are needed for decontamination of black pepper while maintaining product quality.

Radiofrequency (RF) heating is a novel thermal processing method which utilizes electromagnetic energy within the frequency range of 1–300 MHz to dielectrically heat food products. RF heating transfers the electromagnetic energy directly into the food product to generate heat by molecular friction resulting in volumetric heating. RF heating has advantages in terms of large-scale processing, rapid and uniform heating of food materials, and provides a greater penetration depth (Boreddy et al., 2016; Boreddy and Subbiah, 2016; Chen et al., 2015, 2017; Lau et al., 2016; Lau and Subbiah, 2017; Piyasena et al., 2003). Recently, RF heating has been successfully applied for decontamination or disinfection of many low-moisture foods, such as wheat flour (Villa-Rojas et al., 2017; Wang et al., 2008), in-shell almond (Li et al., 2017) and walnut (Mitcham et al., 2004; Wang et al., 2003). Additionally, it has been shown that around 3 log CFU/g reduction can be achieved for whole black pepper after 50 s of RF heating (Kim et al., 2012).

According to FDA Food Safety Modernization Act, processors are required to validate their process controls to ensure food safety (Food and Drug Administration, 2015a). Since it is not often practical for the food industry to validate their process using pathogenic bacteria like *Salmonella*, it is necessary to find a nonpathogenic surrogate that can be inoculated onto the product and subjected to the actual process. *Enterococcus faecium* NRRL B-2354 has been evaluated as a suitable surrogate for

Salmonella spp. in many low-moisture food matrices, such as extrusion of carbohydrate-protein meal (Bianchini et al., 2014), extrusion of oat flour (Verma, 2017), RF heating of wheat flour (Villa-Rojas et al., 2017) and in some selections of low-moisture foods (Rachon et al., 2016).

The objectives of this study were to evaluate RF heating for inactivation of *Salmonella* spp. and *E. faecium* in whole black peppercorn and determine the effect of RF heating on the quality of whole black peppercorn based on color, total phenolics, antioxidant activity, total volatile compounds and piperine content.

3.2 Materials and Methods

3.2.1 Whole black peppercorn

Three batches of commercially steam sterilized whole black peppercorns were obtained from McCormick (Sparks, MD, USA) and stored in a freezer at -12°C to prevent the loss of volatile compounds. The background microflora of all three batches was quantified before conducting further experiments. Three random 10 g samples from each batch were diluted in 90 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD), plated on 3M™ Petrifilm™ (3M Microbiology, St Paul, MN) Aerobic Count Plates and incubated for 24 h at 37°C. The results showed < 10 CFU/g of aerobic bacteria on whole black peppercorn of all three batches.

3.2.2 Moisture and water activity determination

The moisture content of the samples was determined before and after RF heating. A Halogen Moisture Analyzer HR73 (Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) was used to measure the moisture content.

The water activity (a_w) of the samples was measured using a dew point water activity meter (Aqualab Series 4TE, Decagon Devices Inc., Pullman, WA, USA) at 25°C before treatment and only samples within ± 0.02 of the target a_w were used for RF heating treatments.

3.2.3 RF heating treatment

RF heating was conducted in a 6 kW, 27.12 MHz pilot-scale parallel-plate RF heating system (Model SO-6B, Monga Strayfield Pvt. Ltd., Pune, India). The electrode gap was adjusted by moving the top electrode which regulated the RF power. A total of 400 ± 0.1 g of inoculated sample was placed uniformly in a laminated paper tray (Fig. 1) acquired from Conagra Foods (Conagra Brands, Inc., Omaha, NE, USA) and placed inside the RF heater at the center of the bottom electrode. A small polyethylene bag (6 cm x 8 cm) was used to pack 20 g of inoculated sample and placed in the cold spot which was determined later in this study to represent the worst-case scenario; the remaining 380 g peppercorns in the tray represented an average of the whole sample. Subsequently, the tray was sealed by using Press'n Seal (The Glad Products Company, Oakland, CA, USA) to minimize heat and moisture loss from the surface and a vented nut was fixed onto the center of the film for controlled release of water vapor. An infrared camera (Thermal CAMTM SC-640, FLIR Systems, Inc., North Billerica, MA) with an accuracy of ± 2°C was used to obtain temperature profiles of the top surface of RF treated samples immediately after RF heating, after removing the film. Three fiber optic temperature probes (Neoptix, Inc., Quebec City, Quebec, Canada) with an accuracy of ± 0.6°C were inserted at the

geometric center of the top, middle and bottom layer to acquire temperature data during RF heating. The bottom layer was above the bottom of the tray at a distance of 1 cm and each layer had a 1 cm distance interval (Fig. 3.1).

Heat treatment of the inoculated samples was conducted using RF conditions that gave the fastest heating rate at an electrode gap of 10.5 cm to prevent too much quality deterioration. Because heating uniformity is important in the context of food safety, a heating uniformity index (λ) was used to evaluate the RF heating of whole black peppercorn, defined as the ratio of standard deviation to average temperature rise during heating obtained from surface thermal images, using the following equation (Wang et al., 2008):

$$\lambda = \frac{\sqrt{\sigma^2 - \sigma_0^2}}{\mu - \mu_0}$$

where μ and μ_0 are final and initial average sample top surface temperatures (°C), and σ and σ_0 are final and initial standard deviation of sample top surface temperatures.

During RF heating, moisture will evaporate from the sample due to heat. This loss of moisture will be undesirable in a food production setting due to a lower final product weight. In order to offset this moisture content loss, whole black peppercorn samples were adjusted to a higher water activity and moisture content before RF heating. However, according to the American Spice Trade Association (American Spice Trade Association, 2011), the U.S. guideline for black pepper moisture content is a maximum value of 12% (wb). Therefore, the moisture content of the black pepper was adjusted to higher than the maximum moisture content, after RF heating which would then bring the sample moisture content to the required level. Due to the

different RF heating times, the initial moisture content and water activity was determined by preliminary trial-and-error method.

3.2.4 Microbial challenge studies of RF heating

3.2.4.1 Bacteria strains

Five different strains of *Salmonella enterica* were selected to conduct the microbiological studies, namely, *Salmonella* Agona 447967; *Salmonella* Enteritidis PT30; *Salmonella* Tennessee K4643; *Salmonella* Montevideo 488275; *Salmonella* Mbandaka 698538. *Enterococcus faecium* NRRL B2354 was selected as the non-pathogenic surrogate for validations. The *Salmonella* strains were chosen because of their association to low moisture food outbreaks or high resistance to thermal inactivation. *Salmonella* Agona 447967, *Salmonella* Montevideo 488275 and *Salmonella* Mbandaka 698538 were obtained from FDA, ORA Regional Lab in Jefferson, AR. They were associated with foodborne outbreaks caused by roasted oats cereal (Centers for Disease Control, 1998), black and red pepper (Centers for Disease Control, 2010), and sprouts (Jackson et al., 2013), respectively. *Salmonella* Enteritidis PT 30 was related to a raw almond recall (Centers for Disease Control, 2004), while *Salmonella* Tennessee K4643 was associated with a peanut butter recall (Centers for Disease Control, 2007). Both of them were obtained from the University of Georgia, Athens. *Enterococcus faecium* NRRL B2354 which has been identified as a non-pathogenic surrogate for *Salmonella* in almond pasteurization processes (Jeong et al., 2011) was obtained from the USDA, ARS (Peoria, IL). All the bacteria were kept in a stock solution of 40% sterile glycerol stored at -80°C until used. Working stock plates for each bacterium was prepared from the frozen stock and were stored at 4°C.

3.2.4.2 Inoculum preparation and inoculation

For each bacterial strain, a loopful (~10 μL) of one isolated colony was taken from the working stock plate stored at 4°C, transferred into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (YE; Becton, Dickinson and Company, Sparks, MD), then incubated for 24 h at 37°C. After inoculation, 100 μL of the overnight broth culture was spread plated onto a labeled tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (TSAYE) plate and incubated upside down at 37°C for 24 h. Finally, the lawns grown on the plate were harvested by adding 3 mL of 0.1% BPW to the plate and agitating the cells into a suspension with a sterile L-shaped spreader. This process was repeated for each strain. To prepare the *Salmonella* cocktail, 2 mL inoculum of each serovar was aseptically pipetted into a separate sterile conical tube and vortexed for 30 s to ensure uniform distribution of cells. The initial bacterial population of the *Salmonella* cocktail was ca. 10^{10} CFU/mL and *E. faecium* was ca. 10^{9} CFU/mL, respectively.

Whole black peppercorn samples were taken out of the freezer and placed at room temperature one day before the inoculation. Whole black peppercorn samples were aseptically transferred in amounts of 1 kg to a sterile Whirl-Pak style bag and 10 ml of inoculum was sprayed onto the sample inside a biosafety cabinet. Then, the bag was sealed and shaken by hand for 10 min to mix properly. The inoculated samples were aseptically transferred to an equilibration chamber to reach a target water activity. The equilibration chamber had a custom-designed humidity control system. The humidity control system could control relative humidity within ± 0.3% of the set

point, and it consisted of a humidity sensor (AM2303, Aosong Electronics Co., Ltd., Guangzhou, China), a fan for circling air within the chamber, solenoid valves, an air pump (Fusion 700, JW Pet, Teterboro, NJ), a wet column consisting of water and humidifier wicks, a dry column filled with silica beads (640SGO55, Sorbent Systems, Los Angeles, CA), and a microcontroller which could acquire humidity reading from the humidity sensor and switch the appropriate solenoid valves to pump air through the wet or dry column.

3.2.4.3 Stability and homogeneity tests

Upon inoculation, the bacteria require some time to adjust to the low a_w environment and antimicrobial compounds in the whole black peppercorn. In order to measure the time required for the bacteria to reach a stable status and evaluate the homogeneity of inoculation, stability and homogeneity tests were conducted by microbial enumeration of the sample every day for one week. Three 3-g samples were randomly taken from the chamber and placed in Whirl-Pak bags with 27 mL of 0.1% BPW, stomached for 1 min, and then serially diluted in 9 mL 0.1% BPW. Three dilutions of both inoculated samples were duplicate-plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (SIGMA-ALDRICH, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) (mTSA) for *Salmonella* and TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (ACROS, NJ, USA) (eTSA) for *E. faecium*, and incubated for 24 h at 37°C. During enumeration of the plates, colonies with a black center were counted as *Salmonella,* while black colonies were counted as *E. faecium*. During the

week of homogeneity and stability tests, both moisture content and water activity were also measured every day in three different sample, as described in the following section.

3.2.4.4 RF inactivation of *Salmonella* spp. and *Enterococcus faecium*

Before RF heating, the water activity of the samples was measured and only samples within ± 0.02 of the target a_w were used for RF inactivation treatments. Samples were packed and treated by RF heating, as described previously. Immediately after the RF treatment, a thermal image of the top surface was taken by the infrared camera to confirm that the sample achieved the expected temperature before transferring the sample to a sterile bag. The bag was sealed and placed into an ice-water bath for 10 min to cool the sample and prevent further inactivation during cooling period. This represents a conservative estimate of microbial log reduction. In a real industrial setting, the product may not be cooled off rapidly, which would result in higher log reduction and provides an additional factor of food safety.

To enumerate surviving colonies after RF treatment, the sample in the small bag (~20 g) was transferred to a sterile Whirl-Pak bag and the remaining sample was blended and 20 ± 0.1 g of the blended sample was transferred to another Whirl-Pak bag. These samples were first tenfold diluted by using Smart Dilutor W (IUL,S.A., Barcelona, Spain) and then stomached for 1 min in a stomacher (Neutec Group Inc, NY, USA). Stomached samples were serially diluted in 9 mL 0.1% BPW and four dilutions of all samples were duplicate-plated onto mTSA for *Salmonella* and eTSA for *E. faecium* and incubated for 24 h at 37°C. Three biological replicates (batches of

whole black peppercorn from different production lots inoculated with independently frozen stock) and two technical replicates (RF treatments the each biological replicate) were used in the experiments. Two untreated inoculated samples were enumerated as the control and used to obtain the reduction fraction (log R) by subtracting the log count at the end of the treatment (log N) from the log of the initial population count in the control (log N_0).

3.2.5 Quality analysis

The uninoculated samples was treated by using the same RF heating time which provided a more than 5 log reduction of *Salmonella*, and then cool down in the room temperature to evaluate the worse-case scenario of quality deterioration. Then, the samples were grinded by Waring Commercial Spice Grinder (WSG60, Conair Corporation, CT, USA) and passed through U.S. No. 20 sieve (0.841 mm sieve opening) to achieve a consistent particle size for quality analysis. The 0.4 g of ground samples was mixed with 100 mL of ethanol (Decon Labs, Inc., 200 proof) and magnetically stirred overnight. The solution was filtered, and the ethanol extract was stored at 4°C under dark condition until analysis. These extracts were used for determination of piperine, total phenolics, and antioxidant activity of the samples. All samples were analyzed in triplicate.

3.2.5.1 Color

The color of the ground black pepper sample was measured using a colorimeter (Model BC-10, Minolta Co. Ltd., Osaka, Japan) and the color values of L* (lightness and darkness), a* (redness and greenness), and b* (yellowness and blueness) were recorded. The instrument was calibrated each time by placing the flat tip of the

colorimeter over a white calibration plate. The sample was spread into a plastic container, and the top surface was flattened. The color of the sample was measured at five random locations. The total color difference (ΔE) is a useful tool for detecting differences in human perception of color and has been used to evaluate the color change of some thermally processed foods such as RF treated egg white powder (Boreddy et al., 2016) and steam treated canned food (Ramaswamy et al., 1993). The ΔE with reference the control sample was calculated for each treatment using the following formula (Robertson, 1977):

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

3.2.5.2 Piperine content

The piperine content in black pepper ethanol extracts were determined by an Agilent 1100 Series High Performance Liquid Chromatograph equipped with variable wavelength detector. An Eclipse Plus C18 (4.6 x 100 mm, 3.5 µm) column was used in the stationary phase. Isocratic elution was performed with mobile phase of 50% of methanol and 50% water mixture. The flow rate was 0.7 mL/min and detection wavelength was at 341 nm. The samples were diluted 1:1 with ethanol before injection into the HPLC. The 10 µL portion of black pepper ethanol extract was injected into the column using an automatic injector. Standard piperine (Alfa Aesar, 98% purity) solutions were prepared in ethanol in a range of 25-400 µg/mL to generate the standard curve.

3.2.5.3 Total volatile compounds

The volatiles compositions of the ground black pepper samples were determined by a Thermoscientific headspace gas chromatography equipped with ISQ Mass

Selective Detector (GC-MS). For the measurement, 1 g black pepper was incubated for 20 min at 75°C agitator temperature. Then, the 0.7 mL of gas mixture released from black pepper during incubation was injected into GC-MS with 1:80 split ratio. The GC column used was a TG-5MS 30 m x 0.25 mm ID x 0.25 dF capillary column. The oven temperature used was as follows: from 40°C to 290°C with 30°C/min heating rate and maintained at this temperature for 2 min. The ionization energy was 70 eV and mass range was 10-650 amu. The compositions of the samples were identified by NIST 11 mass spectral library.

3.2.5.4 Total phenolics

The total phenolic content of the black pepper ethanol extract was determined by using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). In this method, 1 mL of the ethanol extract and standard solutions of gallic acid (3, 4, 5, 6, 7 µg/mL) were used in the assay and ethanol was used as blank. Two milliliters of Folin-Ciocalteu's phenol reagent was added to each sample tube and vortexed for 5 s. After 10 min, 2 mL of Na_2CO_3 was added to the mixture and vortexed for 5 s. After incubation for 2 hours at room temperature in the dark, the absorbance against the prepared blank was measured at 765 nm using a UV-1800 Shimadzu spectrophotometer (Shimadzu Corp., Kyoto, Japan).

3.2.5.5 DPPH radical-scavenging assay

1,1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is commonly used to determine antioxidant activity (Bersuder et al., 1998). Briefly, a volume of 1 mL DPPH (40 µg/mL) was mixed with 0, 2.0, 2.5, 3.0, 3.5, 4.0 mL of black pepper ethanol extract in test tubes and ethanol was added to make up a 5 mL solution in all

the tubes. Then, the mixtures were shaken vigorously and incubated at room temperature in darkness for 30 min. The absorbance of the remaining DPPH radicals was read at 517 nm using the spectrophotometer. The scavenging activity of the DPPH radical in each sample was calculated according to the following equation (Bersuder et al., 1998):

DPPH Radical - scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}] X 100$

Where $A_{control}$ and A_{sample} are the absorbances of the control and the sample. The values are presented as the average of triplicate analysis.

3.3 Results

3.3.1 Determination of the RF heating pattern

The RF heating times were determined to be 120, 150 and 180 s which give a final average surface temperature around 80, 95 and 105°C, respectively. From preliminary trial-and-error experiments, an initial water activity and moisture content of 0.60 ± 0.02 and 12.7 ± 0.1% (wb) would result in a final water activity and moisture content of 0.57 ± 0.01 and 11.8 ± 0.1%, respectively after 150 s of RF heating.

3.3.2 Determination of the cold spot

Comparison of temperature histories of different layers during RF heating is shown in Figure 3.2. The temperature of the bottom layer increased faster than the other layers and the top layer was the slowest. Also from the Figure 3.3, the temperature profile shows that the central area heated up slower than the peripheral area, with the hot spots located around the edge. The cold spot of RF treated whole black peppercorn was located at the center of the top surface and the small

polyethylene bag was placed in the center and slightly below the top surface to evaluate the worst-case scenario of RF heating.

3.3.3 Stability test

After the inoculation, stability and homogeneity tests of both microorganisms were conducted for seven days to evaluate the effect of low a_w environment on these two bacteria. A total of 1.3 log CFU/g reduction was seen in the samples inoculated with *Salmonella* after a week of equilibration (Fig. 3.4). Most of the reduction occurred one day after inoculation, ca. 0.8 log CFU/g reduction was achieved and less than 0.5 log CFU/g reduction was found during the rest of the week (Fig. 3.4). As for *E. faecium*, a relatively stable survival curve was observed: less than 0.3 log CFU/g reduction was observed after one week of equilibration (Fig. 3.5). In Figure 3.6 and Figure 3.7, water activity of the sample reached a stable status one day after the inoculation while the sample moisture content took two days to reach equilibration. According to the results of stability test, both microorganisms showed a good adaptive ability to the environment of black peppercorn and low a_w condition. High initial microbial populations of both microorganisms were achieved and the fifth day after inoculation was chosen for conducting RF heating inactivation studies.

3.3.4 RF inactivation

The inoculated whole black peppercorn samples reached an average surface temperature of 81.1°C, 96.5°C and 107.3°C after RF treatment for 2, 2.5 and 3 min, respectively (Table 3.1). The longer treatment times had a lower heating uniformity index which indicated a better heating uniformity.

The survival curve of *Salmonella* spp. during RF heating treatment is shown in

Figure 3.8 Survival of *Salmonella* decreased with increasing treatment time and a higher population of *Salmonella* survived in the bag than in the whole sample. After 2 min of RF heating, a reduction of 2.94 log CFU/g was achieved for the whole sample while only 1.08 log CFU/g reduction was obtained for the sample in the bag, indicating a significant difference ($P < 0.05$). Upon applying a 2.5 min RF heating treatment to the inoculated sample, the levels of *Salmonella* were reduced by 5.31 and 5.08 log CFU/g in the whole sample and sample in the bag, respectively, resulting in an insignificant difference ($P > 0.05$). Extending the RF heating to 3 min resulted in no recovery from the plates (< 10 CFU/g), suggesting that more than 6 log reduction was obtained. Therefore, the RF heating treatment of 2.5 min was chosen for the studies with the surrogate.

The population of *E. faecium* was reduced by 5.26 and 4.80 log CFU/g at 2.5 min of treatment time in the whole sample and the sample in the bag, respectively. Figure 3.9 compares the log reduction of *Salmonella* and *E. faecium* after 2.5 min of RF heating treatment, where less log reduction was attained for *E. faecium* than *Salmonella* in both whole sample and sample in the bag. The log reduction obtained for E. *faecium* and *Salmonella* showed no significant difference ($P > 0.05$) for the whole sample while samples in the bag for these two microorganisms showed a significant difference ($P < 0.05$).

3.3.5 Quality analysis

The quality analysis was done with samples treated by RF for 2.5 min. The moisture, color and flavor components (total phenolics, piperine and total volatile

compounds) of black peppercorn samples before and after RF heating treatment are summarized in Tables 3.2 and 3.3. Both moisture content and water activity were significantly reduced after the RF heating treatment ($P < 0.05$) and moisture content dropped to less than 12% (wb). L*, a*, and b* values of RF treated samples were not significantly ($P > 0.05$) different from those of untreated samples. The color difference (ΔE) between the control and treated samples had a value of 1.5 which represents "very small difference, only obvious to a trained eye "(Hunterlab, 1976). There were also no significant ($P > 0.05$) differences for total phenolics and piperine, between untreated and treated whole black peppercorn.

The total volatile compounds of whole black peppercorn that were RF treated and control are shown in Table 3.3. In the BLACK PEPPER GAS MIXTURE, 29 compounds were identified, the major components (> 5% peak area) were β-Ocimene (16.46%), followed by Caryophyllene (11.70%), D-Limonene (11.59%), β-Phellandrene (9.40%), β-Pinene (9.10%), Camphene (8.43%), α-Phellandrene (6.86%), Sabinene (5.29%), 3-Carene (5.17%) and α-Pinene (5.07%). Therefore, the composition showed the presence of 10 major compounds accounting for 89.07% of the total amount. All the compounds detected in the untreated peppercorns were found in the RF treated sample also, and RF treatment did not produce any detectable new component. Among all the volatile compounds, 10 compounds showed significantly different changes after RF treatment, and only half of them belonged to the major compounds (Fig. 3.10; Table 3.3).

Figure 3.11 shows the DPPH radical scavenging activity of control and RF

treated whole black peppercorn sample. It was concluded from Figure 3.11 that the scavenging activity of both samples was concentration dependent. The result indicated that control samples were not significantly different from the RF treated sample. At a final concentration of 3.2mg/mL, both control and RF treated samples showed a high antioxidant activity of 61.37% and 58.51%, respectively

3.4. Discussion

Some studies have reported the effect of RF heating treatment for reducing microorganisms in whole black peppercorn (Kim et al., 2012) and ground black pepper (Jeong and Kang, 2014). In whole black peppercorn, it was reported that 50 s of RF treatment greatly reduced the levels of *S.* Typhimurium by 3.18 log CFU/g (Kim et al., 2012). However, no studies have evaluated the effect of inoculation and equilibration on black pepper before RF treatment. For inactivation studies, it is important to consider the methods used for inoculation and equilibration before processing (Bowman et al., 2015). Liquid inoculum sprayed onto a dry environment of black pepper could cause a shock to the microorganism and the release of water-soluble antimicrobials from black pepper, which may artificially reduce microbial numbers before the RF heating. From the results of stability test, although *E.faecium* maintained a stable population level, concentration of *Salmonella* was significantly reduced one day after inoculation.

When exposed to different stressors and processing, the survival of *Salmonella* would be improved if the sample was previously exposed to a low a_w environment (Bowman et al., 2015). When exposed to desiccated environment, *S. enterica*

serotypes Enteritidis, Hadar, Infantis, and Typhimurium exhibited enhanced tolerance to chemical disinfectants, dry heat, and UV irradiation (Gruzdev et al., 2011). In this study, the equilibration process after sample inoculation simulated the industry process and allowed microorganisms to adjust to the dry environment and become heat resistance.

Although RF heating has been evaluated as a heat treatment to control pathogens in many low a_w foods (Jeong and Kang, 2014; Li et al., 2017; Mitcham et al., 2004; Rachon et al., 2016; Villa-Rojas et al., 2017), the non-uniform temperature distribution is still a major challenge for this technology (Jiao et al., 2014). Due to the non-uniform heating, some parts of the food sample are overheated, while others may be still cold which poses a threat to the inactivation process. Therefore, it is necessary to determine the cold spot during RF heating and evaluate the pasteurization result specifically for the cold spot. In this study, the cold spot was identified to be at the center of the top surface. Therefore, a small inoculated-pack sample was placed at the cold spot to evaluate the worst-case scenario of RF heating process. From the result of 2 min RF heating inactivation, the packed sample showed a significantly lower log reduction of *Salmonella* than the whole sample which suggested that the cold spot identified using fiber optic sensors and thermal imaging camera was accurate. However, the heating uniformity improved upon increasing treatment time, resulting in no significant difference in log reduction of *Salmonella* between the packed sample and whole sample when the heating time increased to 2.5 min or beyond.

RF heating is a complex physical process and can be affected by the sample size,

salt content and moisture content (Tang et al., 2005). Moisture content significantly affects RF heating and microorganism inactivation of black peppercorn sample. Therefore, it is important to control the moisture content of the whole black peppercorn sample before RF treatment. RF heating can be effectively used not only to control pathogens but also to reduce moisture levels in black pepper (Jeong and Kang, 2014). However, it is not feasible to adjust the moisture content to optimize the RF heating inactivation for industrial applications without considering the impact on its quality. From experiment, the moisture content of black peppercorn after RF heating dropped below the U.S. guideline with a maximum of 12% (wb) to ensure the food quality. High moisture content could potentially provide a suitable environment for the growth of mold and yeast during the storage. In this study, the moisture content of black pepper was adjusted to 12.7% (wb) before RF heating. After the 2.5 min RF heating treatment, it dropped back to 11.8% (wb), meeting the U.S. guideline of less than 12% (wb) moisture for black pepper.

The 2.5 min of RF heating provided an average of 5.31 log CFU/g reduction of *Salmonella* for whole black peppercorn which meets the 5-log pathogen reduction performance standard (Food and Drug Administration, 2015b). While 2 min RF heating did not provide enough inactivation, and 3 min RF treatment provided a ca. 6.8 log CFU/g reduction. It reported that an average log reduction of 6.10 ± 0.64 log CFU/g was observed for *Salmonella* PT 30 after 1 min of vacuum steam treatment of whole black pepper at 75°C (Shah et al., 2017). When applying a 50 s RF heating, level of *S.* Typhimurium was reduced by 3.18 log CFU/g in whole black pepper with

an average final temperature of 60°C (Kim et al., 2012). Compared to those two studies, the *Salmonella* spp.in this RF heating inactivation studies showed a higher heat resistance which could be caused by the inoculation and equilibration procedure or the use of five *Salmonella* cocktail instead of one single strain. Compared to the ozone treatment which provided a 4 log CFU/g reduction for 10 min treatment (Zhao and Cranston, 1995) and a remote plasma treatment that achieved a 4.1 log CFU/g reduction after 30 min (Hertwig et al., 2015), the RF heating treatment investigated here provided higher decontamination ability in a shorter treatment time.

E. faecium has been widely used as a surrogate for *Salmonella* as it has similar thermal resistance in many food matrices and its safety for use in processing facilities has been evaluated (Kopit et al., 2014)**.** However, it is important to evaluate the applicability of using *E. faecium* as a surrogate for *Salmonella* in whole black peppercorn for RF heating treatment because it may behave differently in different food matrices and for different processes. In this study, *E. faecium* showed a higher survival than *Salmonella* at all RF heating treatments. Thus, *E. faecium* is a good surrogate for *Salmonella* spp. for RF treatment in whole black peppercorn as it would indicate *Salmonella* inactivation with a margin of safety.

The quality of black pepper is greatly determined by moisture content, color, volatiles, and piperine content. The color of black pepper was evaluated using Hunter's color values and the calculated color difference (ΔE) indicated a non-significant color change during the RF heating treatment. Piperine represents the major component of black pepper and has been reported to have an anti-inflammatory

and an analgesic effect (Gupta et al., 2000). Also, black pepper is recognized as a source of natural antioxidants. Thus, total phenolics and antioxidant activity were evaluated. The DPPH is a stable free radical and is one of the most commonly used substrates to evaluate antioxidant activity in foods. The volatile compounds in black peppercorn has been shown to have antimicrobial activity and therefore was also assessed. Five major volatile compounds out of ten major volatile compounds (> 5% peak area) showed significant difference after RF heating. For those major compounds that experienced significant changes, except D-Limonene, RF heating caused on change or a noticeable increase in their proportions in the total volatiles which was also observed during the ozone treatment of whole black peppercorn (Zhao and Cranston, 1995). D-Limonene was the only major compound which experienced a significant decrease after RF heating. D-Limonene is known as a pepper terpenoid and is considered to have fairly low toxicity (Sun, 2007). Although it is one of the major compounds in black pepper volatiles, it has not been reported to contribute to the flavor for black pepper. Sabinene and terpinene were reported to be the most important volatile compounds which contribute to the characteristic odor of black pepper (Pino et al., 1990).

This study showed color parameters (L*, a*, b*), piperine content, total phenolics and scavenging activity were not affected by 2.5 min of RF heating as those values were not significantly different from the control samples. RF heating only caused slight changes to the volatile constituents, while effect to the flavor of black pepper was negligible. Also, the moisture content and water activity dropped as expected

after RF heating.

In conclusion, RF heating is a promising technology for inactivation of *Salmonella* spp. in whole black peppercorn. A 2.5-min RF heating treatment provides more than 5 log reduction for *Salmonella* spp. without causing significantly quality deterioration. Also, heating the whole black peppercorn for 2.5 min showed that the level of reduction for *E. faecium* was always lower than *Salmonella* spp. reduction. Therefore, *E. faecium* was found to be a good surrogate for *Salmonella* spp. for RF heating of whole black peppercorn.

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Table 3.1. Temperature profile of the top surface of RF heated whole black peppercorn for different treatment times.

Matrices on top surface	Treatment time (min)		
	2.0	**2.5**	**3**
Average Temperature (°C)	81.1 ± 4.5	96.5 ± 1.5	107.3 ± 0.8
Maximum Temperature (°C)	116.1 ± 3.5	134.2 ± 2.5	148.4 ± 2.2
Minimum Temperature (°C)	60.3 ± 0.5	74.8 ± 2.0	76.8 ± 0.4
Heating uniformity index (λ)	0.135 ± 0.006	0.091 ± 0.007	0.092 ± 0.002

Table 3.2. Moisture content, water activity, color parameters, total phenolics and piperine content of untreated and treated black pepper samples subjected to RF.

Parameter	Control	RF treated
Moisture content (%)	12.7 ± 0.1 a	11.8 ± 0.1 b
Water activity	0.60 ± 0.02 a	0.57 ± 0.01 b
Color (L*)	53.6 ± 0.7 a	52.4 ± 0.4 a
Color (a*)	2.9 ± 0.8 a	2.6 ± 0.1 a
Color (b*)	10.8 ± 0.6 a	9.9 ± 0.6 a
Color difference (ΔE)	0	1.5
Total phenolics (mg/g)	22.5 ± 0.9 a	21.2 ± 0.7 a
Piperine (ug/g)	33.9 ± 0.3 a	32.8 ± 1.0 a

[1] Means ± standard deviations from three replications. Values followed by different letters within the row are significantly different ($P < 0.05$).

Table 3.3. Total volatile compounds of untreated and treated black pepper samples subjected to RF heating.

Compound	Area (%)	
	Control	RF treated (% difference)
α-Thujene	1.55 ± 0.02 a	1.08 ± 0.18 b (- 30.32%)
α-Pinene	5.07 ± 0.15 a	5.74 ± 0.23 b (+ 13.21%)
Camphene	1.76 ± 0.02 a	1.28 ± 0.14 b (- 27.27%)
Sabinene	5.29 ± 0.09 a	6.13 ± 0.39 b (+ 15.88%)
β-Pinene	9.10 ± 0.12 a	6.95 ± 1.98 a
α-Phellandrene	6.86 ± 0.22 a	7.61 ± 0.68 a
β-Mycene/4(10)-Thujene	1.15 ± 0.05 a	1.16 ± 0.06 a
β-Phellandrene	9.40 ± 0.17 a	7.24 ± 1.91 a
Camphene, (1R, 4S)	8.43 ± 0.04 a	9.07 ± 0.62 a
γ-Terpinene	0.90 ± 0.08 a	1.26 ± 0.33 a
3-Carene	5.17 ± 0.38 a	7.24 ± 0.66 b (+ 40.04%)
2-Carene	1.03 ± 0.01 a	1.47 ± 0.38 a
β-Ocimene	16.46 ± 0.68 a	17.47 ± 0.32 b (+ 6.14%)
D-Limonene	11.59 ± 0.04 a	10.29 ± 0.56 b (- 11.22%)
p-Menth-3-en-1-ol	0.51 ± 0.04 a	0.38 ± 0.04 b (- 25.49%)
Terpineol, cis-β-	0.4 ± 0.02 a	0.33 ± 0.04 b (- 17.50%)
p-Mentha-1,4(8)-diene	0.23 ± 0.09 a	0.16 ± 0.05 a
β-Linalool	0.24 ± 0.01 a	0.31 ± 0.13 a
p-Menth-3-ene, 2-isopropenyl-1-vinyl-, (1S, 2R)	0.48 ± 0.03 a	0.69 ± 0.28 a
β-Copaene	0.06 ± 0.02 a	0.08 ± 0.03 a
α-Copaene	0.81 ± 0.08 a	1.14 ± 0.59 a
β-Elemene	0.19 ± 0.03 a	0.29 ± 0.17 a
Caryophyllene	11.70 ± 0.99 a	10.98 ± 1.96 a
Humulene	0.48 ± 0.07 a	0.66 ± 0.41 a
trans-α-bargamotene	0.28 ± 0.04 a	0.18 ± 0.04 b (- 35.71%)
δ-Cadinene	0.08 ± 0.01 a	0.13 ± 0.07 a
Isocaryophyllene	0.41 ± 0.06 a	0.37 ± 0.13 a
(Z,E)-α-Farnesene	0.30 ± 0.04 a	0.26 ± 0.10 a
Caryophyllene oxide	0.06 ± 0.01 a	0.06 ± 0.02 a

[1] Means ± standard deviations from three replications. Values followed by different letters within the row are significantly different ($P < 0.05$).

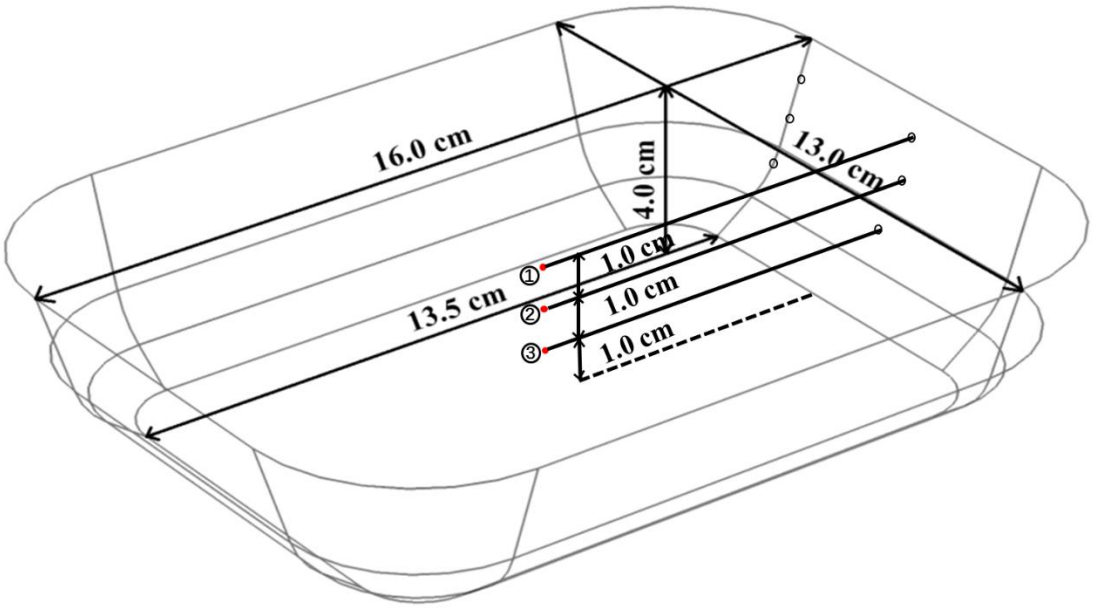

Figure 3.1. Dimensions for the laminated paper tray.

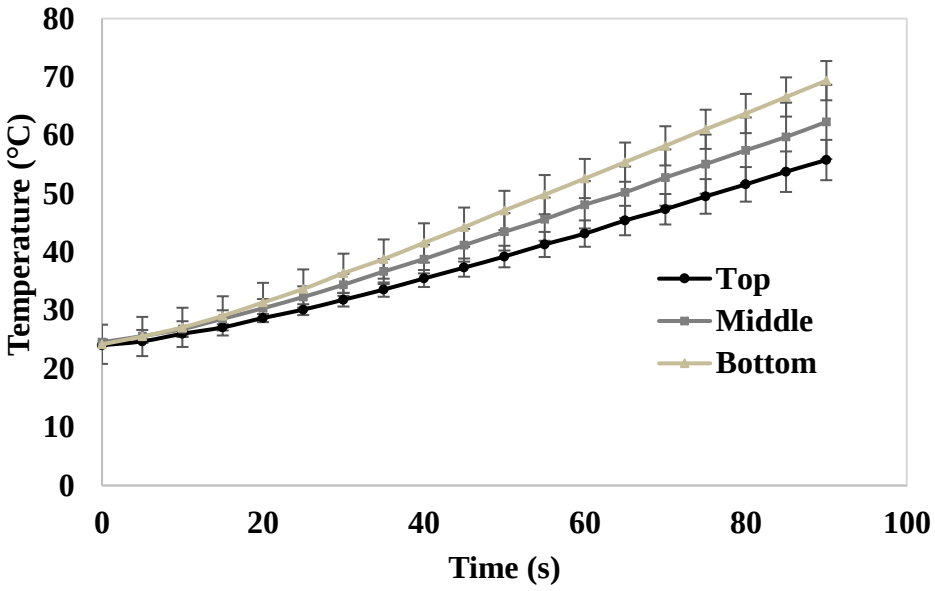

Figure 3.2. Comparison of temperature histories of different layers during RF heating.

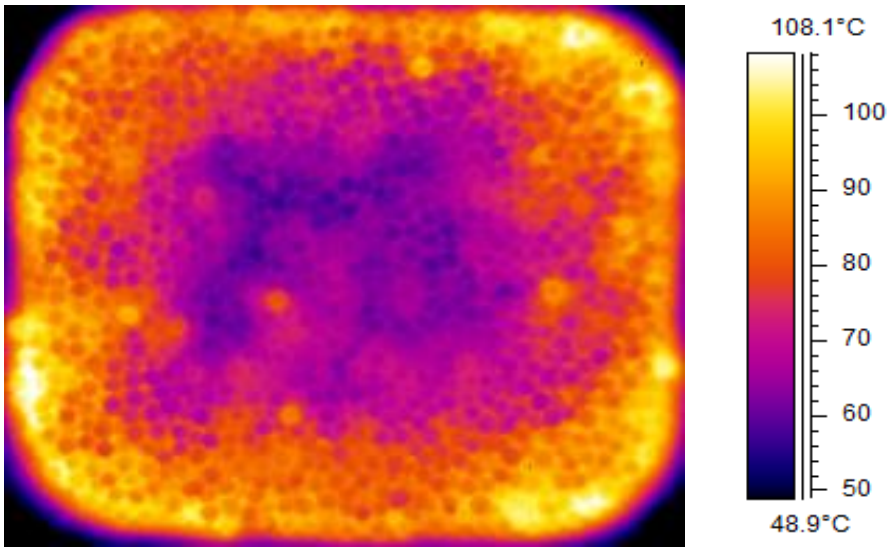

Figure 3.3. Top surface temperature profile of 2-min RF treated whole black peppercorn.

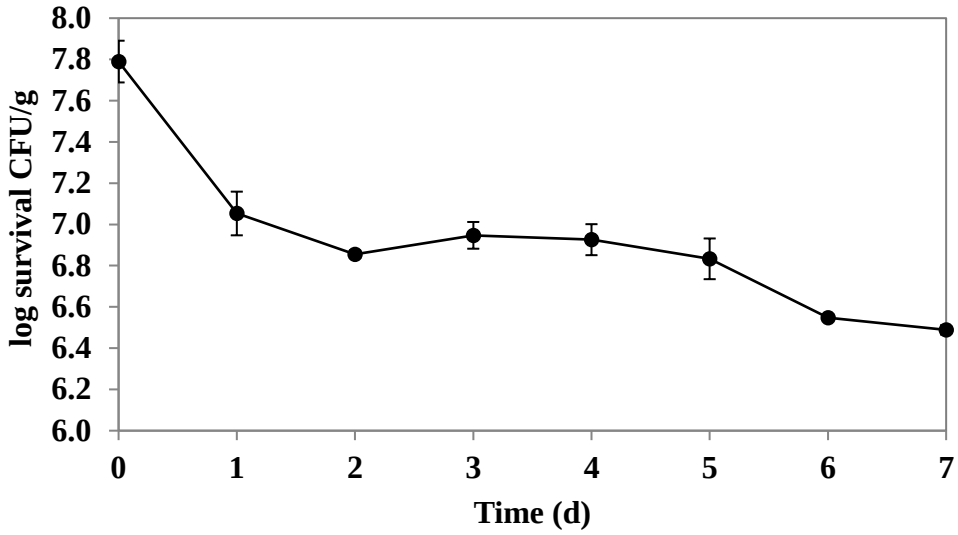

Figure 3.4. Stability test of *Salmonella* spp. survival for 7 days in equilibration chamber.

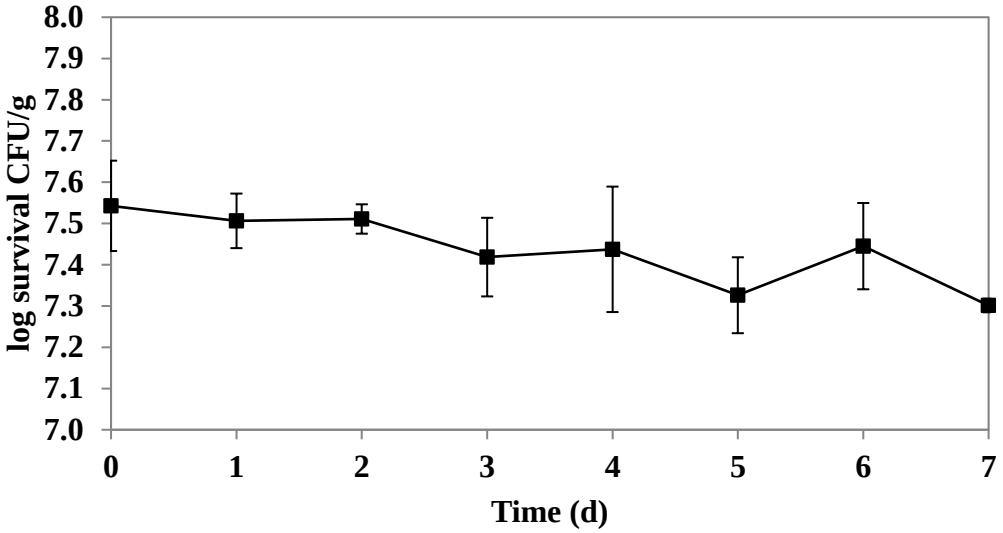

Figure 3.5. Stability test of *E. faecium* survival for 7 days in equilibration chamber.

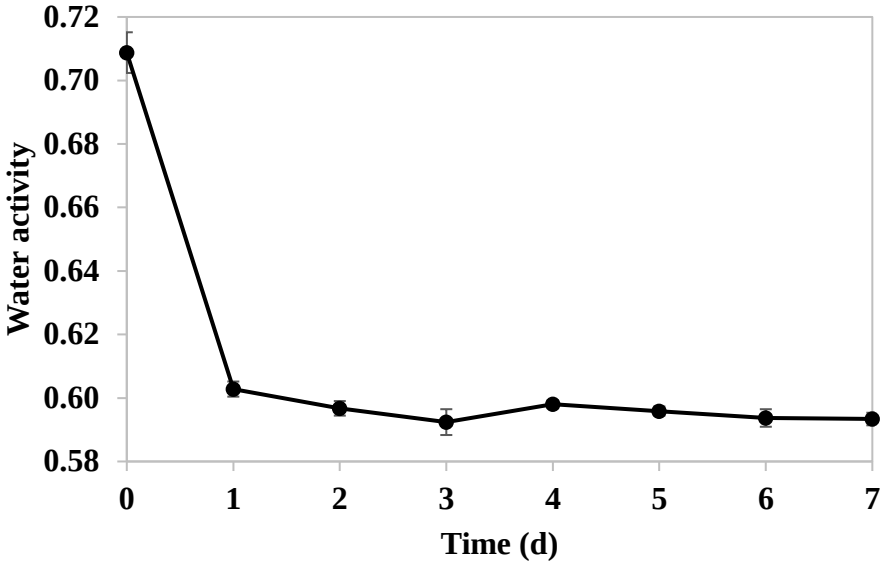

Figure 3.6. Stability test of water activity for 7 days in equilibration chamber.

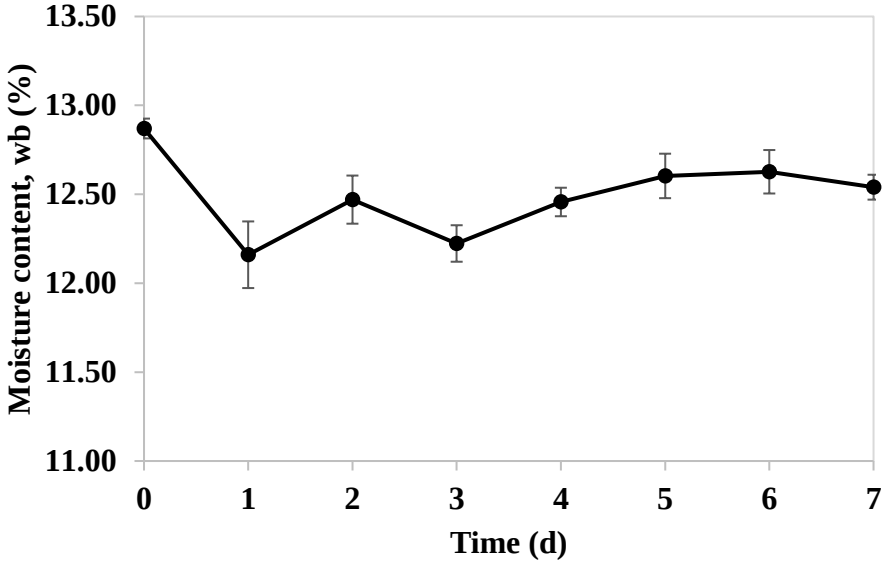

Figure 3.7. Stability test of moisture content for 7 days in equilibration chamber.

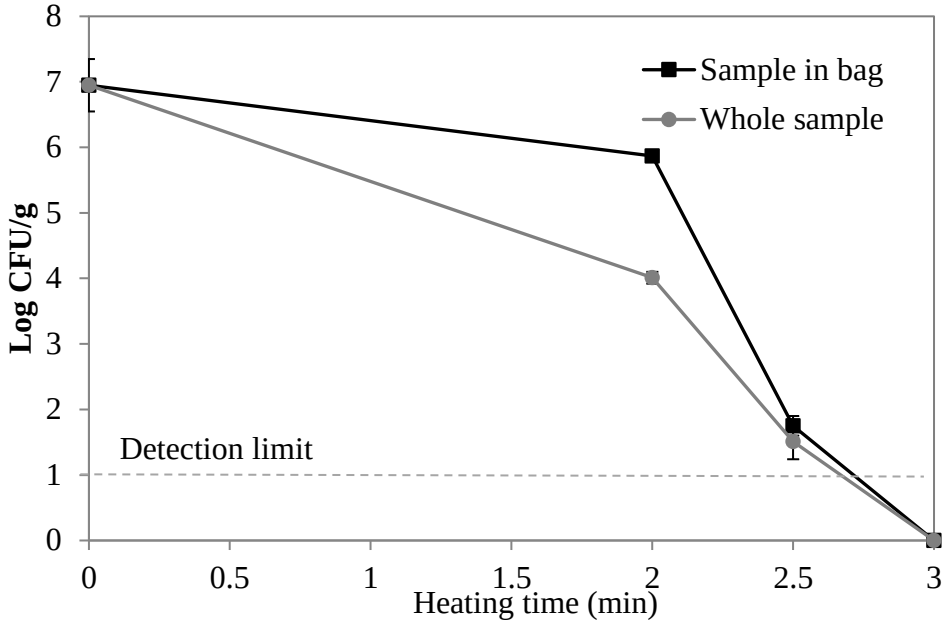

Figure 3.8. Survival curve for *Salmonella* spp. during RF heating. Error bars indicate the ± 1 standard deviation between the biological replications

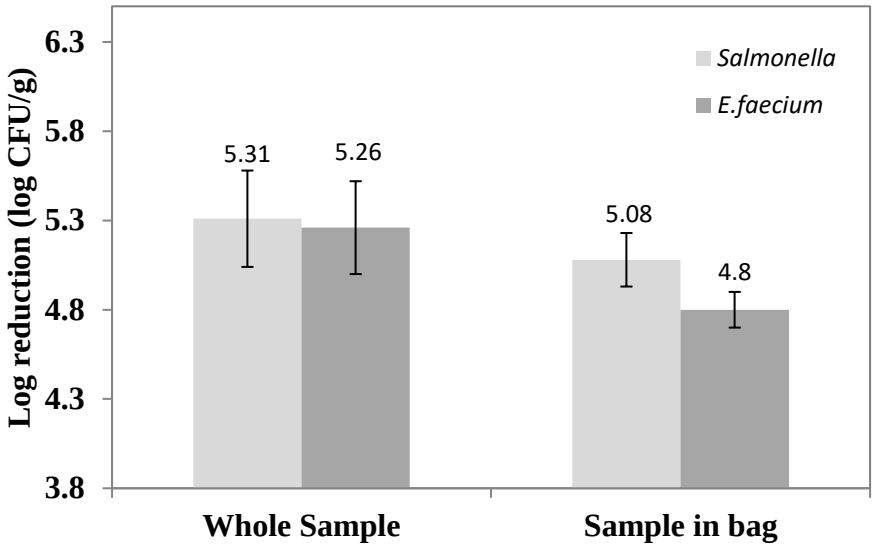

Figure 3.9. Comparison of the log reduction between *Salmonella* spp. and *E. faecium* at 2.5 min of RF heating in whole black peppercorn. Error bars indicate the ± 1 standard deviation between the biological replications

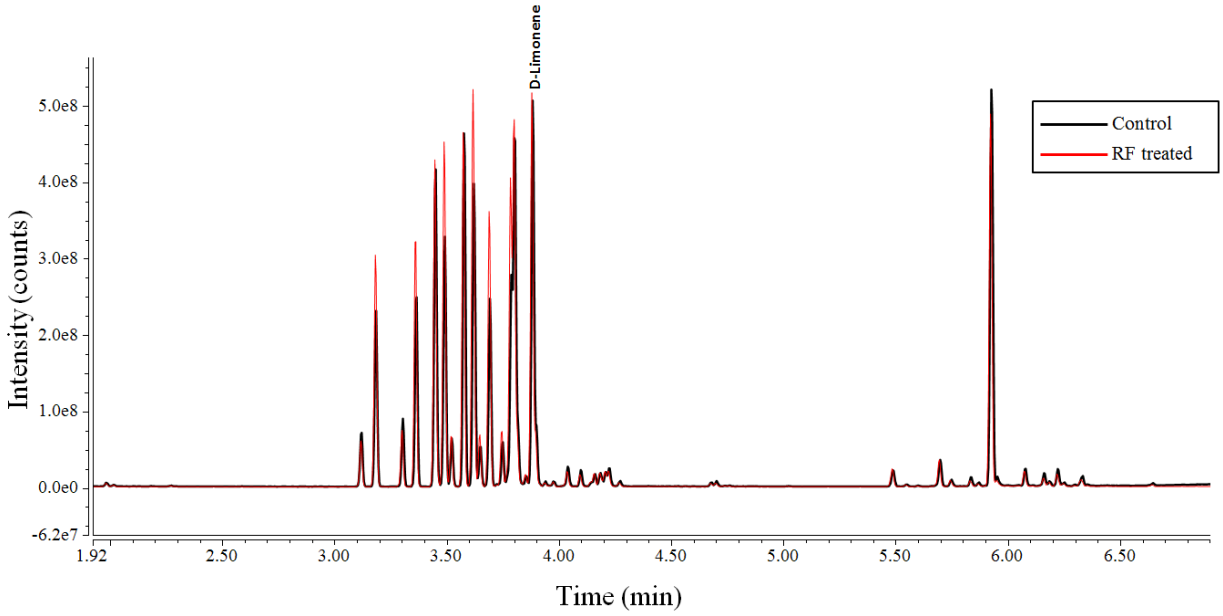

Figure 3.10. GC chromatograms of black peppercorn volatile compounds. Labeled peaks represent major compounds experienced significantly decrease during RF heating.

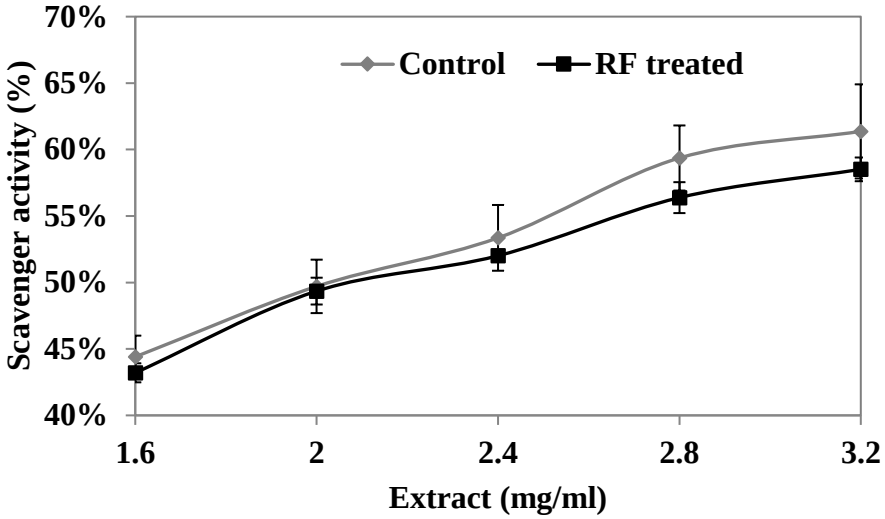

Figure 3.11. Antioxidant activities of untreated and treated black peppercorn samples subjected to RF heating.

Chapter IV. Radiofrequency pasteurization process for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 on ground black pepper

Abstract

Salmonella persistence in ground black pepper has caused several foodborne outbreaks and created public concern about the safety of low water activity ($a_{w)}$ foods. In this study, radiofrequency (RF) processing was evaluated for pasteurization of ground black pepper. Stability and homogeneity tests were done for both *Salmonella* spp.and *E. faecium* during moisture equilibration before RF heating to evaluate the inoculation method. Moisture content and a_w measurements of ground black pepper determined after RF heating indicated that the ground black pepper was dried by the process to the optimal storage moisture. RF heating was shown to provide more than 5.98 log CFU/g reduction of *Salmonella* spp. and the reduction of 3.89 log CFU/g for *E. faecium* with a 130 s treatment time. The higher thermal resistance of *E. faecium* indicated its suitability as surrogate for *Salmonella* spp. during RF heating of ground black pepper. Piperine, total phenolic, volatile oil, and antioxidant activity were assessed as the quality parameters for ground black pepper. The results demonstrated that the RF processing provided effective inactivation of *Salmonella* spp. with insignificant quality deterioration.

4.1 Introduction

Known as “King of spice” or “Black Gold”, black pepper is the fruit of *Piper nigrum* and one of the most popular spices in the world (Nisha et al., 2009). Because of its pungency and aromatic odor, black pepper is commonly used as food flavoring

and seasoning agent to enhance food flavor. The pungency of black pepper is attributed to piperine and phenolics (Srinivasan, 2007), while the volatile oils contribute to the characteristic aromatic odor of black pepper (Murthy and Bhattacharya, 2008). In addition to its pungency, piperine provides black pepper with high antioxidant activity which protect against oxidative damage by inhibiting or quenching free radicals (Srinivasan, 2007). These beneficial properties have been reported to give black pepper pharmacological effects such as anti-diarrheal (Bajad et al., 2001) and hepatoprotective effect (Koul and Kapil, 1993).

Moisture content is an important parameter to access the quality of black pepper and need to be continuously monitored during the manufacturing and storage of black pepper (Dhas and Korikanthimath, 2003). Black pepper could be classified as a low water activity (a_w) food with typical a_w lower than 0.70. Foods with a_w levels lower than 0.70 provide a natural barrier for growth of many microorganisms (Blessington et al., 2013). The minimum a_w for growth of most bacteria is approximately 0.87 and minimum a_w for mycotoxin production by molds is 0.80 (Beuchat et al., 2013), so the low a_w foods have historically been considered to be microbiologically safe (Beuchat, 1981).

However, black pepper contaminated with *Salmonella* has been reported to be the cause of several outbreaks (Centers for Disease Control, 1982; Control and Prevention, 2010) and numerous recalls (Dey et al., 2013) which not only causes food safety issues, but also results in enormous economic losses. Although it has been reported that *Salmonella* can only reproduce themselves in black pepper at a_w higher than 0.94,

they are still able to survive for several years at a low a_w storage conditions (Keller et al., 2013). As a_w decreases, *Salmonella* exhibits increasing thermal resistance which builds up the difficulties to inactivate them (Beuchat et al., 2013). Because black pepper is frequently added as an ingredient in many ready-to-eat foods which are not subjected to further thermal treatment, *Salmonella* persistence could potentially create a public health risk (Little et al., 2003, p.). Thus, it is necessary for the spice industry to develop effective decontamination methods to ensure the food safety of black pepper.

Current decontamination methods such as ozone treatment, fumigation by ethylene oxide, irradiation with gamma rays and steam treatment have been developed to reduce microbial load in black pepper, but these methods come with limitations. Ozone treatment has been reported to reduce the microbial load in ground black pepper by 3-6 log CFU/g at ozone concentration of 6.7 mg/L for 10 min, but resulted in the oxidation of certain volatile oil constituents (Zhao and Cranston, 1995). Ethylene oxide has been shown to significantly reduce microbial load in black pepper (Leistritz, 1997; Toofanian, 1986), however, it has been banned in the European Union due to usage of ethylene which is highly toxic and extremely inflammable (Uijl, 1992). Gamma ray irradiation is not well-accepted by consumers, although it has been proven to significantly reduce *S.* Typhimurium in black pepper (Song et al., 2014). Steam treatment is commonly applied to black pepper as a decontamination method in the European spice industry (Schweiggert et al., 2007), but it has been reported to cause significant color loss and quality deterioration in addition to increased moisture

levels which affect the shelf life of black pepper (Schneider, 1993; Waje et al., 2008). Therefore, the development of an innovative technology becomes necessary for decontamination of black pepper while maintaining product quality.

Radiofrequency (RF) processing is a novel thermal processing method based on dielectric heating using electromagnetic waves. RF heating is based on volumetric heating as it transfers the electromagnetic energy directly into the food product which generates the heat by water molecular friction. The advantages of RF heating compared to conventional thermal treatments are the rapid heating rate, better heating uniformity and higher penetration depth (Boreddy et al., 2016; Boreddy and Subbiah, 2016; Chen et al., 2017, 2013; Lau et al., 2016; Piyasena et al., 2003). RF heating has been applied by the food industry for post-baking of cookies (Palazoğlu et al., 2012). It has also been investigated in the research laboratories for various applications such as drying of grain product (Jumah, 2005) and pet food (McCulloch and Nelson, 1977), pest control of walnuts (Mitcham et al., 2004) and grain (Nelson and Whitney, 1960), and food pasteurization (Al-Holy et al., 2005; Bengtsson et al., 1970; Houben et al., 1991) and inactivation of *Salmonella* in many low-moisture foods (Kim et al., 2012; Li et al., 2017; Liu et al., 2017; Villa-Rojas et al., 2017).

As required by the Food Safety Modernization Act, food manufacturers are responsible for the validation of their process controls to ensure food safety which means showing scientific proof that their process is effective in controlling hazards (Food and Drug Administration, 2013). Food manufacturers cannot directly introduce food pathogen like *Salmonella* into their facilities for process validation as it can

become very hard to eliminate them from the facilities. Therefore, it is necessary to find a surrogate which behaves the same as or has a higher resistance than *Salmonella* to be subjected to validation studies within food facilities. *Enterococcus faecium* NRRL B-2354 has been commonly applied as a surrogate for *Salmonella* in testing of thermal treatments (Almond Board of California, 2007), extrusion of carbohydrate-protein meal (Bianchini et al., 2014) and oat flour (Verma, 2017), RF heating of wheat flour (Villa-Rojas et al., 2017), and infrared pasteurization of raw almonds (Bingol et al., 2011). However, it is important to evaluate the surrogate for its suitability for a specific food product and process treatment (Food and Drug Administration, 2015a).

This study aimed to develop a practical RF heating process which could effectively pasteurize ground black pepper with minimal deterioration in product quality. The specific objectives of this study were to: 1) investigate RF heating for inactivation of *Salmonella* spp. in ground black pepper, 2) evaluate *E. faecium* as a suitable surrogate for this process and 3) assess the effect of RF heating on the quality of ground black pepper.

4.2 Materials and Methods

4.2.1 Ground black pepper

Three batches of commercially steam-sterilized whole black peppercorns were obtained from three different production lots from McCormick & Company, Inc (Sparks, MD, USA) and stored in a walk-in cooler at -12°C to maintain the quality. To obtain ground black pepper, whole black peppercorn samples were ground using a Waring Commercial Spice Grinder (WSG60, Conair Corporation, CT, USA) for 30 s

and passed through a U.S. No. 20 sieve (0.841 mm sieve opening) to achieve a consistent particle size.

4.2.2 Sample preparation

Whole black peppercorn samples were placed into an equilibration chamber which consisted of a small glove box and a humidity control system custom-designed and built at Michigan State University. The humidity control system consisted of a humidity sensor (AM2303, Aosong Electronics Co., Ltd., Guangzhou, China), a fan, solenoid valves, an air pump (Fusion 700, JW Pet, Teterboro, NJ), a wet column consisting of water and humidifier wicks, a dry column filled with silica beads (640SGO55, Sorbent Systems, Los Angeles, CA), and a microcontroller (Mega 2560 R3, SainSmart Technology, Inc., Lenexa, KS) which maintained relative humidity within ± 0.3% of the set point.

Moisture content and water activity of the samples were measured using a Halogen Moisture Analyzer HR73 (Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) and a dew point water activity meter (Aqualab Series 4TE, Decagon Devices Inc., Pullman, WA, USA) at 25°C, respectively. The whole black peppercorn had an initial moisture level of 11.61 ± 0.29% (wet basis, wb) and a natural water activity of 0.581 ± 0.006.

The Spices Board (2007) defined that the optimal storage moisture content of ground black pepper must be less than 10.5% (wb). Because RF heating is expected to reduce the moisture content, the industry would lose the mass. During RF heating experiments, a typical amount of moisture loss was identified, and the samples were

preconditioned to a such moisture content level that after RF treatment, the moisture content would be just below 10.5%. This should also assist in reducing the thermal resistance of microorganisms resulting in higher log reductions (Syamaladevi et al., 2016).

By adjusting the relative humidity within the chamber, the samples were equilibrated to the target water activity and moisture content which resulted in decrease of moisture content to the optimal storage condition after RF heating. The target water activity and moisture of the samples were determined depending on the results of the RF heating time. Three 3-g samples were randomly taken from the equilibration chamber for measurements. Stability tests of moisture content and water activity were conducted by monitoring moisture content and water activity every day for one week to determine the grinding day on which the moisture content and water activity reached the stable status. On the grinding day, whole black peppercorn samples were grinded to obtain ground black pepper using the grinding method described in the previous section.

4.2.3 RF heating of ground black pepper

A total of 400 ± 0.1 g of ground black pepper sample was placed uniformly in a laminated paper tray (Fig. 1) sealed with a plastic film (Press'n Seal, The Glad Products Company, Oakland, CA, USA) to minimize heat and moisture loss from the surface. A vented nut was fixed onto the center of the film for controlled release of water vapor. Six fiber optic temperature probes (Neoptix, Inc., Quebec City, Quebec, Canada) with an accuracy of ± 0.6°C were inserted into the tray though pre-drilled

holes on the short edge of the tray as described below: (Fig. 4.1):

- probes 1, 2, 3 were inserted to the center and arranged from the top to the bottom vertically with a 1 cm interval to each other;
- probes 4, 5, 6 were inserted close to both edge from the top to the bottom vertically with a 1 cm interval to each other.

Each fiber optic temperature probe was held in place with a plastic holder in the tray to ensure the temperature of same location was measured each time. The temperature history during RF heating was recorded by the probes every 5 s.

A 6 kW, 27.12 MHz pilot-scale parallel-plate RF heating system (Model SO-6B, Monga Strayfield Pvt. Ltd., Pune, India) was used to heat ground black pepper in this study. The sample tray was placed inside the RF heating chamber at the center of the bottom electrode and the electrode gap was adjusted to 10.5 cm which gave the fastest heating rate without arcing. RF heating was conducted for 120 s, 130 s and 140 s in duplicates. Immediately after RF heating, the top surface temperature profiles were recorded by an infrared camera (Thermal CAMTM SC-640, FLIR Systems, Inc., North Billerica, MA) with an accuracy of ± 2°C after removing the film. The cold spot was determined according to the results from the fiber optic temperature probes and infrared camera for the subsequent microbial challenge pack studies, which involves placing a small bag of inoculated samples at the cold spot. To evaluate whether the placement of bag affected the heating pattern, a small heating study was conducted by heating the product with or without the bags for 120 s in duplicates. A small polyethylene bag (60 mm x 80 mm x 5 mm) was filled with 20 ± 0.1 g of

ground black pepper sample, then placed in the determined cold spot in a tray filled with 380 ± 0.1 g of ground black pepper sample so that the total mass was the same. To allow the release of vapor from the bag, a tiny hole was cut at the corner of the bag. Then, the tray was sealed and processed by RF using the same settings described above. One fiber optic temperature probe was inserted into the bag to trace the temperature history during RF heating.

From the results of Chapter III, the packed inoculated sample showed a significantly lower bacterial reduction compared to the average of the whole sample. Therefore, the evaluation of only the cold spot could be adequate to estimate the worst-case scenario. Therefore, in the subsequent microbial challenge pack studies, the inoculated sample was packed and placed at the cold spot to estimate the inactivation of the target microorganism after RF heating.

To evaluate the RF heating of ground black pepper, a heating uniformity index (λ) was used, defined by the following equation:

$$\lambda = \frac{\sqrt{\sigma^2 - \sigma_0^2}}{\mu - \mu_0}$$

where μ and μ_0 are final and initial average sample top surface temperatures (°C), while σ and σ_0 are final and initial standard deviation of sample top surface temperatures (Wang et al., 2008).

4.2.4 Microbial challenge pack studies

4.2.4.1 Background microflora

Upon receiving the whole black peppercorn sample, background microflora tests were performed to quantify the aerobic bacteria. The test was conducted by first

diluting three random 10 g samples from each batch into 90 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD). The diluted sample was then stomached for 1 min in a stomacher (Neutec Group Inc, NY, USA), diluted in 9 mL 0.1% BPW blanks, plated on 3M™ Petrifilm™ Aerobic Count Plates (3M Microbiology, St Paul, MN) and incubated for 24 h at 37°C for enumeration.

4.2.4.2 Bacterial strains

Five different strains of *Salmonella enterica* were selected for these the microbiological studies, namely, *Salmonella* Agona 447967; *Salmonella* Enteritidis PT30; *Salmonella* Tennessee K4643; *Salmonella* Montevideo 488275; and *Salmonella* Mbandaka 698538. *Enterococcus faecium* NRRL B2354 was selected as the non-pathogenic surrogate for the validation. *Salmonella* Agona 447967, *Salmonella* Montevideo 488275 and *Salmonella* Mbandaka 698538 were obtained from FDA, ORA Regional Lab in Jefferson, AR. *Salmonella* Enteritidis PT 30 and *Salmonella* Tennessee K4643 were obtained from the University of Georgia, Athens. *Enterococcus faecium* NRRL B2354 was obtained from USDA, ARS (Peoria, IL). All the bacteria were kept in trypticase soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) with 0.6% (w/v) yeast extract (YE; Becton, Dickinson and Company, Sparks, MD) (TSBYE) supplemented with 40% glycerol and stored at -80°C until used.

4.2.4.3 Inoculum preparation and inoculation

For each bacterial strain, 1 mL of the frozen stock was transferred into 10 mL of TSBYE and vortexed for 10 s, then incubated for 24 h at 37°C. For isolation, the overnight culture was streaked onto the surface of tryptic soy agar (TSA; Becton,

Dickinson and Company, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (TSAYE) using a 10 µL sterile loop, then the plates were incubated upside down at 37°C for 24 h. After incubation, a loopful (~10 µL) of one isolated colony was taken from the overnight plate, transferred into TSBYE and then incubated for 24 h at 37°C. Next, 100 µL of the overnight broth culture was spread plated onto a TSAYE plate and incubated upside down at 37°C for 24 h. Finally, the bacterial lawn on TSAYE was harvested with 3 mL of 0.1% BPW. This process was repeated for each strain. To prepare the *Salmonella* cocktail, 2 mL of the inoculum of each *Salmonella* strain was aseptically pipetted into a sterile conical tube, and vortexed for 30 s. The prepared *Salmonella* cocktail and *E. faecium* inoculum would be used for inoculation within 2 hours. The initial microbial load of the *Salmonella* cocktail and *E. faecium inoculum was* ca. 10^{10} CFU/mL and ca. 10^{9} CFU/mL, respectively.

One day before the inoculation, 500g of whole black peppercorn stored in the walk-in cooler were taken out, aseptically transferred to a sterile Whirl-Pak style bag, and left in at room temperature overnight. Then, 10 mL of the *Salmonella* cocktail was sprayed onto the whole black peppercorn sample and the bag was shaken for 10 min to achieve a proper homogeneity. The inoculated sample was transferred into the equilibration chamber to reach the target water activity. On the previously determined grinding day, the inoculated sample was grinded into ground black pepper, then returned to the equilibration chamber to re-equilibrate until the RF heating treatment. The same procedure was used to prepare samples inoculated with *E. faecium*.

4.2.4.4 Microbial stability and homogeneity tests

The stability and homogeneity tests were conducted by microbial enumeration of the inoculated samples every day for one week. Briefly, 3 g of sample was randomly taken from the inoculated batch in the equilibration chamber, aseptically transferred into Whirl-Pak bags, diluted with 27 mL of 0.1% BPW, and stomached for 1 min. The diluted sample was then tenfold serially diluted in 9 mL 0.1% BPW blanks and spread plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (SIGMA-ALDRICH, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) (mTSA) for *Salmonella* spp. or TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (ACROS, NJ, USA) (eTSA) for *E.faecium* , and incubated for 24 h at 37°C. These steps were repeated for a total of three separate random samples from each inoculated batch in the equilibration chamber. Colonies with a black center were enumerated as *Salmonella* spp., while black colonies were enumerated as *E.faecium.* The moisture content and water activity were also monitored during the microbial stability.

4.2.4.5 Inactivation of *Salmonella* spp. and *Enterococcus faecium*

On the day of RF heating challenge studies, the water activity of the inoculated ground black pepper sample was measured to confirm that it reached the target water activity before RF heating. The RF heating of ground black pepper sample was conducted for 120 s and 130 s. Immediately after RF heating, the packed sample was soaked into an ice-water bath for 3 min to prevent further thermal inactivation. The packed inoculated sample was then transferred to a sterile Whirl-Pak bag and serially diluted and spread plated onto mTSA for *Salmonella* spp. and eTSA for

E.faecium and incubated for 24 h at 37°C. Three biological replicates (batches of different whole black peppercorn production lots inoculated with independently frozen stock) and two technical replicates (RF heating for the single biological replicate) were conducted in this experiment. Untreated inoculated samples were enumerated as the control (log N_0). The total bacterial reduction (log R) was obtained by subtracting the number of survivors to the RF heating (log N) from the control. The RF heating time which gave more than 5-log reduction of *Salmonella* spp. was chosen as the optimal RF heating configuration and was used for quality analysis of uninoculated samples.

4.2.5 Quality analysis

Three batches of uninoculated ground black pepper were equilibrated to the target water activity and heated by RF using the optimal RF heating configuration which provides a more than 5 log reduction of *Salmonella* in ground black pepper. After RF heating, the samples were transferred to separate Ziploc bags and sealed. Then, the samples were allowed to cooling down in the room temperature instead of chilling in the ice-water bath for estimating the worst-case scenario of quality deterioration.

To prepare the ethanol extract for the quality analysis, 0.400 ± 0.001 g of sample was diluted by 100 mL of 200 proof ethanol (Decon Labs, Inc., PA, USA) and stirred for 12 h overnight. The solution was filtered, and the ethanol extract was stored in the dark condition at 4°C until analysis.

4.2.5.1 Color measurement

A colorimeter (BC-10, Minolta Co. Ltd., Osaka, Japan) was used to determine the color of black pepper by measuring the color values of L* (lightness and darkness), a*

(redness and greenness), and b* (yellowness and blueness). Before measurement, the colorimeter was calibrated with a white calibration pad. A petri dish was filled with ground black pepper to attain a flat surface, and then five random locations were measured by the colorimeter. The total color difference (ΔE) was calculated to assess any effect of the heating by using the following formula (Robertson, 1977):

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

4.2.5.2 Total volatile compounds

The volatiles compositions of ground black pepper samples were determined by a Thermoscientific headspace gas chromatography equipped with ISQ Mass Selective Detector (GC-MS). At the agitator temperature of 75°C, 1 g of ground black pepper sample was incubated for 20 min. An amount of 0.7 mL of gas mixture released from black pepper was injected into GC-MS with 1:80 split ratio. The GC column used in this test was TG-5MS 30 m x 0.25 mm ID x 0.25 dF capillary column. The oven temperature setting was as follows: first it was heated from 40 to 290°C at a 30°C/min heating rate, and then maintained at 290°C for 2 min. The mass range was 10-650 amu and ionization energy was 70 eV. The NIST 11 mass spectral library was used to identify the compositions of the samples.

4.2.5.3 Total phenolics

The total phenolic content of the ethanol extract was determined by using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). The 1 mL of extracts and standard solutions of gallic acid (1, 2, 3, 4, 5 μg/mL) were used in the assay. The blank was ethanol. Two milliliters of Folin-Ciocalteu's phenol reagent were pipetted into the

sample tubes and vortexed for 5 s. After 10 min, 2 mL of Na_2CO_3 was added to the mixture and vortexed for 5 s. The sample tube was incubated for 2 h in the dark at 25°C. An UV-1800 Shimadzu spectrophotometer (Shimadzu Corp., Kyoto, Japan) was used to quantitate the absorbance of each sample solution at 765 nm wavelength.

4.2.5.4 Antioxidant activity

The antioxidant activity was determined by using the 1,1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. An amount of 3 mg DPPH (Alfa Aesar, 95% purity) was diluted with 100 mL ethanol to obtain a 30 µg/mL of DPPH solution. The 1 mL of DPPH solution was mixed with 0, 2.0, 2.5, 3.0, 3.5, and 4.0 mL of ground black pepper ethanol extract in test tubes and the tubes were filled up to 5 ml using ethanol. The mixtures were vortexed for 5 s, and incubated in the dark for 30 min at room temperature. The tube without sample ethanol extract was used as control and ethanol was used as blank. An UV-1800 Shimadzu spectrophotometer was used to determine the absorbance of the remaining DPPH radicals in the solutions after incubation. The scavenging of DPPH radical was calculated according to the following equation (Bersuder et al., 1998):

DPPH Radical - scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$,

where $A_{control}$ and A_{sample} are the absorbances of the control and the sample, respectively.

4.2.5.5 Piperine content

An Agilent 1100 Series High Performance Liquid Chromatograph (HPLC) equipped with variable wavelength detector was used to determine the piperine content of ground black pepper ethanol extracts. Standard piperine (Alfa Aesar, 98%

purity) solutions were prepared in ethanol at the concentrations of 25, 50, 100, 200 and 400 μg/mL to create the standard curve. For the test, Eclipse Plus C18 (4.6 x 100 mm, 3.5 μm) column was used as stationary phase and isocratic elution was performed with mobile phase of 50% of methanol and 50% water mixture. The flow rate was 0.7 mL/min and detection wavelength was at 341 nm.

4.3 Results and Discussion

4.3.1 Temperature profile of RF heating

RF heating of ground black pepper was conducted for 120, 130, and 140 s, which resulted in a final average surface temperature of 78.1, 80.1, and 81.1°C, respectively (Table 4.1). From trial-and-error experiments, the initial moisture content and water activity were adjusted to 12.8% (wb) and 0.664, so that the final moisture content and water activity would drop to 10.5% (wb) and 0.592 after the RF heating. The final moisture of 10.5% (wb) was selected because the Spice Board identified this as the optimal moisture content for long term storage of ground black pepper to prevent fungal deterioration (Spices Board, 2007).

The fast heating rate provided by RF heating reduced the come-up-time considerably compared to conventional heating. Faster come-up time allows for high-temperature and short time processing, which could potentially minimize the quality deterioration for achieving the desired lethality. The fast RF heating of ground black pepper was also observed in other studies. For example, ground black pepper reached 62°C after 50 s of RF heating (Kim et al., 2012) and the temperature of ground black pepper with a moisture content of 17.2% (db) increased from 22.2 °C to 90.2°C after 63 s of RF heating (Jeong and Kang, 2014). The variance of the heating

rate in different studies could result from the different moisture content of the samples (Jeong and Kang, 2014), different RF heating configurations (Chen et al., 2017; Villa-Rojas et al., 2017) and different sample sizes (Piyasena et al., 2003).

Fig. 4.2 shows that during RF heating there was a linear heating rate until the temperature reaches around 80 -100°C. After that, the heating rate levels off due to evaporative cooling and also due to heat transfer to cold spot by movement of steam. At first, the bottom edge location (6) reached the highest temperature, over 100°C, in 80 s. When the bottom edge reached the levelling state, there was a drastic increase in heating rate of edge center (5) which is just above the edge bottom (6). Thus the steam from edge bottom was rising up and then heated the product at the edge center location. Similarly the top edge then heated up with a higher heating rate within few seconds after the middle center heated with a higher heating rate. The same pattern was shown for the probes in the middle location, where the middle bottom (3) heated faster than middle center (4), while the middle top was the coldest. In general the middle layers heated slower than the edges at all vertical distances.

The small deviation of the average temperature measured at the surface indicated that the RF heating is highly replicable (Table. 4.1). The uniformity indexes (UI) were calculated and the results are similar to other RF heating uniformity optimization studies (Chen et al., 2015; Jiao et al., 2015; Wang et al., 2007) which means RF heating provides a good heating uniformity during heating of ground black pepper. According to the results of RF heating pattern, the cold spot was located at (1) which is the center of the top layer. This location has also been reported in other

studies to be a cold spot (Chen et al., 2017; Liu et al., 2017; Tiwari et al., 2011; Villa-Rojas et al., 2017). A packed sample was placed at (1), and the temperature of the bag was traced during the RF heating. Its temperature history showed no considerable difference from the sample without a pack (Fig. 4.2), which indicated that the slim plastic bag did not affect RF heating. Similar results have also been reported in other RF studies (Liu et al., 2017). Thus, for the microbial challenge pack studies, packed inoculated samples were placed at (1) for each tray to account for the worst-case scenario.

4.3.2 Stability and homogeneity tests of *Salmonella* spp. and *E. faecium* at low a_w condition

The presence of < 10 CFU/g of aerobic bacteria was detected for all three batches of whole black peppercorn. Therefore, the added population of *Salmonella* spp. and *E. faecium* would not be affected by background microflora (Morey and Singh, 2012).

The stability tests for *Salmonella* spp. and *E. faecium* after inoculation were conducted at a_w of 0.660 ± 0.025 for one week. In Fig. 4.3 and Fig. 4.4, it can be observed that the moisture content and water activity reached equilibrium on the second day after the inoculation. Therefore, the whole black peppercorn samples were grinded on the second day, then transferred back to the equilibration chamber. The results of the stability tests for both bacteria are shown in Fig. 4.5 and Fig. 4.6. The initial population of *Salmonella* spp. on the whole black peppercorn samples was more than 8 log CFU/g and it dropped by around 1 log CFU/g after two days. The grinding caused a further reduction of 0.3 log CFU/g but the *Salmonella* spp. population subsequently stabilized, with only 0.2 log CFU/g change after 5 days. *E.*

faecium showed a better stability on black pepper samples with less than 0.5 log CFU/g of reduction in one week including a reduction of 0.2 log CFU/g from the grinding. Both bacteria showed a good stability after the third day and thus the fifth day was selected for microbial challenge studies of RF heating. The observed stability of *Salmonella* was not surprising as *Salmonella* has been previously reported to survive on ground black pepper samples for a long period at low a_w environment (Keller et al., 2013). Small standard deviations of both bacteria (< 0.1 log CFU/g) were observed during these tests and indicating that the inoculation method provided a good homogeneity for the ground black pepper samples.

It is important to evaluate the inoculation method and monitor the equilibration process especially for low a_w food. When the liquid inoculum is mixed with black pepper samples, the added moisture could result in the release of water-soluble antimicrobials which may decrease the population level before the microbial challenge studies (Waje et al., 2008). After inoculation, the change in environment could cause a shock to the bacteria (Palipane and Driscoll, 1993). The bacteria need to adapt themselves to the low a_w environment and it is necessary for the bacteria to reach a stable status before conducting microbial challenge studies to remove the influences brought by the new environment (Bowman et al., 2015). When exposed to a low aw environment, *Salmonella* has been reported to develop enhanced tolerance to heat treatment (Gruzdev et al., 2011) which increases the difficulty for thermal inactivation. In general, black pepper could get contaminated during cultivation, harvesting, and drying (Nair, 2004) before processing which gives bacteria enough

time to adjust themselves to the low a_w environment and build up thermal resistance. Thus, it is necessary to equilibrate the sample until the bacteria reach a stable status to simulate the real scenario.

4.3.3 Microbial challenge pack studies of RF heating

The RF heating treatments were conducted for both ground black pepper samples inoculated with *Salmonella* spp. or *E. faecium* for 120 and 130 s, respectively. This kind of pack studies have been shown to provide a conservative validation of RF heating (Liu et al., 2017). The inoculated black pepper samples in the small packs were enumerated, and the comparison of log reduction between *Salmonella* spp. and *E. faecium* at 120 s and 130 s of RF heating is shown in Fig. 4.7. The error bars indicate the ± 1 standard deviation between the biological replications.

At the RF heating time of 120 s, the error introduced by RF processing systems and associated microbial enumeration method can be estimated by calculating the average of standard deviation between two technical replications for three batches and was found to be 0.47 and 0.04 log CFU/g for *Salmonella* and *E. faecium*. The error introduced by samples batches (replications) and associated microbial enumeration method can be estimated by the standard deviation between three biological replications (after averaging the values for two technical replications) and was found to be 0.30 and 0.06 log CFU/g. Therefore, the error introduced by RF processing is similar to the error introduced by different batches of samples and the error might be introduced primarily by the microbial enumeration method rather than RF processing or samples.

At 120 s of RF heating, reductions of 3.98 and 2.30 log CFU/g were achieved for *Salmonella* spp. and *E. faecium*, respectively. At 130 s of RF heating, more than 5.93 log CFU/g reduction was obtained for *Salmonella* spp., while *E. faecium* only experienced a reduction of 3.89 log CFU/g. For both bacteria, the log reductions at 130 s were significantly higher than 120 s ($P < 0.05$) and within this 10 s, an extra 2 log CFU/g and an extra 1.6 log CFU/g was effectively attained for *Salmonella* spp. and *E. faecium*, respectively, due to the high temperature achieved. It has been reported that 50 s of RF heating with a final average temperature of 60 °C could provide a 4.29 log CFU/g reduction of *Salmonella* Typhimurium in ground black pepper (Kim et al., 2012) and 1 min of vacuum steam treatment of black pepper at 75 °C would result in 6.10 ± 0.64 log CFU/g reduction of *Salmonella* PT 30 (Shah et al., 2017). The *Salmonella* strains in those studies seem to be less thermally resistant compared to the ones used here which could be the result from the different inoculation methods, moisture equilibration (Bowman et al., 2015). different *Salmonella* strains used and different initial a_w of the samples (Jeong and Kang, 2014; Kim et al., 2012). Specifically, both studies (Jeong and Kang, 2014; Kim et al., 2012) inoculated the ground black pepper samples, and immediately treated the samples using RF heating without any equilibration. In addition, they also used only one serotype of *Salmonella*, while this study used a *Salmonella* cocktail of five strains.

RF heating was shown to effectively inactive *Salmonella* spp. in ground black pepper by providing more than 5.93 log CFU/g reduction within 130 s. This RF heating adequately reduced the presence of *Salmonella* spp. by 5 logs which meets the

5-log pathogen reduction performance standard (Food and Drug Administration, 2015b, 2012) and thus the quality analysis of ground black pepper was conducted at this condition. Because of the short come-up time, RF heating could be considered as a high-temperature-short-time processing which potentially minimizes the quality loss. RF heating was shown to provide a more rapid inactivation of *Salmonella* spp. than other methods like ozone treatment which took 10 min to achieved a 4 log CFU/g reduction (Zhao and Cranston, 1995) and cold plasma which provide a 4.1 log CFU/g reduction after a treatment of 30 min (Hertwig et al., 2015). In this study, ground black pepper samples were immediately cooled down after RF heating which represents a conservative estimate of microbial log reduction, because the products may not be chilled immediately after processing in the real industrial setting and thus RF heating could potentially provide additional inactivation.

At both heating times, a significantly higher log reduction was obtained for *Salmonella* spp. than *E. faecium* which indicated that *E. faecium* is more thermally resistant during RF heating of ground black pepper. Hence, *E. faecium* is a suitable surrogate for *Salmonella* spp. for RF heating of ground black pepper. Although *E. faecium* has been demonstrated to be a good surrogate for *Salmonella* in many thermal processing of low-moisture food (Almond Board of California, 2007; Bianchini et al., 2014, 2014; Kopit et al., 2014; Ma et al., 2007), it still necessary to conduct the validation because under different product matrixes and processing methods, the surrogate of choice may not acc accurately represent the behavior of the pathogen of interest (Ceylan and Bautista, 2015; Rachon and Gibbs, 2015).

4.3.4 Quality analysis

The quality of the ground black pepper samples was mainly accessed by its moisture content, a_w, color, piperine, total phenolics, antioxidant activity and volatile compounds. The a_w of ground black pepper sample was equilibrated to 0.660 ± 0.025 with a moisture of 12.8 ± 0.1% (wb) which would result in a_w significantly dropped to 0.595 ± 0.025 with a moisture of 10.5 ± 0.1% (wb) after RF heating for 130 s (Table. 4.2). According to American Spice Trade Association (2011), the optimal storage moisture content of ground black pepper is below 10.5% (wb). RF heating has shown to adequately reduce the moisture of ground black pepper to the optimal storage moisture of 10.5% (wb). Moisture content not only affects the quality but also safety of black pepper as microbial resistance changes with water activity (a_w). Water activity assesses the amount of available free water that could be used to support food spoilage, thus it plays an important role in microbial control and food safety (Beuchat, 1981).

The color of ground black pepper samples did not experience a significant change after the RF heating. The calculated ΔE of 0.65 indicated a normally invisible difference between the control and RF treated samples (Hunterlab, 1976). The color of black pepper is important to be accessed for thermal process, because the thermal decontamination process like commercial steam treatment has been reported to cause significant color loss of black pepper (Schneider, 1993). Although RF heating heated up the samples to a high temperature, it did not induce significant color loss to black pepper.

The main odor, flavor and antioxidant activity contributors to black pepper:

piperine, total phenolic and volatile oils, were evaluated in this study. Table. 4.2 shows that the total phenolics and piperine content of RF treated samples were not significantly different from the control samples. In a previous study, the piperine in black pepper was shown to be non-volatile at room temperature and less sensitive to thermal treatment. However, the heating resulted in a change of relative composition of the major volatile compounds that could change the flavor of black pepper (Chacko et al., 1996). In this study, there were 29 volatile compounds identified and the 9 major components (> 5% peak area) were β-Ocimene (16.76%), followed by Caryophyllene (11.46%), D-Limonene (11.17%), β-Phellandrene (9.62%), β-Pinene (8.57%), Camphene 1R, 4S (8.22%), α-Phellandrene (6.1%), 3-Carene (5.88%) and Sabinene (5.57%) which accounted for 83.3% of the total amount of the volatiles in the untreated ground black pepper samples (Table. 4.3). In Fig. 4.8, it shows that the same 29 volatile compounds were detected in both control and RF treated samples, and there was no any new compounds found after RF processing compared to the volatiles analysis tests in other studies (Ferreira et al., 1999; Zhao and Cranston, 1995). Among those major compounds, only camphene experienced a significant decrease with a decline of 17.64% after RF heating. The other major compounds especially sabinene and terpinene which are the major contributors to the characteristic odor of black pepper (Pino et al., 1990) did not show any significant difference after RF heating.

The DPPH is commonly used to determine the free radical-scavenging activity of an antioxidant, and it has a characteristic absorbance at 517 nm (Yamaguchi et al.,

1998; Zarai et al., 2013). The antioxidant activity of ground black pepper was evaluated using the DPPH radical-scavenging assay and the results are shown in Fig. 4.9. As it can be deduced from this figure, there is no significant difference between RF treated sample and control and thus RF heating did not affect the overall antioxidant activity of ground black pepper.

4.4 Conclusion

In this study, RF heating was shown to effectively inactivate *Salmonella* spp. in ground black pepper and provided more than 5.93 log CFU/g reduction with only 130 s of heating. *E. faecium* was concluded to be as a suitable surrogate for *Salmonella* spp. during RF heating of black pepper. Results showed a 3.98 log CFU/g reduction of *E. faecium* with a 130 s of RF heating indicating that more than 5 log CFU/g reduction of *Salmonella* spp. would be achieved. At the same RF heating time of 130 s, it was also demonstrated that there was no significant quality loss and RF heating was able to dry the ground black pepper to the optimal storage moisture.

4.5 References

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Table 4.1. Temperature profile of the top surface of RF heated ground black pepper for different treatment times.

Matrices on top surface	120 s	130 s	140 s
Average Temperature (°C)	78.1 ± 0.8	80.1 ± 0.8	81.1 ± 0.3
Maximum Temperature (°C)	99.9 ± 1.0	101.1 ± 0.5	100.1 ± 0.5
Minimum Temperature (°C)	69.2 ± 0.9	69.5 ± 0.8	69.3 ± 1.2
Heating uniformity index (λ)	0.075 ± 0.021	0.076 ± 0.004	0.097 ± 0.034

Table 4.2. Moisture content, water activity, color parameters, total phenolics and piperine content of untreated and treated black pepper samples subjected to RF heating.

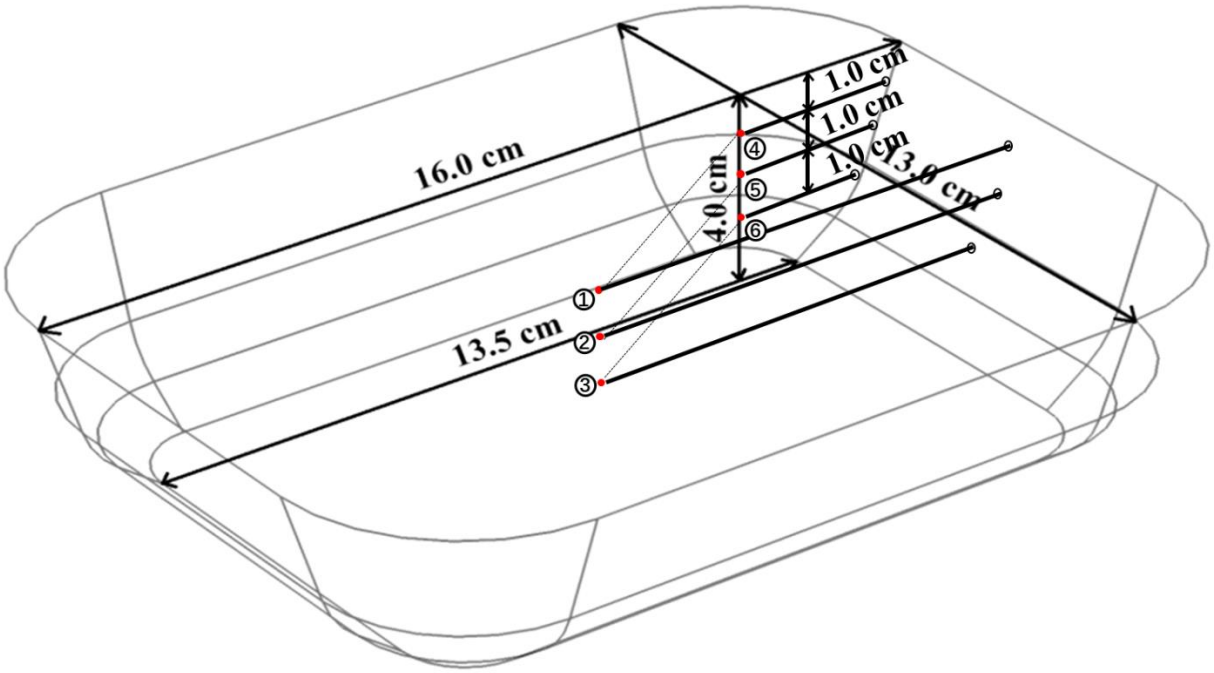
Quality Parameter	Control	RF treated
Moisture content (wb) (%)	12.8 ± 0.1 [a]	10.5 ± 0.1 [b]
Water activity	0.664 ± 0.003 [a]	0.592 ± 0.007 [b]
Color (L*)	55.8 ± 0.8 [a]	55.2 ± 0.4 [a]
Color (a*)	4.1 ± 0.1 [a]	4.0 ± 0.1 [a]
Color (b*)	10.8 ± 0.4 [a]	11.0 ± 0.3 [a]
Color difference (ΔE)	0	0.65
Total phenolics (mg/g)	19.5 ± 0.1 [a]	19.4 ± 0.2 [a]
Piperine (ug/g)	37.5 ± 0.3 [a]	37.7 ± 0.4 [a]

[1]Within a row, the numbers with the same alphabet in the superscript are not significantly different from each other ($p > 0.05$)

Table 4.3. Total volatile compounds of untreated and treated black pepper samples subjected to RF heating.

Compound	Area (%)	
	Control	RF treated (% difference)
α-Thujene	1.37 ± 0.13 [a]	0.91 ± 0.04 [b] (- 33.58%)
α-Pinene	4.89 ± 0.42 [a]	3.68 ± 0.15 [b] (- 24.77%)
Camphene	1.76 ± 0.14 [a]	1.07 ± 0.02 [b] (- 39.20%)
Sabinene	5.57 ± 0.48 [a]	4.9 ± 0.5 [a]
β-Pinene	8.57 ± 0.63 [a]	7.99 ± 0.15 [a]
α-Phellandrene	6.1 ± 0.75 [a]	5.56 ± 0.35 [a]
β-Mycene/4(10)-Thujene	1.06 ± 0.09 [a]	1.08 ± 0.02 [a]
β-Phellandrene	9.62 ± 0.8 [a]	8.06 ± 1.26 [a]
Camphene, (1R, 4S)	8.22 ± 0.62 [a]	6.77 ± 0.2 [b] (- 17.64%)
γ-Terpinene	0.94 ± 0.06 [a]	0.91 ± 0.06 [a]
3-Carene	5.88 ± 0.55 [a]	5.72 ± 0.05 [a]
2-Carene	1.23 ± 0.15 [a]	1.06 ± 0.04 [b] (- 13.82%)
β-Ocimene	16.76 ± 1.64 [a]	17.92 ± 0.28 [a]
D-Limonene	11.17 ± 1.17 [a]	12.34 ± 0.49 [a]
p-Menth-3-en-1-ol	0.63 ± 0.09 [a]	0.61 ± 0.02 [a]
Terpineol, cis-β-	0.55 ± 0.08 [a]	0.49 ± 0.02 [a]
p-Mentha-1,4(8)-diene	0.22 ± 0.02 [a]	0.25 ± 0.02 [b] (+ 13.64%)
β-Linalool	0.33 ± 0.04 [a]	0.3 ± 0.02 [a]
p-Menth-3-ene, 2-isopropenyl-1-vinyl-, (1S, 2R)	0.75 ± 0.12 [a]	0.76 ± 0.02 [a]
β-Copaene	0.09 ± 0.01 [a]	0.09 ± 0.01 [a]
α-Copaene	1.2 ± 0.21 [a]	1.14 ± 0.04 [a]
β-Elemene	0.26 ± 0.02 [a]	0.25 ± 0.01 [a]
Caryophyllene	11.46 ± 8.18 [a]	16.78 ± 0.82 [a]
Humulene	0.55 ± 0.08 [a]	0.53 ± 0.03 [a]
trans-α-bargamotene	0.2 ± 0.01 [a]	0.19 ± 0.02 [a]
δ-Cadinene	0.09 ± 0.01 [a]	0.09 ± 0.01 [a]
Isocaryophyllene	0.31 ± 0.04 [a]	0.33 ± 0.03 [a]
(Z,E)-α-Farnesene	0.22 ± 0.03 [a]	0.21 ± 0.02 [a]
Caryophyllene oxide	0.03 ± 0.01 [a]	0.03 ± 0.01 [a]

[1]Within a row, the numbers with the same alphabet in the superscript are not significantly different from each other ($p > 0.05$)



Figure

Figure 4.1. Dimensions for the laminated paper tray; locations of the six fiber optic sensors

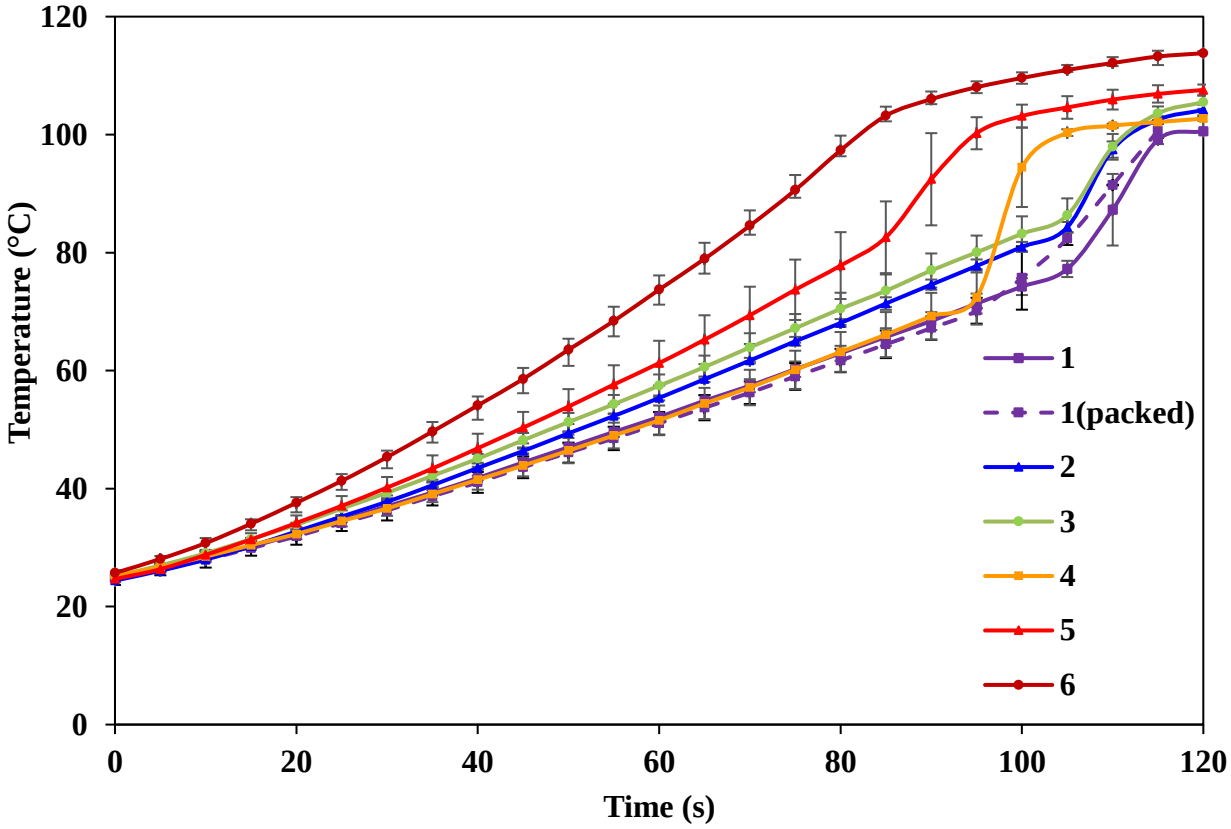


Figure 4.2. Temperature histories of ground black pepper during RF heating.

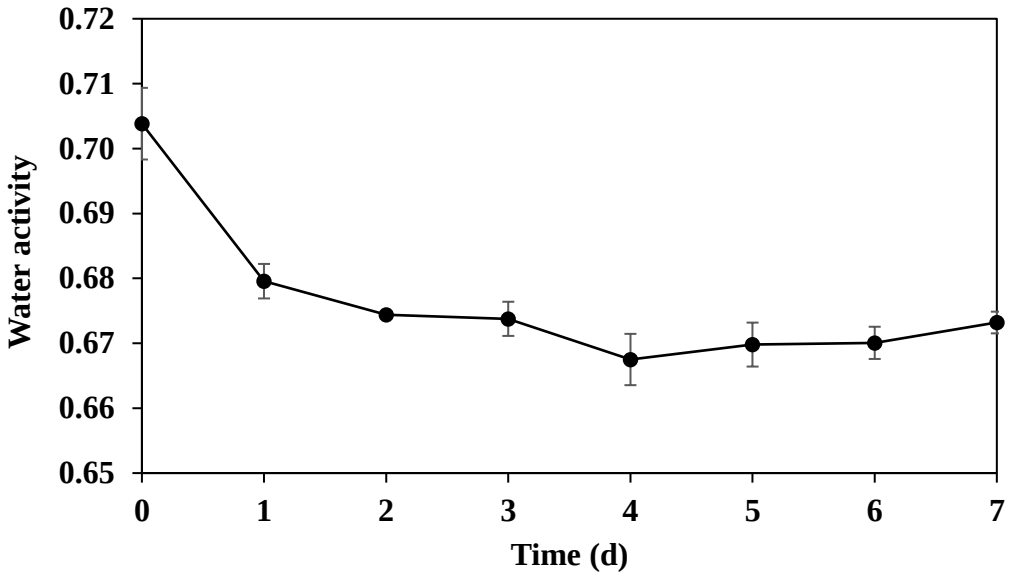


Figure 4.3. Stability test of water activity for 7 days in equilibration chamber.

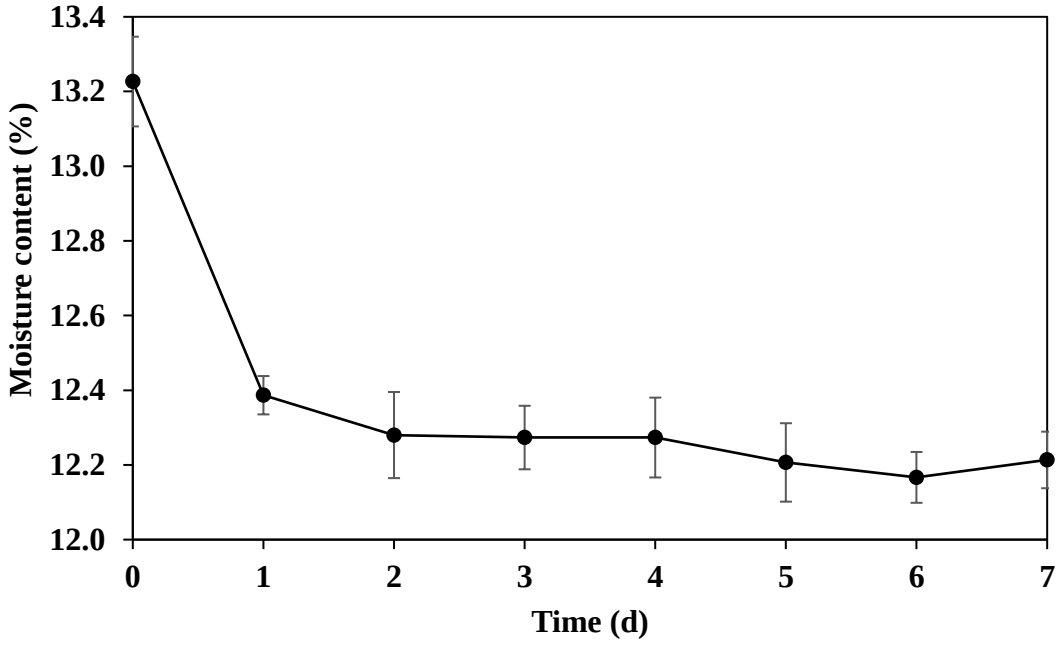


Figure 4.4. Stability test of moisture content for 7 days in equilibration chamber.

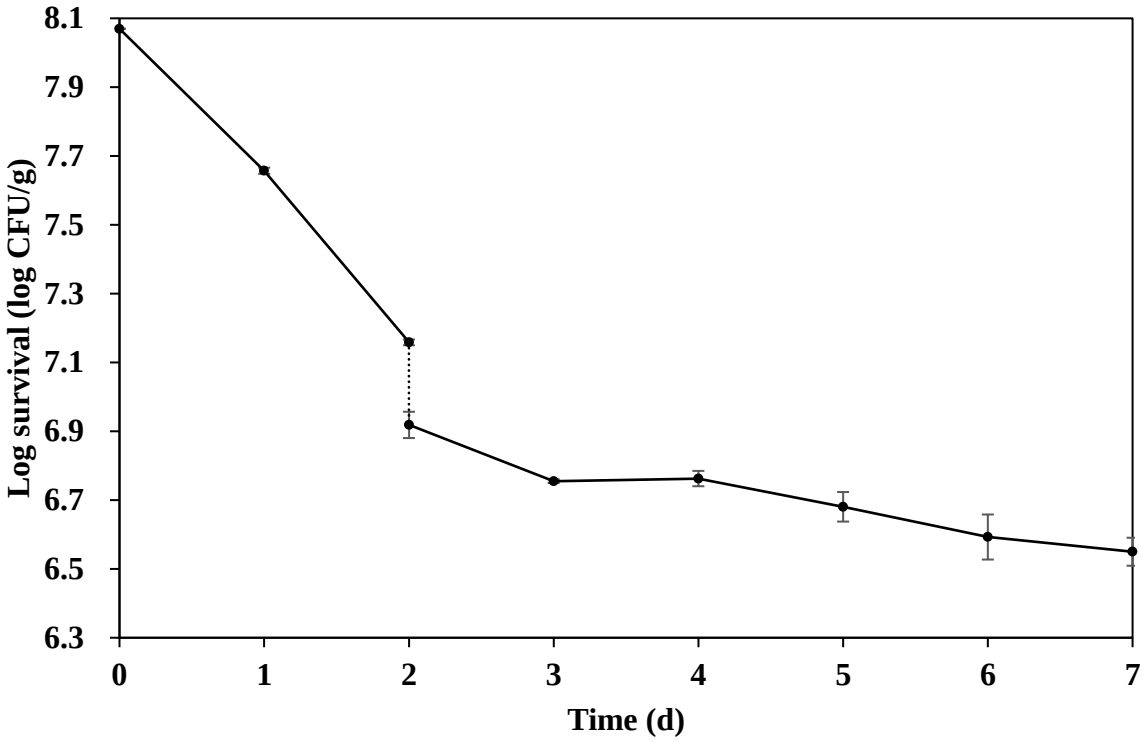


Figure 4.5. Stability test of *Salmonella* in ground black pepper. On Day 2 grinding was performed which resulted in a slight log reduction.

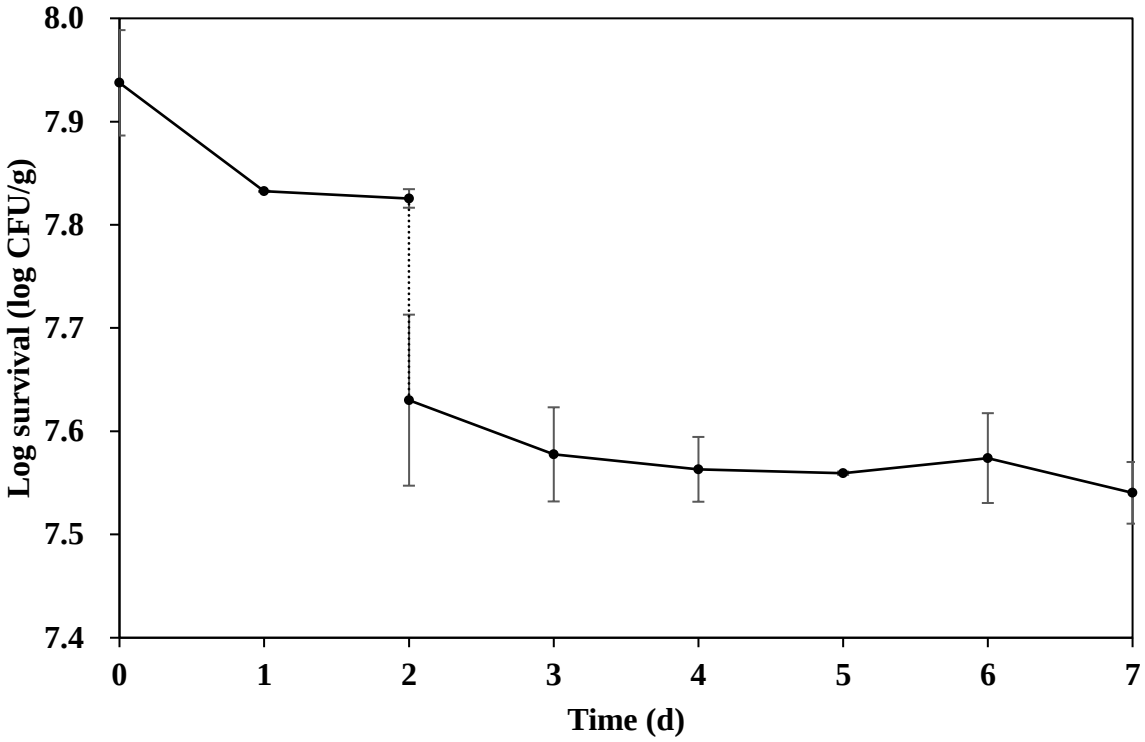


Figure 4.6. Stability test of *E. faecium* in ground black pepper. On Day 2 grinding was performed which resulted in a slight log reduction.

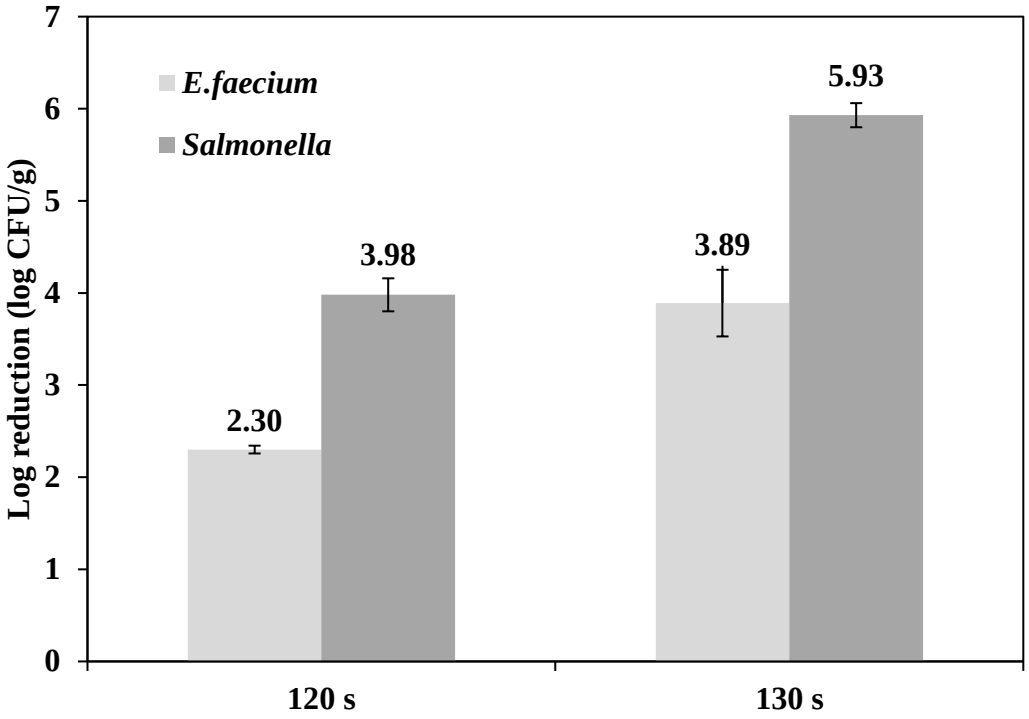


Figure 4.7. Comparison of the log reduction between *Salmonella* and *E. faecium* at 120 s and 130 s of RF heating in ground pepper. Error bars indicate the ± 1 standard deviation between the biological replications.

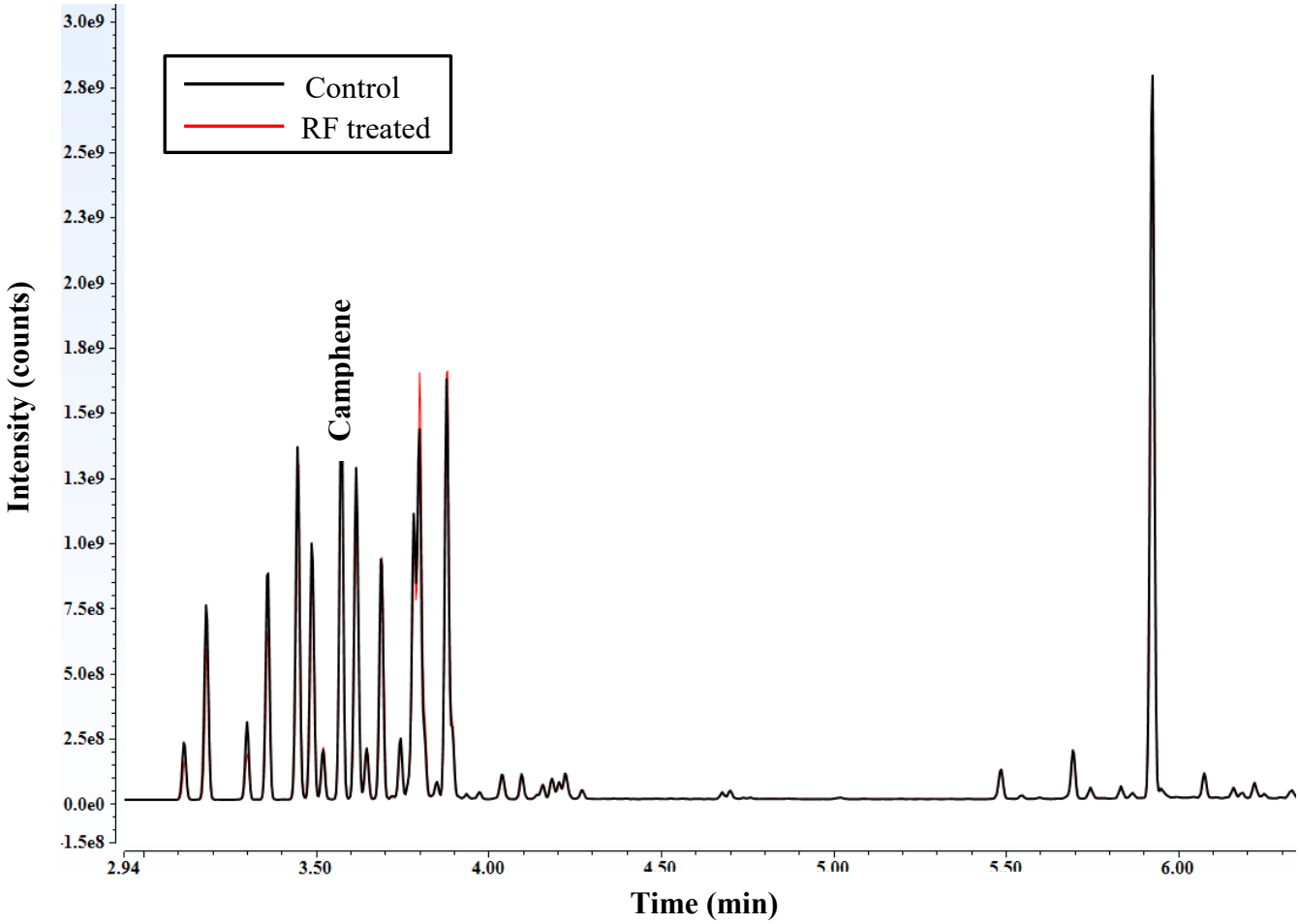


Figure 4.8. GC chromatograms of ground black pepper volatile compounds. Labeled peaks represent the major compound which significantly dropped during RF heating.

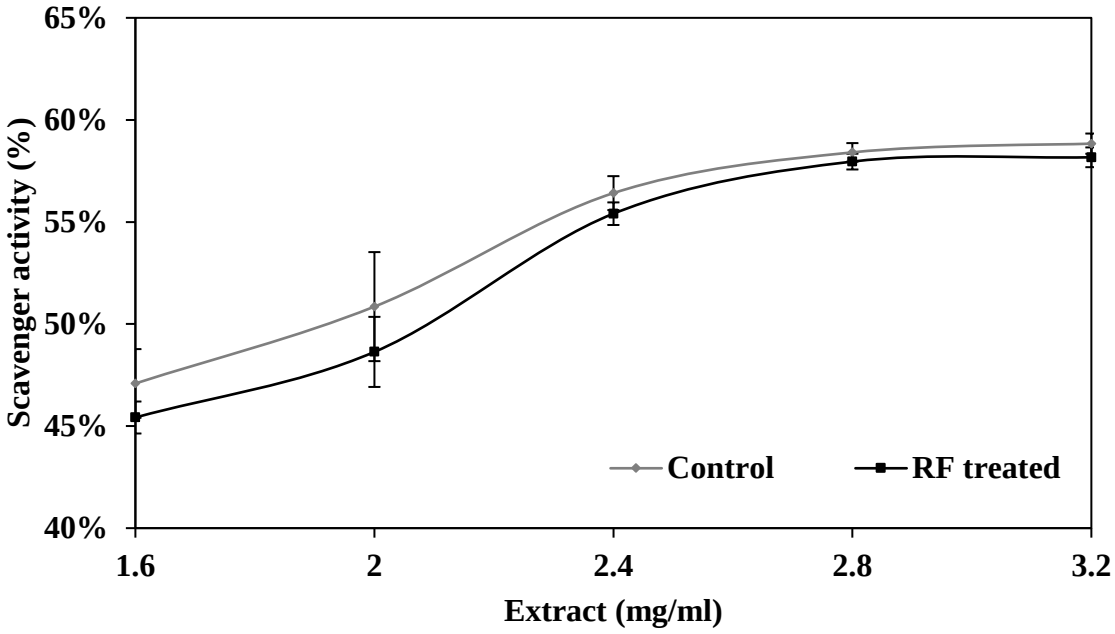


Figure 4.9. Antioxidant activities of untreated and treated black peppercorn samples subjected to RF heating.

Chapter V: Conclusions and Future Research

5.1 Conclusions

In this study, RF heating was shown to be a promising technology for inactivation of *Salmonella* spp. in both whole black peppercorn and ground black pepper. Before RF heating, the moisture contents of whole black peppercorn and ground black pepper samples were adjusted to a higher level to compensate for the moisture loss, so that the final moisture contents would meet the optimal storage condition after the RF heating. The inoculation method was evaluated and the stability tests were conducted to ensure that *Salmonella* properly survive and adapt in black pepper before RF treatment.

The results of RF heating challenge studies suggested that RF heating could rapidly heat up both whole black peppercorn and ground black pepper samples and achieve adequate reduction in a short treatment time. *E. faecium* was evaluated to be a suitable surrogate for *Salmonella* during the RF heating. The cold spot during RF heating of both whole black peppercorn and ground black pepper samples was located at the center of the top layer. The determination of the cold spot could simplify the microbial analysis by conducting a conservative estimation. The quality analysis in terms of color, piperine, volatile oils, total phenolics and antioxidant activity suggested that quality of black pepper samples did not experience a considerable difference after RF heating.

5.2 Future research

In this study, the RF heating pasteurization has been developed to effectively inactivate *Salmonella* in black pepper without causing quality loss, and a proper

surrogate has been evaluated for validation of RF heating. However, there are some further research could be explored.

In this study, the proper RF heating time was determined by using trial-and-error method which was time-consuming, and a lot of samples were wasted. It would be beneficial to collect the D and z values of *Salmonella* in black pepper samples at the same water activity for the pasteurized temperature range. A RF heating inactivation model of *Salmonella* in black pepper could be developed by combing the temperature history during RF heating and the D and z values of *Salmonella*. The model could predict the inactivation of *Salmonella* in black pepper samples during RF heating and help in determining the heating time. Eventually, the proper RF heating time could be confirmed by microbial challenge studies.

A multiphysics model for RF processing of various food products could be developed. This will predict temperature histories, when combined with thermal inactivation kinetics parameters, could predict microbial destruction. The model can be used to optimize package and electrode configuration to further improve heating uniformity that would result in further lower quality deterioration.

A conservative estimate of *Salmonella* inactivation was used to assess the effectiveness of RF heating by immediately cooling down the samples after RF heating. However, it is impractical for food manufacturers to cool the product down immediately after the pasteurization, and thus there would be a further inactivation because of the remaining heat from the process. It would be

interesting to observe the inactivation of *Salmonella* during the period of the black pepper samples naturally reducing the temperature. If we consider the microbial inactivation during cooling, it is possible to further reduce the heating time that would minimize quality deterioration.

In recent years, several novel technologies like superheated steam, cold plasma, electro-beam and blue-LED light have been developed and show their potential applications for food manufacturers to ensure the food safety. Thus, the evaluation of those technologies for inactivation *Salmonella* in black pepper would be helpful to come up with the most effective, economic and applicable process for the spice industry.