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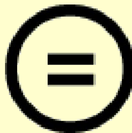

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A Thesis for the Degree of Doctor of Philosophy

Application of Radio-Frequency Heating for Inactivation of Foodborne Pathogen

식중독균 제어를 위한 고주파 가열의 활용

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Abstract

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The specific objectives of this study were, (i) to evaluate the efficacy of RF heating for inactivating foodborne pathogens, such as *Salmonella enterica* serovar Enteritidis, Typhimurium, and Senftenberg in raw shelled almonds compared to conventional convective heating as well as its effect on product quality, (ii) investigate the effect of salt content of samples, packaging material, and electrode gap on the antimicrobial efficacy of RF heating, (iii) evaluate the antimicrobial effects of the combination treatment of RF heating with ultraviolet (UV) radiation and organic acid spray against foodborne pathogens on dried foods, (iv) develop a computer simulation model and predict the behavior of RF heating in spice products.

RF heating can be applied to control internalized pathogens as well as surface-adhering pathogens in raw almonds without affecting product quality. As the salt content of pistachios increased, treatment time required to achieve 4-log reduction of *S. enterica* decreased and then was maintained when the salt content exceeded a level corresponding to the peak heating rate. PEI film reduced the treatment time required to reduce *S.* Typhimurium and *E. coli* O157:H7 by more than 7 log CFU/g (below the

detection limit, 1 log CFU/g) in red and black pepper powders. The dielectric constant of PEI film was similar to that of target sample, and the dielectric loss factor of PEI film was relatively low. The heating rate of the sample increased with decreasing electrode gap. RF heating for the treatment time required to reach 90 °C achieved 2.85-, 2.17-, and 2.09-log reductions of *C. sakazakii* without generating heat-injured cells at the electrode gaps of 8 cm, 10 cm, and 12 cm, respectively.

The RF-UV combined treatment showed synergistic effects: the total microbial log unit reduction of the combined treatment was significantly ($P < 0.05$) different from the sum of the reductions obtained from individual treatments. Qualitative (transmission electron microscopy) and quantitative (leakage of intracellular substances and propidium iodide uptake) analyses provide evidence that damage to the cell membrane was identified as the main factor contributing to the synergistic lethal effect of the combination treatment of RF heating and UV irradiation. RF-UV combined treatment for 60 s did not significantly ($P > 0.05$) affect the color, moisture content, and sulfhydryl activities of powdered infant formula. As another available hurdle combination, combined treatment of RF heating and LA sprays for 40 s caused 4.94 and 5.48 reductions of *S.* Enteritidis PT 30 and *S.* Typhimurium, respectively. The RF-LA combined treatment did not change color and oxidative rancidity of almonds significantly ($P > 0.05$).

A computer simulation was studied to predict the influence of various factors on the inactivation of foodborne pathogens on food samples by RF heating. A finite

element-based commercial software, COMSOL Multiphysics, were used to predict electric potential, electric field distribution, and temperature distribution of red pepper powder during RF heating. The computer simulation model was validated by comparing with the experimental temperature profiles of powdered red pepper spices and applied to predict the effect of frequency, electrode gap, and dielectric properties of packaging materials on the antimicrobial effect of RF heating. The simulated results demonstrated that the efficacy of RF heating in reducing foodborne pathogens could be improved using a higher frequency, a bigger electrode area, a similar dielectric constant of packaging material as target sample, and a lower dielectric loss factor of packaging material.

The results of this thesis are helpful to establish treatment conditions for maximizing the antimicrobial efficacy of RF treatment, and by extension, to commercial practical application of RF heating. The combination treatment of RF heating with other technology suggest alternatives to conventional decontamination treatments. In conclusion, application of RF heating in the food industry is expected to represent a novel and innovative thermal process for the production of safe foods.

Keywords: radio-frequency heating, salt content, dielectric properties, packaging material, electrode gap, ultraviolet irradiation, organic acid, lactic acid, spray, foodborne pathogen, computer simulation, dry powdered food, nut kernel

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Chapter I.

Evaluation of radio-frequency heating in controlling foodborne pathogens in raw shelled almonds

I-1. Introduction

Consumption of nut products has continually increased from 2000 to 2011, as consumers have taken greater interest in health and nutrition (Statista, 2014). Almonds are among the most popular nuts, accounting for 28% of the nut market in 2011 (Almond Board of California, 2012). However, outbreaks of salmonellosis have been associated with the consumption of raw almonds in the United States, Canada, and Sweden in 2001, 2004, and 2006, respectively (Centers for Disease Control and Prevention, 2004; Ledet et al., 2007). *Salmonella enterica* serovar Enteritidis phage type 30 (PT 30) was identified as the outbreak strain. No outbreaks involving serovars Typhimurium and Senftenberg on almonds have been reported; however, other nut-associated outbreaks were caused by these foodborne pathogens. Of the 5 nut-associated outbreaks reported to the Centers for Disease Control and Prevention between 1998 and 2008, 20% were caused by *S.* Typhimurium (Jackson et al., 2013). In March 2009, a multistate outbreak of *S.* Senftenberg infections associated with pistachios occurred in the United States (Centers for Disease Control and Preservation, 2009b).

As of 2007, in response to these outbreaks, the U.S. Department of Agriculture has mandated that almonds be processed to achieve a minimum 4-log reduction of *Salmonella* using a validated process prior to export. To reduce the microbial load of almonds, several processes including propylene oxide (PPO) fumigation, oil roasting,

dry roasting, blanching, and steam heating have been approved by the U.S. Food and Drug Administration (FDA) (U.S. Department of Agriculture, 2007). However, as PPO treatment is controversial due to residues remaining on almonds, the European Union and many other countries have banned its use (Cornucopia Institute, 2007). In addition, with conventional thermal treatments, externally generated heat is only slowly transferred to bulk almonds due to low thermal conductivities, necessitating lengthy treatments, which may result in thermal damage to almonds (Doores, 2002). Several alternative methods, such as high pressure, irradiation, and infrared heating, have been suggested; however, constraints of high installation cost, poor consumer acceptance, and difficulty of scaling up for large volume commercial applications still limit widespread use of these treatments (Goodridge et al., 2006; Prakash et al., 2010; Yang et al., 2010).

For the above reasons, it is necessary to develop new pasteurization technologies for almonds. One of these alternatives is radio-frequency (RF) heating which involves the use of electromagnetic energy at frequencies between 1 and 300 MHz. Among these, only selected frequencies (13.56, 27.12, and 40.68 MHz) are permitted for domestic, industrial, scientific and medical applications so as not to interfere with communication systems (Piyasena et al., 2003). RF generates heat rapidly within food materials due to molecular friction and space charge displacement in response to an externally applied alternating electric field. This technology can deliver thermal energy quickly to every part of the bulk food product in which pathogens may reside

(Kinn, 1947; Zhao et al., 2000). Thus, RF heating could potentially replace conventional heating for solid and semi-solid foods which have low thermal conductivities. In my previous study, the effectiveness of RF thermal processing for pasteurization of powdered foods was investigated (Jeong and Kang, 2014).

Recently there have been some research efforts to apply RF heating as a new thermal intervention for treatment of almonds (Gao et al., 2010; Gao et al., 2011). However, these studies were limited to disinfestation and product quality following RF treatment, and did not assess microbial inactivation. Furthermore, a comparison between the pasteurization efficacy of RF heating and approved methods including conventional heating has not been reported. Accordingly, microbial inactivation rates between RF heating and conventional heating need to be evaluated in order to be approved for industrial use by the FDA. Also, because of the possibility of pathogen internalization within almonds during growing and distribution (Danyluk et al., 2008), more studies on the inactivation of internalized foodborne pathogens in almonds are required.

In this study, the efficacy of RF treatment and conventional convective heating, especially dry roasting which is somewhat comparable to RF heating in treatment condition were comparable for reducing populations of *S.* Enteritidis, *S.* Typhimurium, and *S.* Senftenberg in raw shelled almonds. Also, the effects of RF heating for controlling internalized pathogens and quality of almonds, including color, peroxide

value, and acid value, were investigated through additional experiments utilizing the same treatments.

I-2. Materials and Methods

Bacterial strains. All bacterial strains, namely, *S*. Enteritidis PT 30 (ATCC BAA-1045), *S*. Typhimurium (ATCC 700408), and *S*. Senftenberg (KVCC 0590) were obtained from the Department of Food and Animal Biotechnology bacterial culture collection of Seoul National University (Seoul, South Korea). These strains were isolated from human or animal. Stock cultures were kept frozen at 80 °C in 0.7 ml of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD) containing 0.3 ml of 50% glycerol. For this study, working cultures were prepared by streaking for isolation onto tryptic soy agar (TSA; Dicfo), incubating at 37 °C for 24 h, and storing at 4 °C.

Preparation of pathogen inocula. For each experiment, inoculum was prepared individually for each strain using the method described by (Danyluk et al., 2005). A loopful (ca. 10 μl) from a single isolated colony of each strain of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg was cultured in 30 ml of TSB at 37 °C for 24 h, then a loopful was transferred into 30 ml of TSB, and incubated at 37 °C for 18 h. For production of a bacterial lawn, 1 ml of the overnight culture was spread onto each of three TSA plates and followed by incubation at 37 °C for 24 h. The bacterial lawn was dislodged by adding 9 ml of 0.2 % peptone water (PW; Difco) to each plate and rubbing with a sterile cotton swab. Cell suspensions were collected from the three

plates and pooled, corresponding to approximately 10^9 CFU/ml. These final suspensions of the three pathogenic serovars were used in this study.

Sample preparation and inoculation. Raw shelled almonds of the variety Nonpareil were purchased from a local grocery store (Seoul, South Korea) and sorted to remove any damaged kernels before being used for experiments. For surface inoculation, 25 ml of prepared inoculum (*S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg) was added to 400-g samples inside sterile stomacher bags (Labphas, Inc., Sainte-Juilie, Quebec, Canada), and then mixed by hand for 1 min. The inoculated samples were dried for 24 h inside a biosafety hood (24 ± 2°C) with the fan running until the moisture content and water activity of the samples equaled those of a noninoculated samples (ca. 4.20 %, dry basis and 0.42, respectively). The final cell concentration was 10^6 to 10^7 CFU/g.

Internal inoculation with foodborne pathogens was performed according to the procedure reported by (Niemira, 2007) using a vacuum perfusion method. Twenty-five milliliters of the inoculum was diluted in 500 milliliters of sterile distilled water. Almonds samples sufficient for one replication were immersed in the combined pathogen suspension and placed in a vacuum oven (OV-11; Jeio tech. Co., Ltd., Daejeon, South Korea). A vacuum was drawn to about 96 kPa in order to pull gas from the intercellular spaces of the almonds. After 4 min, the vacuum was quickly broken, thereby drawing the inoculum into the sample. The vacuum perfusion was

repeated three times to fully perfuse the almonds. For the removal of pathogens on exposed surfaces, the fully perfused almonds were agitated in 500 ml of 300 ppm sodium hypochlorite solution for 3 min, washed with sterile distilled water for 3 min. The internally-inoculated almonds were dried for 24 h in order to ensure the properties of the samples were close to the original almonds, resulting in an internalized cell concentration of 10^5 to 10^6 CFU/g. This method was chosen for almonds by comparing dye (Red No. 40 AR6223; Emerald Performance Materials, Cincinnati, OH) internalizations after dipping for 20 min, syringe injection, and the vacuum perfusion method (Fig. I-1).

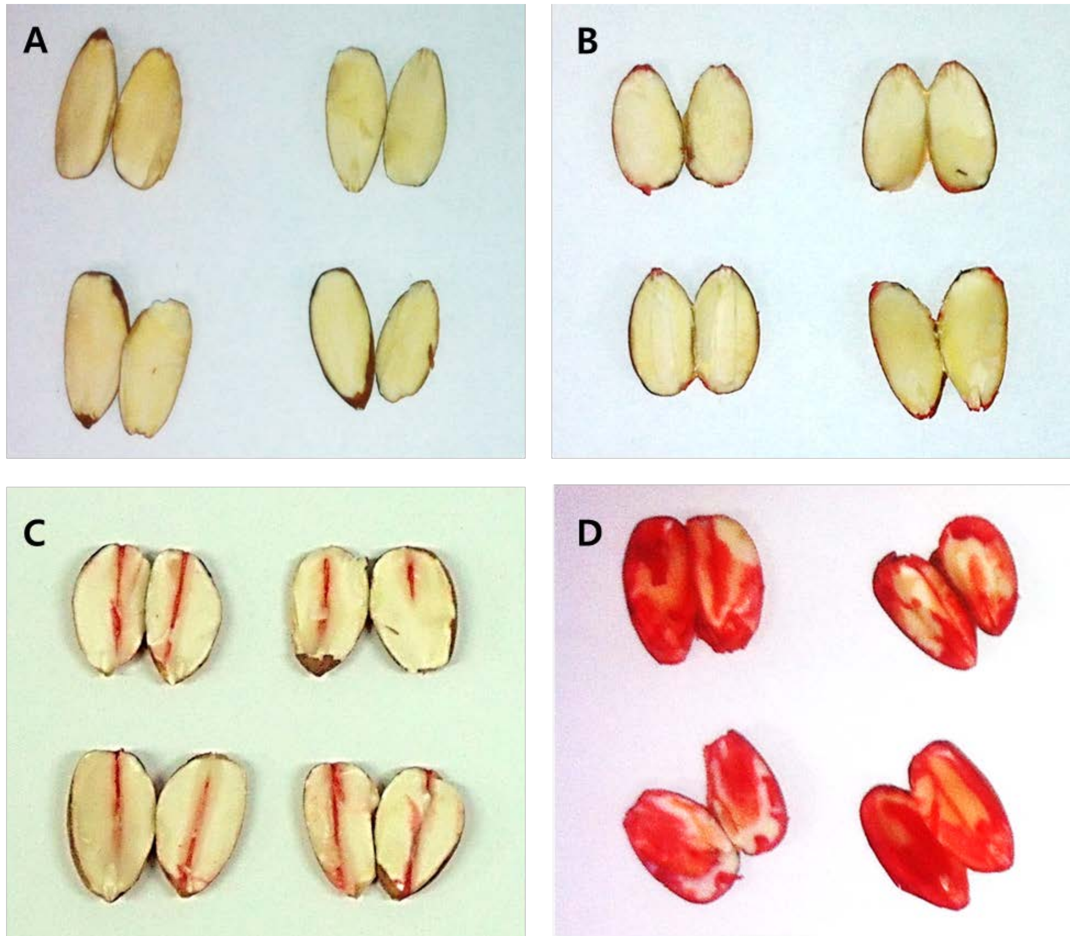


Fig. I-1. Comparison of dye internalization by dipping, syringe injection, and vacuum perfusion method. (A) control; (B) dipping for 20 min; (C) syringe injection; (D) vacuum perfusion at 96 kPa.

Experimental apparatus. The RF heating and conventional convective heating system (Fig. I-2) consisted of a RF heater (Seoul National University, Seoul, South Korea; Dong Young Engineering Co. Ltd., Gyeongnam, South Korea), a convection oven (CK9230; Convex Korea Co. Ltd., Gyeonggi, South Korea), and a temperature signal conditioner (TMI-4; FISO Technologies Inc., Quebec, Canada). The RF electric field with a frequency of 27.12 MHz was generated between two parallel-plate electrodes (30.0 × 35.0 cm; 0.6 cm thick) spaced 5.5 cm apart. The convection oven was composed of four electric resistive emitters arranged horizontally in parallel with the four emitting surfaces facing each other. The heated air in the roasting oven was circulated by fan. The temperature signal conditioner was connected to a computer for control using FISO Commander 2 Control and Analysis Software (FISO Technologies Inc.).

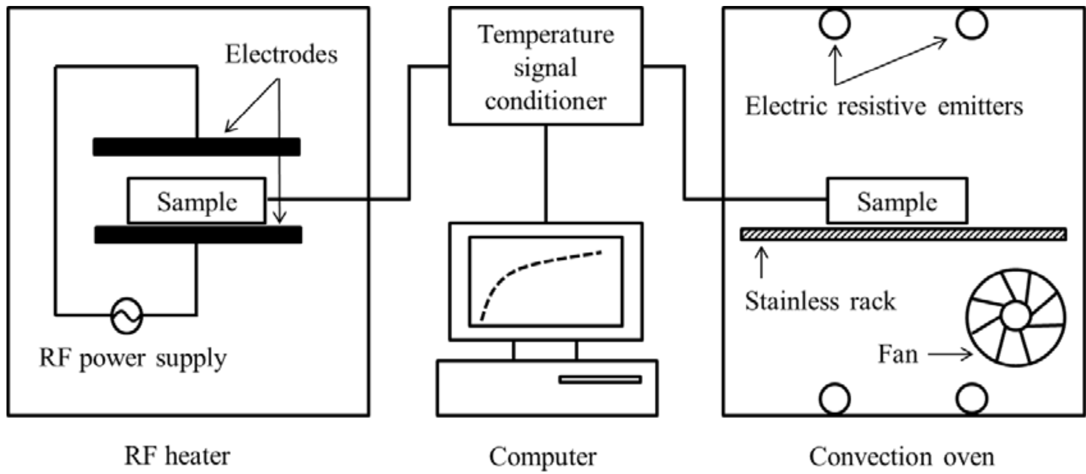


Fig. I-2. Schematic diagram of RF and conventional convective heating system.

RF heating and conventional convective heating treatment. For the RF heating treatment, 25 g of inoculated almonds were placed in a polypropylene jar, 4.5 cm in diameter and 4.0 cm deep (NALGENE 2118-0002; Thermo Scientific, Hudson, NH), which was placed on the center of the bottom electrode. For conventional convective heating, almond samples were spread in a single layer on a sterilized stainless rack located 10 cm from the upper and the lower emitters. A roasting temperature of 150 °C, as conventionally used in industry, was selected (Almond Board of California, 2007). RF and convective heating were applied to each prepared sample and heated to about 100 °C and 150 °C, respectively, in order to maximize the efficacy of pasteurization while maintaining product quality.

Temperature measurement. A fiber optic temperature sensor (FOT-L; FISO Technologies Inc., Quebec, Canada) connected to a temperature signal conditioner was used to measure real-time temperatures in samples during RF and convective heating. The sensor was placed directly on the surface of the non-inoculated almonds or inserted into the center of the kernel located in middle, and the temperature was recorded at 5 s intervals. In the case of convective heating, the temperature was recorded at 10 s intervals after 40 s of treatment. The fiber optic did not interfere with the temperature profile of the treated sample, since it was coated with electric insulating material (Wang et al., 2003). The temperature values of RF and convective heated samples were compared to determine the heating rate.

Bacterial enumeration. For enumeration of pathogens, 25 g of treated almonds were placed at room temperature for 30 s, transferred immediately into sterile stomacher bags containing 100 ml of 0.2% PW pre-chilled in a 4 °C refrigerator (detection limit, 0.7 log CFU/g), and homogenized for 2 min with a stomacher (Easy Mix; AES Chemunex, Rennes, France). After homogenization, 1-ml aliquots of sample were serially diluted in 9-ml blanks of 0.2% PW, and 0.1 ml of sample or diluent was spread-plated onto a selective medium, xylose lysine desoxycholate agar (XLD; Oxoid, Ogdensberg, NY), for enumeration of *S. enterica*. Where low levels of surviving cells were anticipated, 1 ml of undiluted homogenate was equally divided onto four plates of medium and spread-plated. All agar plates were incubated at 37 °C for 24 h, and typical black colonies were counted. To confirm identity of the pathogens, colonies randomly selected from the enumeration plates were subjected to the *Salmonella* latex agglutination assay (Oxoid).

Enumeration of heat-injured cells. The overlay (OV) method was used to enumerate heat-injured cells of *S. enterica* using TSA as a nonselective medium for recovery of injured cells (Lee and Kang, 2001). Appropriate dilutions were spread-plated onto TSA medium and incubated at 37 °C for 2 h to allow heat-injured microorganisms to repair, and then 7 to 8 ml of XLD selective medium was overlaid on the plates. After solidification, plates were further incubated for an additional 22 h at 37°C and black colonies were enumerated. Preliminary experiments verified that

the 2-h incubation recovery period on TSA did not result in multiplication of uninjured cells in the control samples (data not shown).

Quality measurement. To evaluate the effect of RF heating on quality during storage, changes in color, peroxide value, and acid value were measured. The accelerated shelf life tests were conducted in a temperature and humidity chamber (TH-TG-300; Jeio tech. Co., Ltd.) at 35 °C and 30% relative humidity for 10 and 20 days. Storage conditions of 1 and 2 years at 4 °C were chosen based on commercial practices using a Q_{10} value of 3.4 for lipid oxidation and validated by real-time storage experiments (Taoukis et al., 1997; Wang et al., 2006). Kernel skin and core color of RF-treated and untreated uninoculated almonds were measured at random locations using a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan). The values of L*, a*, and b* were used to quantify color attributes and indicate lightness, redness, and yellowness of the sample, respectively.

The peroxide value and acid value were determined by the oil extracted from the almond samples using a solvent recovery extractor (4002842; JP Selecta S.A., Barcelona, Spain) and tested according to AOCS official methods Cd 8-53 and Cd 3a-63, respectively. After titration of the almond oil in acetic acid/chloroform solutions (3:2 [v/v]) with 0.1 N sodium thiosulfate, the peroxide value was calculated by the following equation (1):

$$\mathrm{PV} = \frac{(S-B)\times N_1\times 1000}{W} \qquad (1)$$

where PV is the peroxide value (meq/kg), S and B is consumption of 0.1 N sodium thiosulfate (ml) at the end point for the sample and the blank, respectively, N_1 is the normality of sodium thiosulfate, and W is the weight of the almond oil (g).

The acid value was calculated by equation 2, based on the titration of the almond oil in ether/ethanol solutions (1:1 [v/v]) with 0.1 N potassium hydroxide.

$$\mathrm{AV} = \frac{V \times N_2 \times 56.11}{W} \qquad (2)$$

where AV is the acid value (%), V is consumption of 0.1 N potassium hydroxide (ml) at the end point for the sample, and N_2 is the normality of potassium hydroxide.

Statistical analysis. All experiments were performed in triplicate. Data were analyzed by the analysis of variance procedure and Duncan's multiple-range test of the Statistical Analysis System (SAS Institute, Cary, NC). A P value of < 0.05 was used to determine significant differences.

I-3. Results

Temperature curves of almonds. Average surface and center temperatures of almonds during RF and conventional convective heating are shown in Fig. I-3. The rate of RF heating was much higher than that of conventional convective heating on the almond surface, especially the initial heating rate. The surface temperature increased immediately in response to the RF electric field when the almond samples were subjected to RF heating, while it began to rise after approximately 30 s of convective heating. During 40 s of RF heating, almond surface temperature reached 93°C. For convective heating, the mean time taken to reach 93 °C was ca. 180 s. The overall patterns of temperature increase for almond centers were similar to those for almond surfaces. The center temperature reached 97 °C after ca. 35 s and 320 s of RF and convective heating, respectively. Furthermore, ca. 70 s was required for the initiation of temperature increase during convective heating.

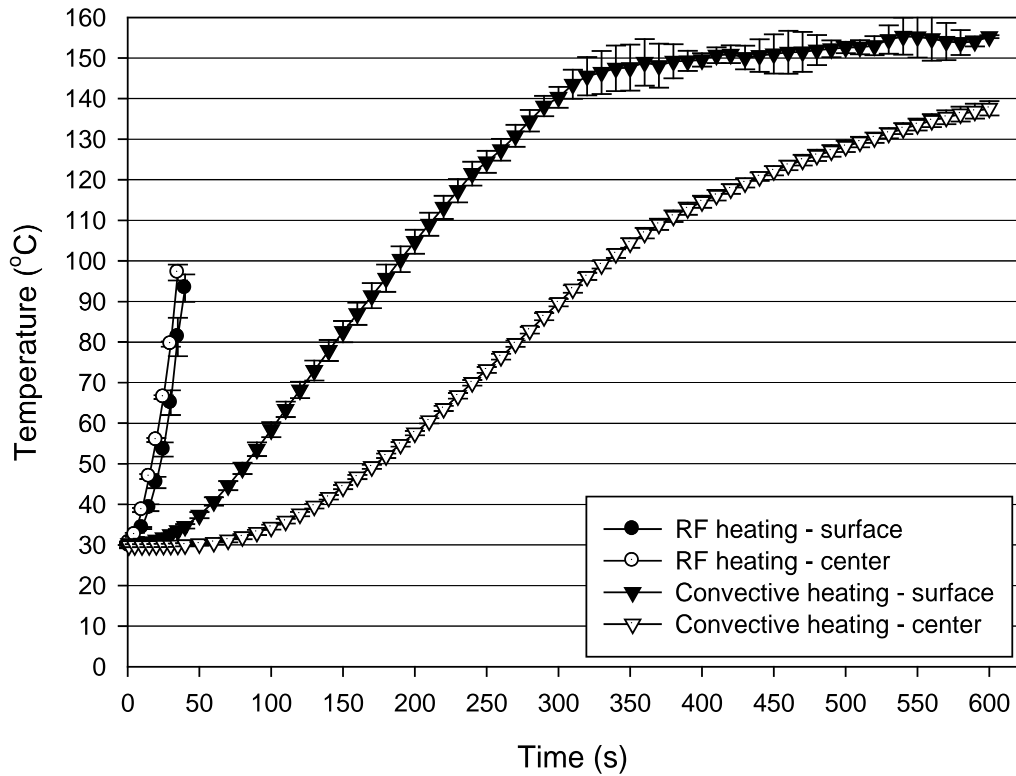


Fig. I-3. Temperature curves of surface and center of almonds during RF and conventional convective heating. The results are means from three experiments, and error bars indicate standard deviations.

Survival curves of foodborne pathogens. Populations (log CFU/g) of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg on almond surfaces during RF and convective heating are shown in Fig. I-4. Significant ($P < 0.05$) log reductions of the three pathogens were observed after 20 s of RF heating and 300 s of convective heating. RF heating for 40 s achieved 3.70-, 5.98-, and 5.59-log reductions in *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg, respectively, whereas convective heating did not attain comparable reductions even at the end of treatment. After 600 s of convective heating, levels of these pathogens were reduced by 1.73, 2.51, and 3.68 log CFU/g, respectively. The reduction of *S*. Enteritidis was significantly ($P < 0.05$) smaller than that of *S*. Typhimurium and *S*. Senftenberg during both heating treatments.

The vacuum perfusion method effectively introduced the inoculum into almond kernels (Fig. I-1). Although dye was an unreliable indicator of bacterial penetration due to the difference in sizes, dye uptake experiments provide a simple and practical means of comparing internalization methods. Fig. I-5 shows the different effect of RF and convective heating on inactivation of internally inoculated *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg. The reduction patterns of the three pathogens in almonds were similar to those of the surface-inoculated pathogens. RF heating yielded about a 290 s shorter initiation time of significant ($P < 0.05$) reduction compared to convective heating. The numbers of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg were greatly reduced to undetectable levels after RF heating for 30 s.

However, convective heating only reduced *S*. Senftenberg to below the detection limit after the maximum treatment of 600 s. Log reductions of 2.55 and 2.98, respectively, were observed in *S*. Enteritidis and *S*. Typhimurium at the same treatment time.

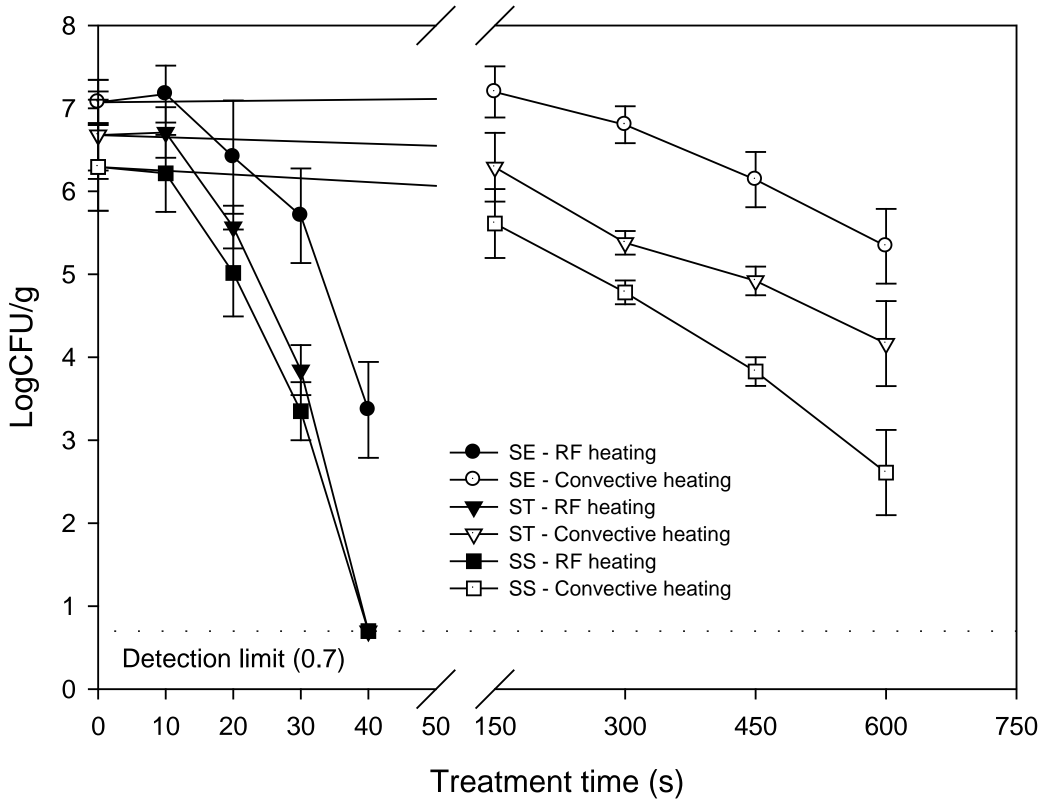


Fig. I-4. Survival curves for *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Senftenberg on almond surfaces treated with RF or conventional convective heating. The results are means from three experiments, and error bars indicate standard deviations.

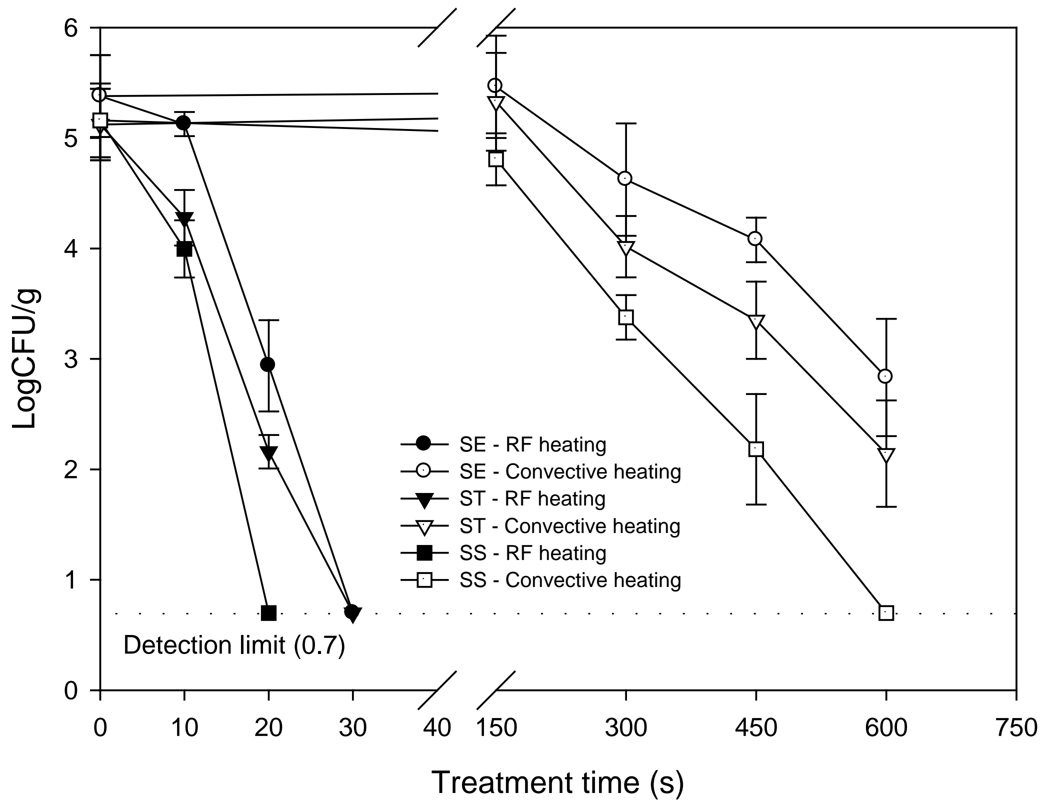


Fig. I-5. Survival curves for *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Senftenberg inside of almonds treated with RF or conventional convective heating. The results are means from three experiments, and error bars indicate standard deviations.

Recovery of heat-injured cells. Table I-1 shows numbers of surviving cells, including heat-injured *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg, on the surfaces and insides of almonds following RF heating. When inoculated almonds were treated with RF heating, slightly higher numbers of these pathogens were detected on the agar for recovery (OV-XLD) than on the selective agar (XLD). Especially, after 20 s corresponding to approximately 50 °C, there were differences in microbial levels between XLD and OV-XLD, resulting in injured populations on surface- and internally-inoculated almonds of 0.26 and 0.21 log CFU/g, respectively, for *S*. Enteritidis; and 0.08 and 0.13 log CFU/g, respectively, for *S*. Typhimurium. Overall, lower reductions of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg were observed at various stages of RF treatment by the injured cell recovery procedure than by direct plating on selective media. However, at all treatment time intervals, no significant ($P > 0.05$) differences between levels of surviving heat-injured and uninjured cells were observed in raw shelled almonds.

Table I-1. Comparison of pathogen populations between uninjured cells and cells including heat-injured cells on surface- or internally-inoculated almonds following RF heating[a]

Inoculation type and treatment time (s)	Population (log_{10} CFU/g) by organism and selective medium					
	S. Enteritidis		*S.* Typhimurium		*S.* Senftenberg	
	XLD[b]	OV-XLD	XLD	OV-XLD	XLD	OV-XLD
Surface						
0	7.07 ± 0.27 Aa	7.54 ± 0.25 Aa	6.68 ± 0.43 Aa	7.02 ± 0.22 Aa	6.29 ± 0.53 Aa	5.97 ± 0.14 Aa
10	7.17 ± 0.34 Aa	7.63 ± 0.44 Aa	6.71 ± 0.31 Aa	7.07 ± 0.30 Aa	6.22 ± 0.46 Aa	5.99 ± 0.31 Aa
20	6.41 ± 0.68 ABa	6.67 ± 0.86 Aa	5.57 ± 0.26 Ba	5.65 ± 0.61 Ba	5.02 ± 0.52 Ba	5.05 ± 0.40 Ba
30	5.71 ± 0.57 Ba	5.52 ± 0.82 Ba	3.85 ± 0.30 Ca	4.27 ± 0.52 Ca	3.35 ± 0.35 Ca	3.13 ± 0.51 Ca
40	3.37 ± 0.58 Ca	3.26 ± 0.42 Ca	ND[c]	ND	ND	ND
Internal						
0	5.38 ± 0.37 Aa	5.60 ± 0.43 Aa	5.12 ± 0.32 Aa	4.96 ± 0.23 Aa	5.16 ± 0.33 Aa	5.15 ± 0.26 Aa
10	5.13 ± 0.11 Aa	5.46 ± 0.19 Aa	4.28 ± 0.25 Ba	4.41 ± 0.37 Aa	4.00 ± 0.26 Ba	4.22 ± 0.45 Ba
20	2.94 ± 0.41 Ba	3.15 ± 0.45 Ba	2.16 ± 0.15 Ca	2.28 ± 0.51 Ba	ND	ND
30	ND	ND	ND	ND	ND	ND

[a] Means ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] XLD, xylose lysine desoxycholate; OV-XLD, overlay XLD agar on TSA.
[c] ND, below detection limit (0.7 log CFU/g).

Effect of RF heating on product quality. Color values of kernel skin and almond cores after RF heating for the time interval (40 s) required to achieve maximum reduction of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg are summarized in Table I-2. The L^*, a^*, and b^* values of RF-treated samples were not significantly ($P > 0.05$) different from those of untreated samples during the entire storage time. Table I-3 shows the lipid oxidation parameters of almonds following RF treatment. There were no significant ($P > 0.05$) differences in PV and AV between untreated and treated almonds. Although they varied slightly in accordance with RF heating at several storage times, no statistically significant differences ($P > 0.05$) were detected between any of the tested samples. Therefore, RF heating for 40 s did not degrade the quality of raw shelled almonds.

Table I-2. Kernel skin and core color values of RF-treated and untreated almonds stored at 35 °C and 30% relative humidity for 20 days[a]

Parameter and treatment type	Storage time (days) at 35 °C and 30% relative humidity[b]		
	0	10	20
Kernel skin color[c]			
L^*			
Control	48.58 ± 0.72 a	48.02 ± 1.27 a	49.29 ± 1.64 a
RF treated	48.25 ± 0.83 a	48.32 ± 0.69 a	48.32 ± 0.87 a
a^*			
Control	15.07 ± 0.41 a	14.55 ± 0.32 a	15.47 ± 0.51 a
RF treated	15.58 ± 0.86 a	15.53 ± 1.11 a	15.72 ± 0.23 a
b^*			
Control	32.08 ± 0.67 a	32.38 ± 0.80 a	32.01 ± 1.52 a
RF treated	32.20 ± 0.93 a	32.35 ± 0.73 a	32.40 ± 0.42 a
Kernel core color (L^*)[d]			
Control	75.43 ± 0.49 a	76.04 ± 1.37 a	75.53 ± 1.45 a
RF treated	75.70 ± 1.05 a	75.40 ± 0.79 a	75.24 ± 0.45 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 10 and 20 days at 35 °C and 30% relative humidity to simulate 1 and 2 years storage at 4 °C, respectively.
[c] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).
[d] Accepted L^* values for good quality are more than 40.

Table I-3. Peroxide values and acid values of RF-treated and untreated almonds stored at 35 °C and 30% relative humidity for 20 days[a]

Parameter and treatment type	Storage time (days) at 35 °C and 30% relative humidity[b]		
	0	10	20
Peroxide value (meq/kg)[c]			
Control	0.40 ± 0.00 a	1.46 ± 0.46 a	1.53 ± 0.58 a
RF treated	0.40 ± 0.00 a	1.12 ± 0.12 a	1.56 ± 0.55 a
Acid value (%)[c]			
Control	0.61 ± 0.08 a	0.62 ± 0.05 a	0.67 ± 0.03 a
RF treated	0.63 ± 0.11 a	0.67 ± 0.02 a	0.67 ± 0.03 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 10 and 20 days at 35 °C and 30% relative humidity to simulate 1 and 2 years storage at 4 °C, respectively.
[c] Accepted PV and AV for good quality are less than 5 meq/kg and 1.5%, respectively.

I-4. Discussion

In the present study, RF heating resulted in a much more rapid heating rate compared with conventional convective heating. This result is in agreement with earlier research by (Wang et al., 2003) who reported that lethality was achieved in macaroni and cheese with the use of RF processing within shorter times than with conventional retort processing. Similar results were observed in ground beef and ready-to-eat aquatic foods such as caviar heated by RF and a water bath (Al-Holy et al., 2004; Guo et al., 2006). Although it has been reported that there was no non-thermal effect of RF energy on microbial inactivation (Geveke et al., 2002; Ponne et al., 1996), we determined that RF treatment produced significantly greater reduction of all tested pathogens even at the same temperature as convective heating. This is in agreement with the effect of heating rate on inactivation of pathogens. The behavior of bacteria in foods that are heated slowly may mimic heat shock response, resulting in increased thermal resistance (McCleery and Rowe, 1995; Stephens et al., 1994; Wesche et al., 2009).

Besides rapid heating, uniformity is one of the advantages of RF heating. Guo et al. (2006) concluded that the temperature variation in RF heating between the surface and center of ground beef was much lower than that achieved in water-bath heating. This result was similar to my data which showed that the gap in treatment time required for the surface and center of almonds to reach the same temperature (93 °C)

was only 5 s for RF heating; however, the time gap within samples was 140 s in conventional convective heating, indicating surface overheating of the samples. Also, maximum temperature of RF-treated almonds was much lower than that of conventionally heated almonds. These patterns reflect the means of RF heating and the nature of heat transfer. Unlike other heat transfer modes, RF heating is an internal heating process resulting from the direct interaction between electromagnetic waves and foods. The energy conversion from electrical energy to heat occurs within the food itself and generates relatively uniform heating (Zhao et al., 2000). Conversely, conventional heating requires heat energy that is generated externally, and heat is transferred to the food product by convection, conduction, radiation, or a combination thereof. Because of these heat transfer modes, heating uniformity is decreased which results in cold spots in the food which could permit survival of harmful microorganisms (Doores, 2002).

In recent years, numerous research investigations on internalization of foodborne pathogens within various forms of fresh produce have been conducted. These studies have reported that pathogenic bacteria can be introduced and possibly infiltrate into fresh fruits and vegetables following pre-harvest and/or post-harvest processes (Barak et al., 2011; Beuchat and Mann, 2010; Danyluk et al., 2008; Franz et al., 2007; Mitra et al., 2009). Internalization of *S.* Enteritidis PT 30 into almond kernels was detected by confocal laser scanning microscopy (Danyluk et al., 2008). In 1999, the FDA declared that conventional surface decontamination treatments are generally

ineffective in reducing internalized pathogens. However, no researchers have proposed any decontamination methods in an effort to inactivate internalized *Salmonella* in almonds. In this study, as with the comparison of efficacy of RF and convective heating for controlling surface-adhering pathogens, RF heating significantly decreased the treatment time required to reduce internalized *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg in almonds to below detectable levels. During RF heating, the heating rate was much higher at the center than on the surface of almonds. These results are in agreement with the internal generation of heat within the product caused by molecular friction.

Strains of *Salmonella* may survive high temperatures within low-moisture foods such as nuts, powdered milk, chocolate, peanut butter, and cereal (Doyle and Mazzotta, 2000). Among them, *S*. Enteritidis PT 30 implicated in the outbreak was found to be quite resistant to dry heat compared to other strains evaluated on almonds. Although the cause of its high resistance is still not fully understood, Parker et al. (2010) determined that *S*. Enteritidis PT 30 metabolized L-aspartic acid, L-glutamic acid, L-proline, L-alanine, and D-alanine amino acids efficiently. Their metabolites are required for protein synthesis which may be involved in enhancing cellular resistance (Wesche et al., 2009; Wu, 2009). Some typical industry roasting processes did not achieve a minimum 4-log reduction of *S*. Enteritidis PT 30 (Danyluk et al., 2006). Therefore, the Almond Board of California (ABC) identified *S*. Enteritidis PT 30 as the target pathogen of almonds for the validation test of dry roasting processes

(Almond Board of California, 2007). In the present study, although *S*. Enteritidis PT 30 showed more resistance to RF heating than did *S*. Typhimurium and *S*. Senftenberg, a greater than 4-log reduction was confirmed in raw shelled almonds after RF heating. For conventional convective heating, *S*. Enteritidis PT 30 populations decreased approximately 2-logs.

Even though RF heating was highly effective in reducing foodborne pathogens in almonds, the occurrence of sub-lethally injured cells in RF-treated samples should be considered. Sub-lethal thermal injury may occur during thermal treatments that require extended come-up time (Bunning et al., 1990). Heat-injured cells are potentially as dangerous as their uninjured counterparts because they can undergo recovery and become functionally normal under favorable environmental conditions (Lee and Kang, 2001; McCleery and Rowe, 1995). Therefore, sub-lethally injured pathogens in almonds were assessed by plating on selective agar with and without a recovery step. There were no significant ($P > 0.05$) differences between injured cells and uninjured cells in surface- and internally-inoculated almonds after the maximum RF treatment of 40 s and 30 s, respectively. This suggests that RF heating effectively inactivated *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg in raw almonds without generating heat-injured cells during the short come-up time.

It is necessary to investigate the quality changes occurring during RF heating for commercial practical application of this highly appealing technology. Because of the potential of almonds to be affected by elevated temperature during RF treatment, the

quality parameters in this study included color values (L^*, a^*, and b^*) of kernel skin and core, PV, and AV used for indicators of possible lipid oxidation. After the maximum treatment applied for inactivation of foodborne pathogens, all tested parameters were not significantly ($P > 0.05$) different from those of the control. These values slightly varied in both untreated and RF-treated almonds during the storage period. However, the quality of almonds stored at 35 °C for 20 days, which simulated two-year storage at 4 °C, remained within the acceptable range ($L^* > 40$, PV < 5 meq/kg, and FA < 1.5%) according to the ABC's standard. These results indicate that RF heating can be applied to control pathogens in raw shelled almonds without affecting product quality during storage.

Industrial scale RF heating for reducing foodborne pathogens in almonds should be based on commercial validation. Pathogen inactivation during RF heating is dependent on sample moisture content, salt content, density, temperature, and certain other factors (Jeong and Kang, 2014; Orsat and Raghavan, 2005). Further studies to enhance the effect of inactivation and shorten the RF treatment time for minimization of quality changes are required. In conclusion, my results indicate that RF heating leads to effective inactivation of *S*. Enteritidis, *S*. Typhimurium, and *S*. Senftenberg in raw shelled almonds without degrading quality. RF heating could be applied to control microbiological contamination in almonds over conventional pasteurization methods.

Chapter II.

Intrinsic and extrinsic factors affecting antimicrobial effect of RF heating against foodborne pathogens

II-1. Effect of salt content on inactivation of foodborne pathogens in pistachios by RF heating

II-1.1. Introduction

In recent years, there have been increasing concerns about the microbial safety of pistachios, because salmonellosis has been known to be linked to pistachios. A multistate outbreak of *Salmonella* serovars Senftenberg and Montevideo infections associated with pistachios occurred in the United States in 2016. During this outbreak, a reported 11 cases in nine states were identified (Centers for Disease Control and Preservation, 2016). In 2009 and 2013, pistachios were recalled after isolation of several serotypes of *Salmonella enterica* (Centers for Disease Control and Preservation, 2009a; U.S. Food and Drug Administration, 2014). *Salmonella* cannot regenerate on low water activity (a_w) foods such as pistachios, but can survive in these environments for prolonged periods of time (Al-Moghazy et al., 2014; Harris et al., 2016; Kotzekidou, 1998).

Following harvest and de-hulling, pistachios are dried to a moisture content of less than 7% wet basis in order to avoid shell staining, decay, and microbial growth (Ferguson et al., 2005). Although various approaches, including sun drying and forced-air drying, are used for commercial postharvest processing of pistachios, these practices do not guarantee microbiological safety in the light of outbreaks and recalls due to *Salmonella* contamination of pistachios (Ghazanfari et al., 2003; Kouchakzadeh and Tavakoli, 2011; Kouchakzadeh, 2013). Several decontamination methods have been investigated to reduce microbial populations on pistachios, such

as chemical sanitizers, irradiation, and various heat processes involving infrared heating and superheated steam. However, these treatments have drawbacks, including unhealthful residues, poor consumer perception, difficulty of scaling up for commercial applications, and complicated operation (Ban and Kang, 2016; McEgan and Danyluk, 2015; Venkitasamy et al., 2017; Zare et al., 1993). Therefore, the development of new pasteurization technologies for pistachios is needed.

Radio-frequency (RF) heating has been studied as a potential novel thermal treatment because of its more rapid and uniform heating than conventional heating (Geveke et al., 2017; Guo et al., 2006; Ha et al., 2013; Kim et al., 2012). RF energy can directly interact with foods and generate heat within the materials due to molecular friction (Marra et al., 2009). Dielectric properties should be taken into account in order to prevent improper heating resulting in cold spots or product burning, since these properties of foods influence their heating rate during RF treatment. These properties are influenced by sample salt content, moisture content, density, temperature, frequency of the applied electromagnetic waves, and a few other factors (Datta et al., 2014). Among them, in the present study, salt content was selected as a factor to investigate the effects of RF heating on pistachios. This is because pistachios are commercially marketed worldwide as light, medium, and strongly salted nuts (Tsantili et al., 2010).

Although several research studies have reported on the dielectric properties of nuts such as hazelnuts, macadamia nuts, and peanuts at different frequencies, temperatures,

and moisture content, very little is known about the impact of salt content on dielectric properties (Boldor et al., 2004; Wang et al., 2013; Zhu et al., 2014). Ling et al. (2015) found that dielectric properties of pistachios was affected by salt content, but that study did not assess heating rate and microbial inactivation. Also, the effect of RF heating on product quality at different salt levels has not been reported. It is necessary to investigate the impact of salt content on the heating rate of pistachios in order to optimize process without loss of product quality when applying RF heating.

The objectives of this research were to investigate the impact of salt content of pistachios during RF heating on heating rate, dielectric properties, and inactivation of *Salmonella enterica*. The effect of RF heating on the quality of pistachios with varying salt content, including color and degree of lipid oxidation was also examined.

II-1.2. Materials and Methods

Bacterial strains. All bacterial strains, namely, *S.* Enteritidis PT 30 (ATCC BAA-1045), *S.* Typhimurium (ATCC 19585), and *S.* Senftenberg (KVCC 0590) were obtained from the Department of Food and Animal Biotechnology bacterial culture collection of Seoul National University (Seoul, South Korea). Stock cultures were kept frozen at – 80 °C in 0.7 ml of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD) containing 0.3 ml of 50% glycerol (vol/vol). For this study, working cultures were prepared by streaking for isolation onto tryptic soy agar (TSA; Difco), incubating at 37 °C for 24 h, and storing at 4°C.

Preparation of pathogen inocula. For each experiment, inoculum was prepared according to the method described by Danyluk et al. (2005) with minor modifications. All strains of *S. enterica* were cultured individually in 30 ml of TSB at 37 °C for 24 h, then a loopful (10 μL) was transferred into 30 ml of TSB and incubated at 37 °C for 18 h. For production of a bacterial lawn, 1 ml of overnight culture was spread onto each of three TSA plates and incubated at 37 °C for 24 h. The bacterial lawn was harvested by adding 9 ml of 0.2 % peptone water (PW; Difco) to each plate and rubbing with a sterile cotton swab. Subsequently, cell suspensions of the three serovars of *S. enterica* were combined to produce mixed-culture cocktails. These cocktails at a final concentration of approximately 10^9 CFU/ml were used in this study.

Sample preparation and inoculation. Three types of commercially processed pistachios were purchased from a retail supermarket (Seoul, South Korea). The composition of these pistachios is shown in Table II-1. These products had the same ingredient contents except for three levels of salt: 0, 100, and 330 mg sodium/serving. For inoculation, 25 ml of prepared inoculum was added to 400-g samples inside sterile stomacher bags (Labphas, Inc., Sainte-Juilie, Quebec, Canada). The inoculated samples were thoroughly mixed by hand for 1 min and dried for 24 h inside a biosafety hood (24 ± 2°C) with the fan running until the moisture content of the samples equaled that of noninoculated samples (ca. 2.2 %, dry basis). The final cell concentration was 10^7 to 10^8 CFU/g.

Table II-1. Formulation of three different pistachios used in this study

Ingredient	No salt	Lightly salted	Highly salted
	Amount[a]	Amount	Amount
Calories (kcal)	180	180	180
Total carbohydrate (g)	9	9	9
Protein (g)	6	6	6
Total fat (g)	13	13	13
Cholesterol (mg)	0	0	0
Sodium (mg)	0	100	330
Potassium (mg)	302	302	302
Calcium (mg)	32	32	32
Iron (g)	1.2	1.2	1.2

[a] Serving size is 30 g.

Experimental apparatus. The RF heating and dielectric measurements were performed in a previously described apparatus (Jeong and Kang, 2014). A RF heater with a maximum power of 9 kW at a frequency of 27.12 MHz was developed by Seoul National University (Seoul, Korea) and Dong Young Engineering Co. Ltd. (Gyeongnam, Korea). The RF electric field was generated between two parallel-plate electrodes (30.0 × 35.0 cm; 0.6 cm thick) and the electrode gap was 7.0 cm. The dielectric measurement system consisted of a liquid test fixture (16452A; Agilent Technologies, Palo Alto, CA) and a precision impedance analyzer (4294A; Agilent Technologies). The liquid test fixture consisted of two parallel-plate electrodes in contact with the liquid or powder sample and the distance between the two electrodes was 1.0 mm. It was connected to the precision impedance analyzer by a port extension cable (16048G; Agilent Technologies). The precision impedance analyzer measured electrical data of the samples which was used to calculate dielectric properties of the sample.

RF heating treatment. For the RF heating treatment, 25 g of inoculated pistachios in a 4.5 cm diameter and 4.0 cm deep polypropylene jar (NALGENE 2118-0002; Thermo Scientific, Hudson, NH), was placed on the center of the bottom electrode. RF heating was applied to each prepared sample and heated to 90 °C in order to maximize the effectiveness of pasteurization while maintaining product quality. The temperature of pistachio samples was measured using a fiber optic temperature sensor

(FOT-L; FISO Technologies Inc., Quebec, Canada) connected to a temperature signal conditioner (TMI-4; FISO Technologies Inc., Quebec, Canada). The sensor was inserted into a pistachio kernel located in the center of the sample through a predrilled hole, and the temperature was recorded every 5 s. The fiber optic sensor did not interfere with the temperature profile of the sample, since it was coated with electric insulating material. The heating rate was calculated by dividing the difference between the beginning and final temperature by the treatment time.

Dielectric properties measurement. The dielectric properties of pistachios were determined by the parallel plate method in ASTM D150. Ground samples were prepared for using this method, since pistachio kernels did not make close contact with the flat plate due to their uneven shape (Guo et al., 2008; Ling et al., 2015). Dielectric measurements of pistachios were taken at 27.12 MHz. The procedure was as follows: the precision impedance analyzer was calibrated using a 100 Ω resistor (04294-61001; Agilent Technologies), and then air capacitance of the test fixture was measured. About 2 g of sample was placed into the test fixture and the sample capacitance and equivalent parallel resistance were measured automatically at room temperature (22 ± 2°C). The dielectric properties of pistachio kernels were calculated as follows (equation 1 and 2):

$$\varepsilon' = \frac{C_p}{C_0} \qquad (1)$$

where ε' is the dielectric constant, C_p is the pistachio capacitance (pF), and C_0 is the air capacitance (pF).

$$\varepsilon'' = \frac{1}{C_0 R_p \omega} \qquad (2)$$

where ε'' is the dielectric loss factor, C_0 is the air capacitance (pF), R_p is the equivalent parallel resistance (Ω), and ω is the angular frequency (2πf).

Bacterial enumeration. For enumeration of pathogens, 25 g of treated pistachios were immediately transferred into sterile stomacher bags containing 100 ml of 0.2% PW pre-chilled in a 4 °C refrigerator (detection limit, 0.7 log CFU/g). After homogenization for 2 min with a stomacher (Easy Mix; AES Chemunex, Rennes, France), 1-ml aliquots of sample were serially diluted in 9-ml blanks of 0.2% PW, and 0.1 ml of sample or diluent was spread-plated onto a selective medium, xylose lysine desoxycholate agar (XLD; Oxoid, Ogdensberg, NY), for enumeration of *S. enterica*. When low levels of surviving cells were anticipated, 1 ml of stomacher bag contents was equally divided onto four plates of medium and spread-plated. All agar plates were incubated at 37 °C for 24 h, and typical black colonies were counted. To confirm identity of the pathogens, colonies randomly selected from the enumeration plates were subjected to the *Salmonella* latex agglutination assay (Oxoid), a serological test.

Enumeration of heat-injured cells. The overlay (OV) method was used to enumerate heat-injured cells of *S. enterica.* TSA was used as a nonselective medium

to recover injured cells (Lee and Kang, 2001). One hundred microliters of appropriate dilutions were spread-plated onto TSA medium and incubated at 37 °C for 2 h to allow heat-injured cells to recover. Plates were then overlaid with 7 to 8 ml of XLD selective medium. After solidification, plates were further incubated for an additional 22 h at 37 °C. Following incubation, presumptive colonies of *S. enterica* with black colonies were enumerated.

Quality measurement. To evaluate the effect of RF heating on product quality, changes in color, peroxide value, and acid value were measured. Color assessments were measured at random locations on treated and untreated pistachios using a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan). CIELAB color space using illuminant C and 2° viewing angle were used to quantify color attributes. The values of L*, a*, and b* indicate lightness, redness, and yellowness of the sample, respectively.

Indicators of lipid oxidation in RF treated pistachios were measured by peroxide value and acid value. These values were determined by the oil extracted from the pistachio samples using a solvent recovery extractor (4002842; JP Selecta S.A., Barcelona, Spain) and tested according to official methods Cd 8-53 and Cd 3a-63 of the American Oil Chemists' Society, respectively.

Statistical analysis. All experiments were performed in triplicate. Data were analyzed by the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC). Means were separated using Duncan's multiple-range test, and a P value of < 0.05 was used to indicate significant differences.

II-1.3. Results

Temperature curves of pistachios with different salt contents. Average temperatures of pistachios with salt content varying from 0 to 330 mg sodium/serving during RF heating at a constant frequency of 27.12 MHz are shown in Fig. II-1. At the same salt level, the temperature rapidly increased with increasing treatment time. The heating rate of pistachios was dependent on salt content up to 100 mg sodium/serving, but there was no significant ($P > 0.05$) difference when salt content exceeded the above levels. Salted pistachios with 100 and 330 mg sodium/serving increased from 29.6 °C to 90.7 °C when exposed to RF energy for 40 s. For the same treatment time, the temperature of non-salted pistachios was 61.3 °C.

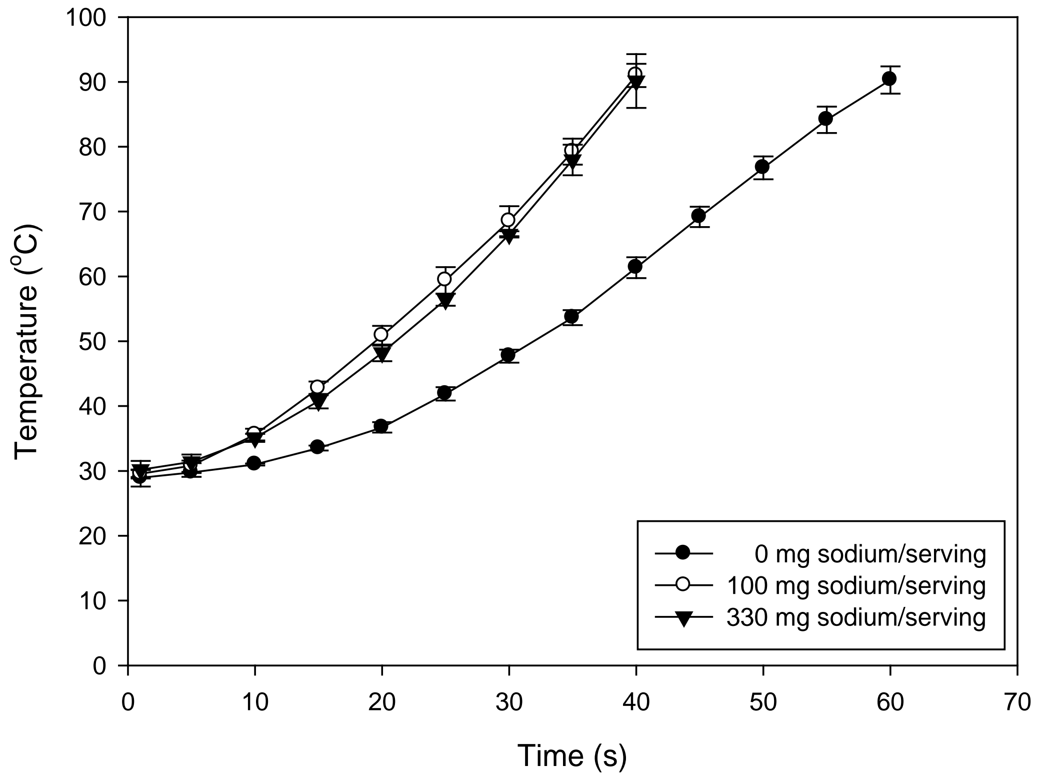


Fig. II-1. Average temperature-time histories of pistachios during RF heating as influenced by salt levels. The results are means from three experiments, and error bars indicate standard deviations.

Effect of salt content on dielectric properties of pistachios. The dielectric properties of pistachios at each of the three salt levels and at a fixed frequency of 27.12 MHz are listed in Table II-2. Both dielectric constants and dielectric loss factors of pistachios significantly ($P < 0.05$) increased with increasing salt content from 0 to 330 mg sodium/serving. High dependence on salt content was shown not in dielectric constants but in dielectric loss factors of the samples. The dielectric loss factors of pistachio kernels varied slightly at the beginning of salt-enriching, however, those greatly increased as salt content increased.

Table II-2. Dielectric properties of pistachios with varying salt content at 27.12 MHz[a]

Salt content (mg sodium/serving)	Dielectric properties	
	ε'[b]	ε''
0	10.37 ± 0.64 c	5.33 ± 0.22 c
100	15.34 ± 0.20 b	15.83 ± 0.29 b
330	23.78 ± 0.38 a	42.83 ± 2.20 a

[a] Means ± standard deviations from three replications. Values followed by different letters within the column are significantly different ($P < 0.05$).
[b] ε' is the dielectric constant and ε'' is the dielectric loss factor.

Relationships between heating rate, dielectric loss factor, and salt content of pistachios. Fig. II-2 demonstrates tripartite relationships between the rate of temperature increase, dielectric loss factor, and moisture contents of pistachios. As shown in Table II-2, the dielectric loss factor was dependent on salt content. The heating rate was significantly ($P < 0.05$) proportional to salt content up to 100 mg sodium/serving for pistachios as shown in Fig. II-1. The patterns of relationship between the heating rate and the dielectric loss factor were similar to those between the heating rate and salt content. The rate of temperature increase was maintained above a dielectric loss factor of 15.83 in pistachios.

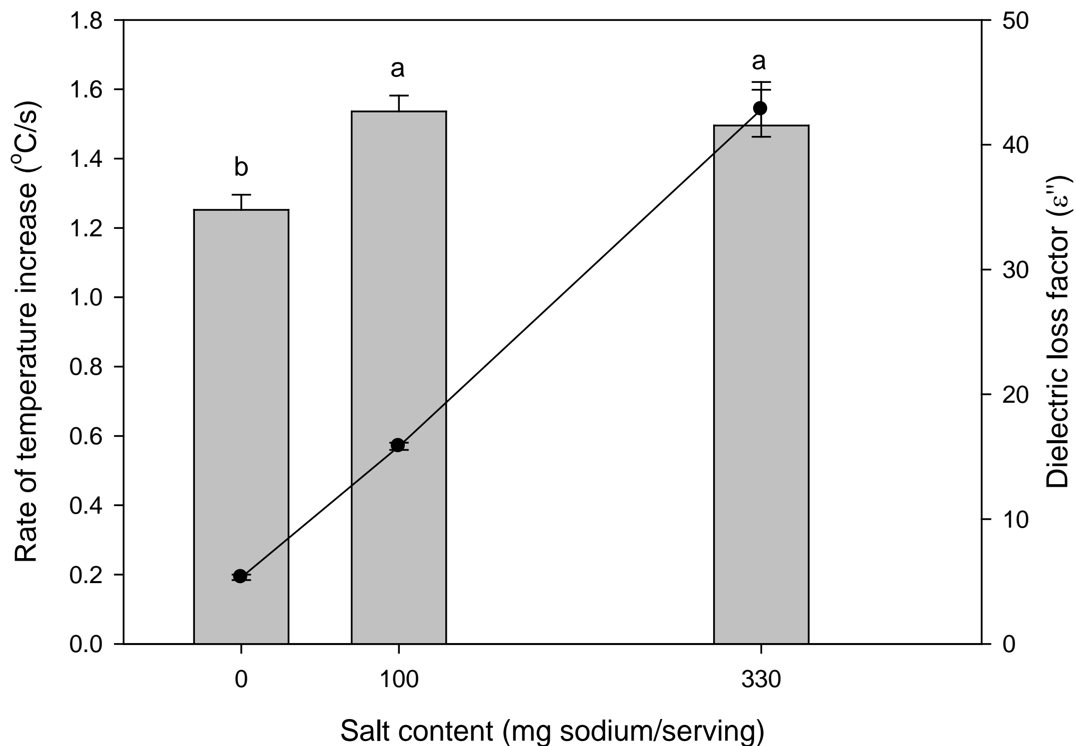


Fig. II-2. Relationship between rate of temperature increase and dielectric loss factor of pistachios with varying salt content during RF heating. The results are means from three experiments, and error bars indicate standard deviations. █, rate of temperature increase; ●, dielectric loss factor.

† Different letters between rates of temperature increase indicate significant differences ($P < 0.05$).

Effect of salt content on inactivation of pathogenic bacteria in pistachios. Populations (log CFU/g) of *S. enterica* in pistachios during RF heating are depicted in Fig. II-3. Survival of this pathogen decreased with increasing treatment time. There were no significant ($P > 0.05$) reductions in microbial levels for 10 s regardless of salt content compared to the control. However, treatment for 20 s significantly ($P < 0.05$) reduced levels of *S. enterica* by 0.61 log CFU/g in salted pistachios. After 30 s of RF treatment, levels of this pathogen were reduced by 1.66 and 1.41 log CFU/g in pistachios with salt contents of 100 and 330 mg sodium/serving, respectively. Populations of *S. enterica* in salted pistachios were inactivated more effectively compared to those in non-salted pistachios. However, treatment time required to achieve 4-log reduction of *S. enterica* was maintained when the salt content exceeded 100 mg sodium/serving. The levels of surviving cells were reduced by 4.02 log CFU/g within 40 s at a salt content of 100 mg sodium/serving. At 300 mg sodium/serving, treatment for 40 s reduced levels of this pathogen by 4.32 log CFU/g. The numbers of *S. enterica* in pistachios were greatly reduced by same levels after 60 s at 0 mg.

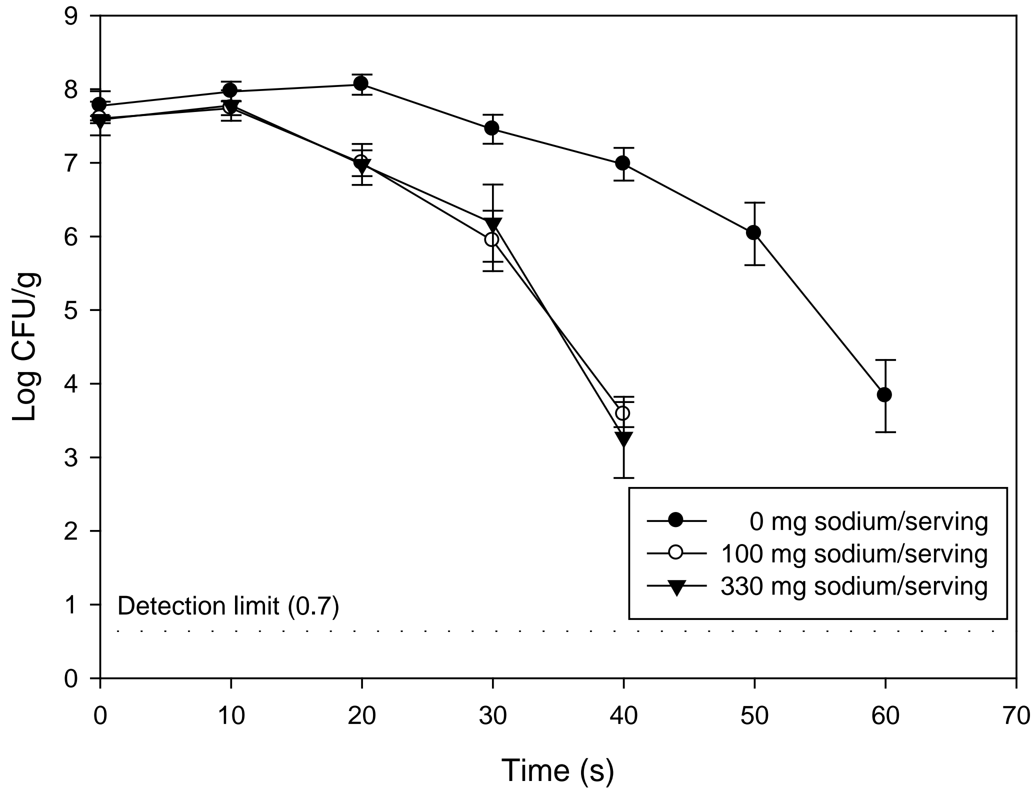


Fig. II-3. Survival curves for *Salmonella enterica* in pistachios with salt contents of 0 mg (●), 100 mg (○), and 330 mg sodium/serving (▼) during RF heating. The results are means from three experiments, and error bars indicate standard deviations.

Recovery of heat-injured cells. Non-injured and heat-injured cells of *S. enterica* from pistachios following RF heating are compared in Table II-3. There were differences in microbial levels between the recovery medium (OV-XLD) and the selective medium (XLD), indicating injured populations on inoculated pistachios of 0.59, 0.16, and 0.29 log CFU/g at salt content of 0, 100, and 330 mg sodium/serving, respectively, after the maximum RF treatment. Slightly lower reductions of *S. enterica* were observed at the final stages of treatment by the injured cell recovery procedure than by direct plating on selective media. However, no significant ($P > 0.05$) differences between levels of surviving *S. enterica* cells were revealed as a result of injured-cell recovery in pistachios with different salt ranges over the entire span of treatment times.

Table II-3. Comparison of pathogen populations between surviving cells and cells including heat-injured cells in inoculated pistachios following RF heating[a]

Salt content (mg sodium/serving)	Treatment time (s)	Population (log_{10} CFU/g)	
		XLD[b]	OV-XLD
0	0	7.77 ± 0.20 ABa	7.82 ± 0.15 Aa
	10	7.97 ± 0.13 ABa	8.02 ± 0.08 Aa
	20	8.06 ± 0.14 Aa	8.07 ± 0.18 Aa
	30	7.46 ± 0.20 BCa	7.53 ± 0.18 ABa
	40	6.98 ± 0.22 Ca	7.06 ± 0.17 Ba
	50	6.03 ± 0.42 Da	6.23 ± 0.31 Ca
	60	3.38 ± 0.49 Ea	3.97 ± 0.71 Da
100	0	7.60 ± 0.23 ABa	7.74 ± 0.20 Aa
	10	7.74 ± 0.10 Aa	7.83 ± 0.09 Aa
	20	6.99 ± 0.18 Ba	7.21 ± 0.35 Aa
	30	5.94 ± 0.41 Ca	6.01 ± 0.34 Ba
	40	3.58 ± 0.17 Da	3.74 ± 0.48 Ca
330	0	7.59 ± 0.05 ABa	7.58 ± 0.06 ABa
	10	7.78 ± 0.21 Aa	7.79 ± 0.24 Aa
	20	6.98 ± 0.28 Ba	7.04 ± 0.22 Ba
	30	6.18 ± 0.52 Ca	6.58 ± 0.52 Ca
	40	3.27 ± 0.55 Da	3.56 ± 0.45 Da

[a] Means ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] XLD, xylose lysine desoxycholate; OV-XLD, overlay XLD agar on TSA.

Effect of RF heating within different salt range on product quality. Color values of pistachio samples of various salt content after RF heating for the time intervals required to achieve 4-log reduction of *S. enterica* are summarized in Table II-4. Within all tested levels of salt content, L^*, a^*, and b^* values of RF-treated pistachios were not significantly ($P > 0.05$) different from those of untreated controls. Table II-5 shows the oxidative rancidity of pistachios of varying salt content following RF treatment. There were no significant ($P > 0.05$) differences in peroxide value and acid value between untreated and treated pistachios. Although these lipid oxidation parameters varied slightly in accordance with RF heating at several salt levels, no statistically significant ($P > 0.05$) differences were detected between any of the tested samples. Thus, RF heating did not degrade the quality of pistachios of differing salt content.

Table II-4. Color values of RF-treated and untreated pistachios of varying salt levels subjected to RF heating[a]

Parameter[b]	Salt content (mg sodium/serving)		
	0	100	330
L*			
Control	53.24 ± 1.85 a	50.29 ± 1.64 a	48.43 ± 0.46 a
RF treated	53.42 ± 0.77 a	49.32 ± 0.87 a	48.73 ± 0.43 a
a*			
Control	2.79 ± 0.48 a	5.47 ± 0.51 a	8.51 ± 0.38 a
RF treated	3.47 ± 0.47 a	5.72 ± 0.23 a	8.13 ± 0.21 a
b*			
Control	13.69 ± 1.13 a	6.01 ± 1.52 a	0.40 ± 0.02 a
RF treated	13.60 ± 2.63 a	6.40 ± 0.42 a	0.43 ± 0.04 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).

Table II-5. Peroxide values and acid values of RF-treated and untreated pistachios of varying salt levels subjected to RF heating[a]

Parameter and treatment type	Salt content (mg sodium/serving)		
	0	100	330
Peroxide value (meq/kg)			
Control	0.71 ± 0.13 a	0.90 ± 0.20 a	0.93 ± 0.02 a
RF treated	0.67 ± 0.07 a	1.01 ± 0.08 a	0.93 ± 0.55 a
Fatty acid (%)			
Control	0.69 ± 0.10 a	0.73 ± 0.09 a	0.86 ± 0.12 a
RF treated	0.72 ± 0.05 a	0.70 ± 0.06 a	0.83 ± 0.40 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).

II-1.4. Discussion

Recently, RF heating has been gaining wider acceptance for solid and semi-solid foods which have low thermal conductivities. These studies have reported that RF heating is a promising food processing technology due to internal heating resulting from the direct interaction between electromagnetic waves and the material (Gao et al., 2011; Ha et al., 2013; Jeong and Kang, 2014; Luechapattanaporn et al., 2004). However, there are no published reports describing the inactivation of foodborne pathogens in pistachios of varying salt content using RF heating. In the present study, salt content of pistachios had a great effect on inactivating *S. enterica*. At a salt content of 0 mg sodium/serving, 60 s was required to achieve 4-log reduction of *S. enterica* in pistachios, but only 40 s was required at 100 and 330 mg sodium/serving. There was an upper limit at which increasing salt content led to a leveling of RF treatment time required to reduce *S. enterica* by 4 log CFU/g. This result was caused by a difference in the salt-dependent heating rate, since RF energy has no non-thermal effect on microbial inactivation (Geveke et al., 2002; Ponne et al., 1996).

RF heating is affected by dielectric properties which determine the dielectric constant and dielectric loss factor. The dielectric constant is a measure of the ability of a material to absorb, transmit and reflect electric energy and a characteristic of the polarizing effect from an applied electrical field; namely, how easily the medium is polarized. The dielectric loss factor indicates the dissipation of electric energy in the

form of heat, which is relevant to how the energy from an external electric field is absorbed and converted to heat (Datta et al., 2014; Piyasena et al., 2003). Among the factors affecting dielectric properties, the effect of processing pistachios having added salt was confirmed by my data which showed slight increases in the dielectric constant and sharp increases in the dielectric loss factor. Similar trends at different salt levels were also reported by Ling et al. (2015). Therefore, an understanding of the dielectric properties of foods relative to their salt content is extremely important in maximizing the effectiveness of RF heating.

The impact of dielectric properties on temperature increase during RF heating is represented by the equation $P = 2\pi f V^2 \varepsilon_0 \varepsilon''$, where P is the electrical power transferred to the food as heat, f is the frequency, V is the electric field strength, ε_0 is the dielectric constant of a vacuum (8.85×10^{-12} F/m), and ε'' is the dielectric loss factor of the sample (Marra et al., 2009). Based on this equation, heat generation increases proportionally to the frequency, the electric field strength, and the dielectric loss factor. In this study, because of the fixed values of frequency and electric field strength at 27.12 MHz and 0.3 kV/cm, respectively, we examined only the effect of the dielectric loss factor which depends on salt content during RF heating. My results are in agreement with the equation; the higher the dielectric loss factor of pistachios resulting from higher salt levels, the greater the heat generation. However, above the upper limit dielectric loss factor which is 15.83 at a salt content of 100 mg sodium/serving, the heating rate remained stable. This result agreed with earlier

research which suggested that if the dielectric loss factor was too high, current leakage occurred through the material resulting in stopping ongoing temperature increase. (Birla et al., 2008a; Orfeuil, 1987). My previous study also concluded that the dielectric loss factor should be within an appropriate range for successful RF heating (Jeong and Kang, 2014). In order to clarify the upper limit dielectric loss factor, further studies concerning the effect of salt content within 100 mg are required.

Following thermal treatment, there is a need to consider the impact of sub-lethally injured foodborne pathogens which are potentially as important as their uninjured counterparts due to the ability of resuscitating and regaining pathogenicity in a favorable environment (Wesche et al., 2009; Wu, 2008). Since heat-injured cells could not grow well on selective agar, in the present study, a repair step for injured cells on nonselective agar was incorporated followed by selective enumeration. After RF heating within a range of salt levels, there were no significant ($P > 0.05$) differences in reduction levels between injured and uninjured cells in pistachios at all treatment time intervals. This suggests that RF heating effectively inactivated *S. enterica* in pistachios without generating many injured cells which could introduce a potential food safety threat.

In order to validate commercial application of this new bactericidal technology, it is essential to investigate quality changes of the processed food. My experimental conditions for evaluating the quality of RF-treated pistachios focused on measurement of color values (L^*, a^*, and b^*), peroxide value, and acid value because of their

potential to be affected by increased temperature during RF heating. Following maximum treatment of pistachios of various salt content, no tested parameters were significantly ($P < 0.05$) different from those of untreated controls. Other studies also reported that RF heating treatment did not affect quality of agricultural products. Gao et al. (2010) found that the RF heating method produced no loss in color and caused little lipid oxidation in almonds. Zheng et al. (2016) reported that RF heated corn maintained levels of moisture content, water activity, ash, color, starch, protein, fat, and fatty acids.

In conclusion, this research demonstrated that RF heating leads to effective inactivation of *S. enterica* in pistachios of varying salt content, as well as preventing quality deterioration. Given my results, the potential utilization of RF heating could be considered as an alternative technology to other interventions which are currently employed. Industrial-scale RF heating for reducing foodborne pathogens in pistachios should be based on appropriate treatment times relative to salt content. This is because improper RF heating can cause insufficient heating or overheating which can enable survival of microorganisms or lead to quality loss of food products. The results of the present study are fundamental to an understanding of the response of pistachios to the RF electromagnetic field at certain salt content, and by extension, to commercial practical application of RF sterilization. Further studies concerning the impact of other factors on the heating rate in pistachios are required to maximize the effectiveness of RF heating as well as guarantee uniform commercial sterilization.

II-2. Effect of packaging materials on inactivation of foodborne pathogens in red and black pepper spices by RF heating

II-2.1. Introduction

Powdered red (*Capsicum* spp.) and black (*Piper nigrum*) pepper spice are non-perishable commodities because of their low moisture content. However, they are natural products and may be burdened with high levels of microorganisms such as *Salmonella* spp., *Escherichia coli*, and *Bacillus cereus* (Little et al., 2003; Schweiggert et al., 2007). Such contaminated spices can lead to severe foodborne illnesses, since they are utilized in ready-to-eat foods which are not subjected to further cooking (Rico et al., 2010). In 2009, a large multistate outbreak of *Salmonella* infections associated with salami products occurred in the United States. The implicated foods were made with contaminated ground pepper. During this outbreak, a reported 272 persons in 44 states and Washington, DC became ill (Centers for Disease Control and Preservation, 2010). In the United States 14 pepper-associated outbreaks were reported to the Centers for Disease Control and Prevention (CDC) between 1998 and 2014. As a consequence, dried powdered spices should be decontaminated to prevent further food spoilage and foodborne diseases.

Cross contaminations due to inappropriate storage or unclean processing conditions may bring the pathogens to food products and cause serious poisoning to people. In order to eliminate the foodborne pathogen, a post-packaging pasteurization process for shelf stable foods would be desirable. In conventional thermal processes that rely on steam or hot water as a heat medium, low moisture food products are

difficult to be heated because of the low thermal conductivities, resulting in a longer heating period, overheating at the edges of the food package, and consequently, a quality deterioration (Gao et al., 2010; Wang et al., 2007c). Furthermore, the slow heating rate may allow bacteria to mimic heat shock response and increase thermal resistance (Chung et al., 2007; Stephens et al., 1994; Wesche et al., 2009).

Radio-frequency (RF) heating is a highly appealing technology by which internal heating as a result of molecular friction is rapidly generated in response to an applied alternating electric field at frequencies between 1 and 300 MHz (Piyasena et al., 2003). It has been applied for drying, baking and thawing in food industry (Farag et al., 2011; Jumah, 2005; Kocadağlı et al., 2012; Koray Palazoğlu et al., 2012; Wang et al., 2014) . Since RF waves can penetrate through packaging materials such as plastic films or conventional cardboard, RF heating does not have any requirement for direct contact between the food product and electrodes (Marra et al., 2009). Therefore, RF heating has the potential for packaged food pasteurization.

Until now plastic films such as polyethylene (PE), polypropylene (PP), polycaprolactam (nylon), polystyrene (PS), and polyethylene terephthalate (PET) have been increasingly used as packaging materials for foods due to low cost, good mechanical performance, and good barrier to oxygen, carbon dioxide, and aroma compound (Siracusa et al., 2008). Among them, PE, PP, and nylon are commonly used for the packaging of foods that are treated with in RF heater because of their high heat resistance (Ozen and Floros, 2001; Risch et al., 1991). Recently, polyetherimide

(PEI), high-performance plastic film, is also used for food packaging (Miller et al., 2009). The effectiveness of RF heating for pasteurization of packaged foods have been reported by Houben et al. (1991); Luechapattanaporn et al. (2004); Luechapattanaporn et al. (2005). However, there are no studies considering the effect of packaging materials of foods on the inactivation efficacy of RF heating.

The objective of this study was to investigate how packaging materials composed of PE, PP, nylon, and PEI influence the heating rate of powdered red and black pepper spices The effects of RF heating for reducing *S.* Typhimurium and *E. coli* O157:H7 in red and black pepper powders surrounded with various plastic films, as well as quality of spices.

II-2.2. Materials and Methods

Bacterial strains. All bacterial strains, namely, *S.* Typhimurium (ATCC 19585, ATCC 43971, ATCC 700408) and *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890) were obtained from the Department of Food and Animal Biotechnology culture collection at Seoul National University (Seoul, South Korea). Stock cultures were stored at −80 °C in 0.7 ml of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. For all experiments, working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37 °C for 24 h, and stored at 4 °C.

Preparation of pathogen inocula. Each strain of *S.* Typhimurium and *E. coli* O157:H7 was cultured individually in 5 ml of TSB at 37°C for 24 h, harvested by centrifugation at 4,000 × g for 20 min at 4 °C, and washed three times with sterile 0.2% peptone water (PW; Difco). The final pellets were resuspended in 3 ml of 0.2% PW, corresponding to approximately 10^8 to 10^9 CFU/ml. This concentration was confirmed by direct plating method in a preliminary experiment. To inoculate red and black pepper spice, suspended pellets of all strains of both pathogens were combined to produce a mixed culture cocktail (six strains). These cocktails at a final concentration of ca. 10^9 CFU/ml were used in subsequent experiments.

Sample preparation and inoculation. Commercially dried red and black pepper powders were purchased from a local grocery store (Seoul, South Korea). For inoculation, 6 ml of culture cocktail was applied to 250 g of samples and thoroughly mixed by hand massaging for 10 min to ensure even distribution of the pathogens and dried for 24 h inside a biosafety hood (22 ± 2 °C) with the fan running until the moisture level of the sample equaled that of a noninoculated sample (ca. 10.6 and 6.8 of red and black pepper spices, respectively). The final cell concentration was 10^7 to 10^8 CFU/g. Aseptic plastic bags (PE, PP, nylon, and PEI) were tailored so that final products had a size of 10 × 15 cm; 0.7 cm thick. Each 25 g portion of inoculated samples was transferred to four different plastic bags and packaged using a packaging machine (Airzero, Ansan, South Korea). The inoculated and packaged samples were immediately subjected to RF heating.

RF heating treatment. RF heating treatment was conducted in a treatment system described previously (Jeong and Kang, 2014). The RF heater generated a RF electric field at a frequency of 27.12 MHz and a maximum power of 9 kW. Its cavity was composed of two parallel-plate electrodes (30.0 × 35.0 cm; 0.6 cm thick) and the distance between the two electrodes was 11.0 cm. A sample was placed on the center of the bottom electrode. RF heating was applied to each prepared sample and heated to 90 °C in order to maximize the efficacy of pasteurization while maintaining product quality.

Temperature measurement. A fiber optic temperature sensor (FOT-L; FISO Technologies Inc., Quebec, Canada) connected to a temperature signal conditioner was used to measure real-time temperatures in samples during RF heating. The sensor was inserted at the center of the treated red and black pepper powder through a predrilled hole, and the temperature was recorded at 1 s intervals. Since the fiber optic sensor was coated with electric insulating material, it did not interfere with the temperature profile of the treated sample (Wang et al., 2003). All experiments were repeated three times, and averages and standard deviations of RF treated sample temperatures were compared to determine the heating rate of samples.

Dielectric properties measurement. Dielectric properties of plastic films were measured according to ASTM D150 on a precision LCR meter (4284A; Agilent Technologies, Palo Alto, CA) with a frequency range of 1 kHz to 1 MHz. For dielectric measurements, the films were cut into small samples of 10 mm × 10 mm, and then the samples were coated with conductive silver paste on both surfaces. Dielectric measurements of spices were taken at 1 MHz.

Bacterial enumeration. At selected time intervals, treated 25 g samples were immediately transferred into sterile stomacher bags (Labphas, Inc., Sainte-Juilie, Quebec, Canada) containing 225 ml of 0.2% PW, which were pre-chilled in a 4°C refrigerator. After homogenization for 2 min with a stomacher (Easy Mix; AES

Chemunex, Rennes, France), 1-ml aliquots of sample were 10-fold serially diluted in 9-ml blanks of 0.2% PW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Xylose lysine desoxycholate agar (XLD; Difco) and sorbitol MacConkey agar (SMAC; Difco) were used as selective media for the enumeration of *S.* Typhimurium and *E. coli* O157:H7, respectively. When low levels of surviving cells were anticipated, 1 ml of undiluted stomacher bag contents was equally divided onto four plates of each medium and spread-plated (detection limit, 1 log CFU/g). All agar media were incubated at 37°C for 24 h and typical colonies were counted. To confirm identity of the pathogens, colonies randomly selected from the enumeration plates were subjected to serological tests. These tests consisted of the *Salmonella* latex agglutination assay (Oxoid, Ogdensberg, NY) and *E. coli* O157:H7 latex agglutination assay (Oxoid) for *S.* Typhimurium and *E. coli* O157:H7, respectively.

Enumeration of heat-injured cells. The overlay (OV) method was used to enumerate heat-injured cells of *S.* Typhimurium using TSA as a nonselective agar and XLD as the selective agar (Lee and Kang, 2001). Appropriate dilutions were spread-plated onto TSA medium and incubated at 37°C for 2 h to allow injured cells to recover, and then 7 to 8 ml of XLD was overlaid on the plates. After solidification, plates were incubated for an additional 22 h at 37°C, and typical black colonies were enumerated. To enumerate heat-injured cells of *E. coli* O157:H7, RF-treated samples were serially diluted and spread-plated onto phenol red agar base with 1% sorbitol

(SPRAB; Difco) at time intervals causing the large temperature change (Rocelle et al., 1995). After incubation at 37°C for 24 h, typical white colonies were enumerated. Random colonies were selected from SPRAB plates and subjected to serological confirmation as *E. coli* O157:H7 (*E. coli* O157:H7 latex agglutination assay; Oxoid), since SPRAB is not a selective media for enumerating *E. coli* O157:H7.

Color measurement. To measure the effect of RF heating on the color of powdered red and black pepper spice surrounded with plastic films, a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan) was used to measure the color changes of RF-treated samples. The values of L^*, a^*, and b^* were used to quantify color attributes and measurements were taken from treated and untreated noninoculated red and black pepper taken at random locations. L^*, a^*, and b^* values indicate color lightness, redness, and yellowness of the sample, respectively.

Volatile flavor component measurement. Capsaicinoids and piperine were measured as volatile flavor components in this study for red and black pepper powder, respectively. The total capsaicinoids content was tested according to the method described by Attuquayefio and Buckle (1987). This method involves capsaicinoid extraction, cleanup, and separation. Red pepper spice samples (4 g) were mixed with 20 ml of acetonitrile for 2 min with a vortex mixer (WiseMix VM-10; Daihan Wisd., Gangwon, South Korea). The capsaicinoids were eluted by passing the sample extract

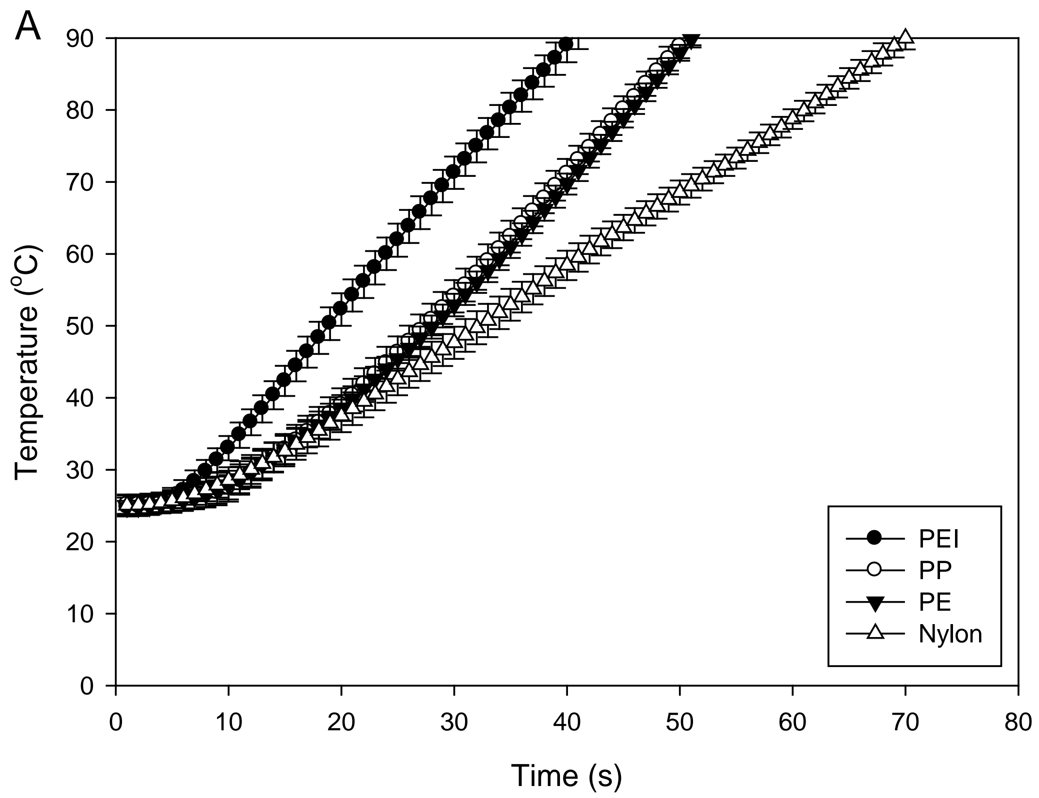
into a conditioned Sep-pak (WAT054945; Waters, Milford, MA) column. The eluent was passed through a Teknokroma 0.45-mm-pore-size membrane and subjected to the following procedure. For capsaicinoid separation, a high-performance liquid chromatography apparatus (HPLC; Waters 2695; Waters) equipped with an autosampler and a photodiode array detector (Waters 996; Waters) was used. The wavelength was set at 280 nm, and a reversed-phase C_{18} column (5-mm particle size, 4.6-mm diameter, 250-mm length; Young Jin Biochrom Co. Ltd., Gyeonggi, South Korea) where temperature was controlled at 35°C was used with these conditions of the mobile phase: methanol and triple-distilled water (70:30 [v/v]) at a flow rate of 1 ml/min. A standard calibration curve was obtained by using capsaicin (Sigma Chemical Co., St. Louis, MO) and dihydrocapsaicin (Sigma Chemical Co.) prepared in acetonitrile.

Piperine concentration in black pepper was also determined using HPLC set at 340 nm. The column which was the same one we used to separate the capsaicinoid in red pepper was utilized. The mobile phase was prepared according to the method reported by Chiang (1986) and passed through the column at 1.5 ml/min. Standard piperine was purchased from Sigma Chemical Co. and prepared in methanol. The samples (0.1 g) were homogenized with 5 ml of methanol by vortexing for 2 min in a 10 ml volumetric tube, and methanol was added to the mark. After the solids settled, the supernatant was filtered through a Teknokroma filter and a 20-μl portion of filtrate was injected into the column using the HPLC autosampler.

Statistical analysis. All experiments were repeated three times with duplicate samples. Data were analyzed by the analysis of variance procedure of Statistical Analysis System (SAS Institute, Cary, NC), and mean values were separated using Duncan's multiple-range test. $P < 0.05$ was used to determine significant differences in the processing treatment.

II-2.3. Results

Temperature curves of powdered red and black pepper spice surrounded with different packaging materials. Average temperatures of red and black pepper powder surrounded with PE, PP, nylon, and PEI films during RF heating at a constant frequency of 27.12 MHz are shown in Fig. II-4. In the same plastic film, the temperature increased with increasing treatment times. The average heating rates were significantly different ($P < 0.05$) in both samples surrounded with different plastic films, samples packaged in PEI film was heated at the most rapid rate, followed by PP, PE, and nylon. There were no significant differences ($P > 0.05$) between the average heating rate of red and black pepper spices packaged in PP and PE films. Red pepper and black pepper surrounded with PEI film increased from 22.2 °C to 90.6 °C when exposed to RF energy for 41 s and 49 s, respectively. For the same treatment time, the temperature of both spices did not exceed 70 °C in nylon film.


A
Temperature (°C)
Time (s)
PEI
PP
PE
Nylon
0
10
20
30
40
50
60
70
80
90
0
10
20
30
40
50
60
70
80

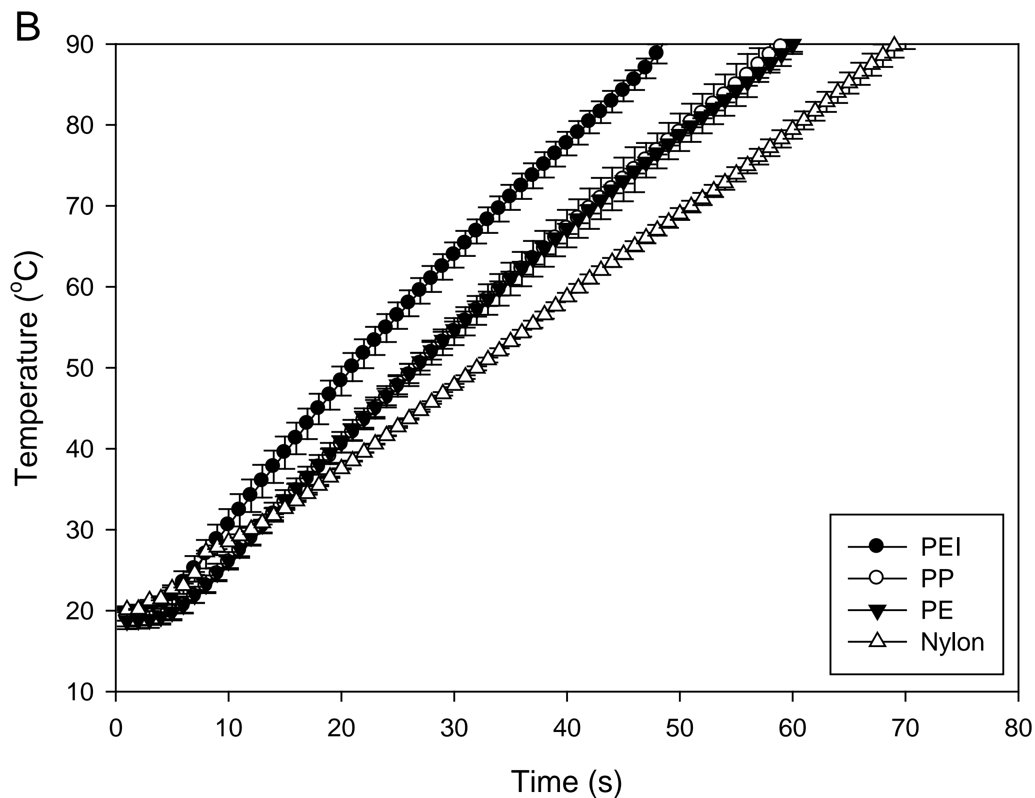


Fig. II-4. Temperature curves of red (A) and black (B) pepper powder during RF heating as influenced by packaging materials. The results are means from three experiments, and error bars indicate standard deviations.

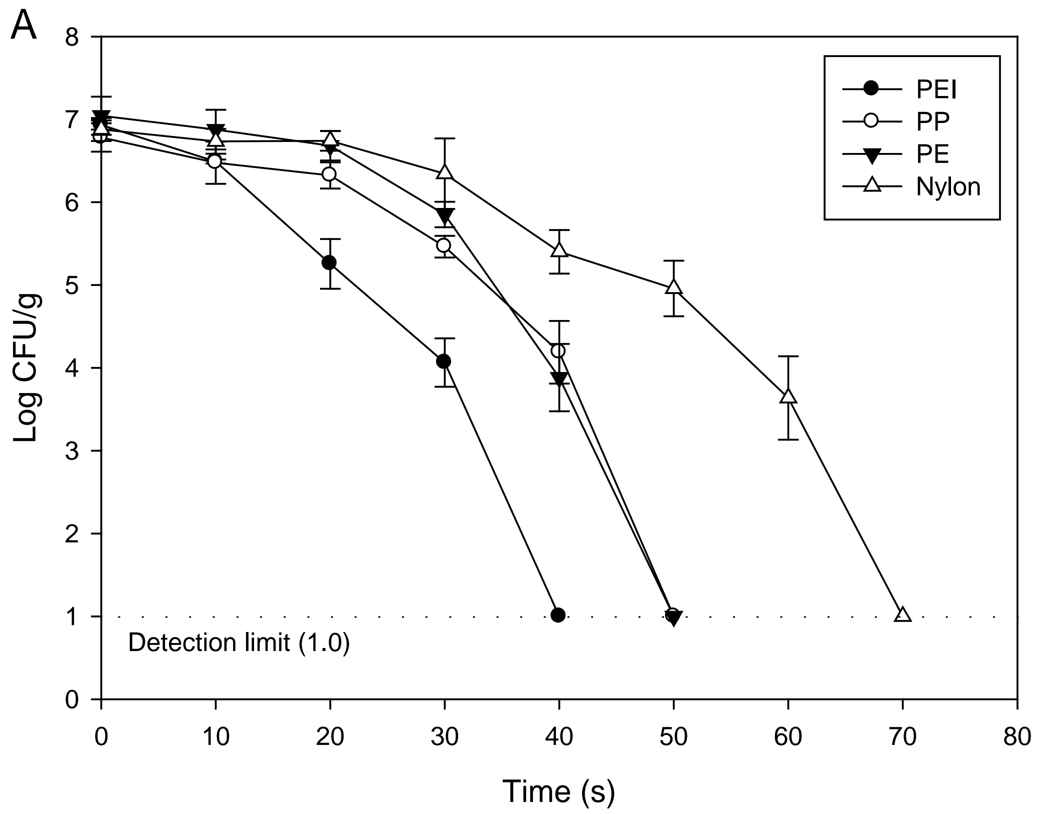
Dielectric properties of different packaging materials. The dielectric properties of PE, PP, nylon, and PEI films at a fixed frequency of 1 MHz are listed in Table II-6. The patterns of dielectric properties were similar to those of the heating rate as shown in Fig. II-4. Both dielectric constants and dielectric loss factors of PP and PE films were not significantly different ($P > 0.05$). The dielectric constant of PEI film was similar that of target sample (ca. 4.26 and 4.45 of red and black pepper spices, respectively). Compared to nylon film which delayed the treatment time required to reduce foodborne pathogens below the detection limit, the dielectric loss factor of PEI film was relative low.

Table II-6. Dielectric properties of various packaging materials at 1 MHz[a]

Packaging materials	ε'[b]	ε''
Polypropylene (PP)	2.10 ± 0.40 b	0.0001 ± 0.0000 c
Polyethylene (PE)	2.26 ± 0.28 b	0.0001 ± 0.0000 c
Polycaprolactam (nylon)	3.07 ± 0.11 a	0.1088 ± 0.0052 a
Polyetherimide (PEI)	3.23 ± 0.34 a	0.0031 ± 0.0002 b

[a] Means ± standard deviations from three replications. Values followed by different letters within the column are significantly different ($P < 0.05$).
[b] ε' is the dielectric constant and ε'' is the dielectric loss factor.

Effect of packaging materials on inactivation of foodborne pathogens in powdered red and black pepper spice. Populations (log CFU/g) of *S.* Typhimurium and *E. coli* O157:H7 in red pepper during RF heating are depicted in Fig. II-5. Survival of both pathogens decreased with increasing treatment time. In the case of sample packaged in PEI films, treatment time required to reduce *S.* Typhimurium and *E. coli* O157:H7 to below the detection limit (1 log CFU/g) was the shortest. The levels of surviving cells of both pathogens were reduced the detection limit within 40 s in red pepper surrounded with PEI film. With PP and PE films, levels of *S.* Typhimurium experienced a significant reduction of 2.59 and 3.16 log CFU/g, respectively, after 40 s and a > 5.91-log reduction to below the detection limit after 50 s of treatment. Cell numbers of *E. coli* O157:H7 were reduced by 3.88 and 4.29 log CFU/g, respectively, after 40 s and to below the detection limit after 50 s of treatment. The numbers of *E. coli* O157:H7 and *S.* Typhimurium in red pepper packaged in nylon film were greatly reduced to undetectable levels after 70 s.


A
Log CFU/g
Time (s)
PEI
PP
PE
Nylon
Detection limit (1.0)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80

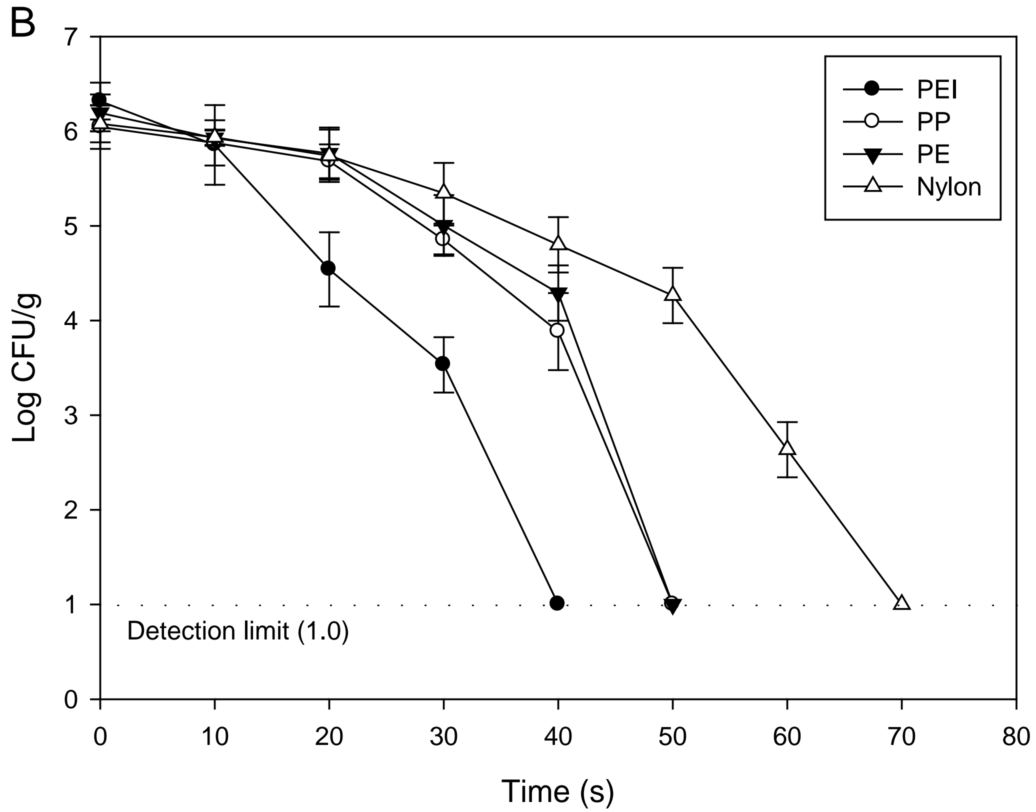
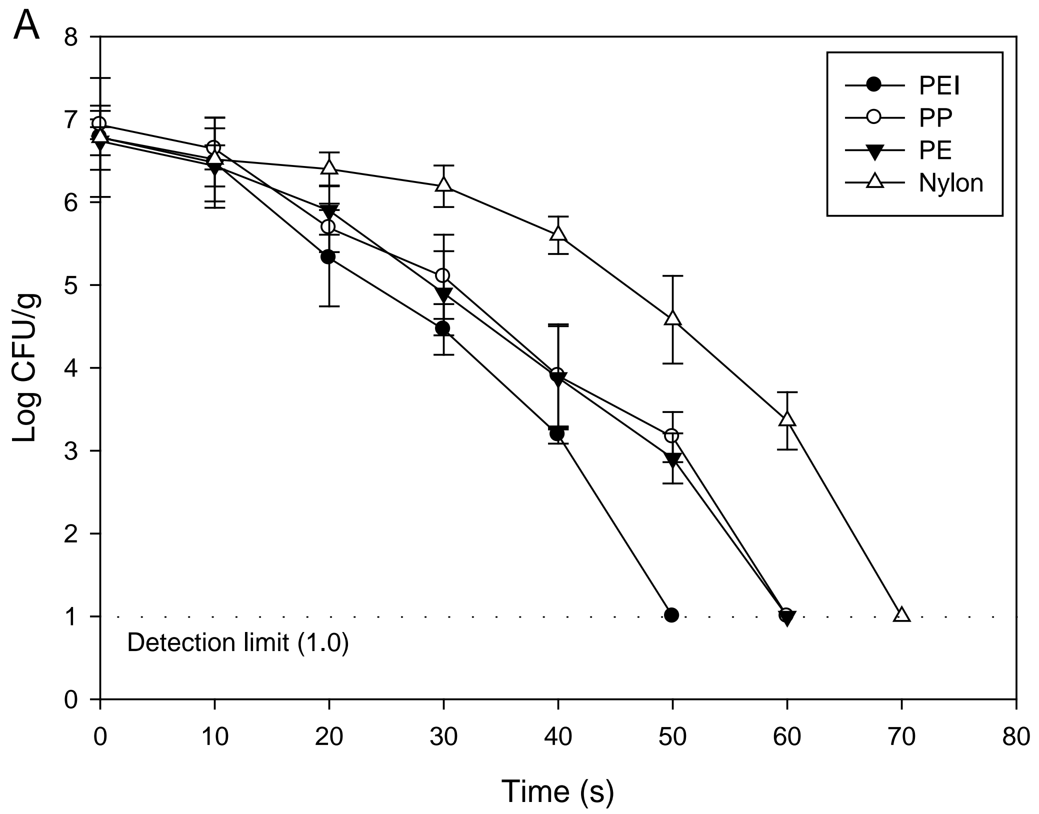


Fig. II-5. Survival curves for *Salmonella* Typhimurium (A) and *Escherichia coli* O157:H7 (B) in red pepper powder with surrounded with polypropylene (PP), polyethylene (PE), polycaprolactam (nylon), and polyetherimide (PEI). The results are means from three experiments, and error bars indicate standard deviations.

Figure II-6 shows the survival of *S.* Typhimurium and *E. coli* O157:H7 of black pepper treated with RF heating. The overall reduction patterns of both pathogens in black pepper were similar to those in red pepper. There were no significant differences ($P > 0.05$) in microbial levels between control and 10 s of treatment regardless of packaging materials. After 20 s, each significant ($P < 0.05$) reduction of both pathogens were observed in the sample surrounded with PEI film. RF heating with PEI packaging plastic film reduced both pathogens to below the detection limit after 50 s. With PP or PE films, *S.* Typhimurium and *E. coli* O157:H7 were reduced to below detectable levels after 60 s of treatment. Population of both pathogens decreased by 5.78 and 6.14 log CFU/g after 70 s.


A
Log CFU/g
Time (s)
PEI
PP
PE
Nylon
Detection limit (1.0)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80

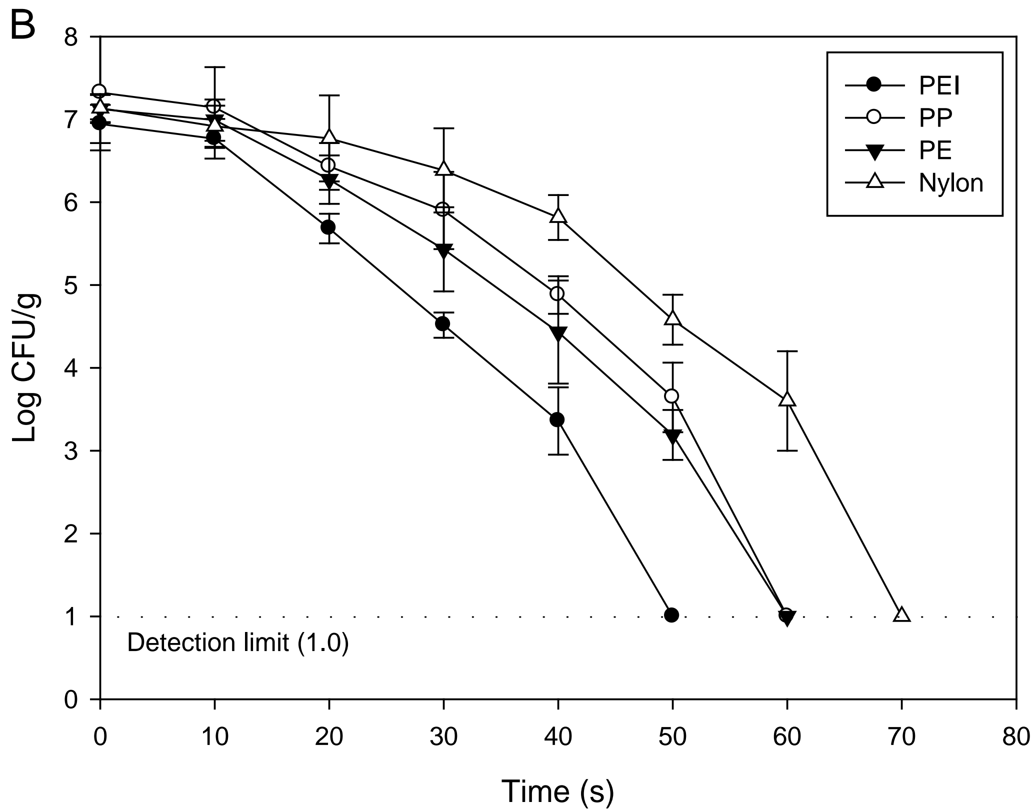


Fig. II-6. Survival curves for *Salmonella* Typhimurium (A) and *Escherichia coli* O157:H7 (B) in black pepper powder surrounded with polypropylene (PP), polyethylene (PE), polycaprolactam (nylon), and polyetherimide (PEI). The results are means from three experiments, and error bars indicate standard deviations.

Recovery of heat-injured cells. Table II-7 and II-8 show cells and heat-injured cells of *S.* Typhimurium and *E. coli* O157:H7 from red and black pepper powder following RF heating, respectively. When inoculated red and black pepper were treated with RF heating, slightly higher numbers of both pathogens were detected on the agar for recovery (OV-XLD and SPRAB) than on the selective agar (XLD and SMAC). However, during the entire treatment time, no significant ($P > 0.05$) differences in the levels of cells enumerated between the agar for recovery and the selective agar were observed in powdered red and black pepper spice surrounded with different packaging materials.

Table II-7. Comparison of pathogen populations between surviving cells and cells including heat-injured cells in inoculated red peppers following RF heating[a]

Packaging materials	Treatment time (s)	Population (log10 CFU/g) by organism and selection medium			
		Salmonella. Typhimurium		*Escherichia. coli* O157:H7	
		XLD[b]	OV-XLD	SMAC	SPRAB
PP	0	6.78 ± 0.17 Aa	6.39 ± 0.36 Aa	6.04 ± 0.24 Aa	6.40 ± 0.37 Aa
	20	6.32 ± 0.16 Aa	5.80 ± 0.48 ABa	5.68 ± 0.18 Ba	5.36 ± 0.22 Ba
	30	5.46 ± 0.13 Ba	5.18 ± 0.32 BCa	4.85 ± 0.15 Ca	4.50 ± 0.23 Ca
	40	4.19 ± 0.38 Ca	4.81 ± 0.75 Ca	3.88 ± 0.41 Da	3.38 ± 0.42 Da
	50	ND[c]	ND	ND	ND
PE	0	7.04 ± 0.23 Aa	6.84 ± 0.19 Aa	6.19 ± 0.19 Aa	6.73 ± 0.28 Aa
	20	6.68 ± 0.18 Ba	6.24 ± 0.30 Aa	5.76 ± 0.28 Ba	5.32 ± 0.18 Ba
	30	5.85 ± 0.15 Ca	5.99 ± 0.45 Ba	5.00 ± 0.32 Ca	5.27 ± 0.37 Ba
	40	3.88 ± 0.41 Da	3.16 ± 0.28 Ca	4.29 ± 0.29 Da	4.51 ± 0.25 Ca
	50	ND	ND	ND	ND
Nylon	0	6.88 ± 0.14 Aa	6.53 ± 0.21 Aa	6.08 ± 0.19 Aa	6.34 ± 0.28 Aa
	40	5.40 ± 0.26 Ba	5.14 ± 0.42 Ba	4.80 ± 0.29 Ba	4.43 ± 0.40 Ba
	50	4.96 ± 0.33 Ca	5.05 ± 0.33 Ba	4.26 ± 0.29 Ca	4.27 ± 0.16 Ca
	60	3.64 ± 0.50 Da	3.64 ± 0.19 Ca	2.64 ± 0.34 Da	2.42 ± 0.42 Da
	70	ND	ND	ND	ND
PEI	0	6.93 ± 0.05 Aa	6.47 ± 0.38 Aa	6.32 ± 0.19 Aa	6.62 ± 0.33 Aa
	10	6.49 ± 0.02 Ba	6.96 ± 0.53 Ba	5.85 ± 0.42 Ba	5.37 ± 0.20 Ba
	20	5.26 ± 0.30 Ca	5.28 ± 0.55 Ca	4.54 ± 0.39 Ca	5.08 ± 0.30 Ba
	30	4.06 ± 0.29 Da	4.15 ± 0.63 Da	3.53 ± 0.29 Da	4.02 ± 0.52 Ca
	40	ND	ND	ND	ND

[a] Means ± standard deviations from three replications. Means with the same capital letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).

[b] SMAC, Sorbitol MacConkey agar; SPRAB; Phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate; OV-XLD, overlay XLD agar on TSA.

[c] ND, below detection limit (1.0 log CFU/g).

Table II-8. Comparison of pathogen populations between surviving cells and cells including heat-injured cells in inoculated black pepper following RF heating[a]

Packaging materials	Treatment time (s)	Population (log10 CFU/g) by organism and selection medium			
		Salmonella. Typhimurium		*Escherichia. coli* O157:H7	
		XLD[b]	OV-XLD	SMAC	SPRAB
PP	0	6.93 ± 0.17 Aa	6.68 ± 0.46 Aa	7.32 ± 0.70 Aa	7.02 ± 0.27 Aa
	30	5.10 ± 0.51 Ga	4.91 ± 0.44 Ba	5.90 ± 0.47 Ba	5.63 ± 0.42 Ba
	40	3.90 ± 0.62 Ca	3.52 ± 0.28 Ca	4.88 ± 0.23 Ca	4.67 ± 0.51 Ca
	50	3.16 ± 0.30 Da	2.87 ± 0.62 Da	3.64 ± 0.42 Da	3.29 ± 0.42 Da
	60	ND[c]	ND	ND	ND
PE	0	6.74 ± 0.17 Aa	6.92 ± 0.39 Aa	7.12 ± 0.17 Aa	6.85 ± 0.36 Aa
	30	4.90 ± 0.51 Ba	4.45 ± 0.41 Ba	5.43 ± 0.51 Ba	5.31 ± 0.29 Ba
	40	3.88 ± 0.62 Ca	3.72 ± 0.27 Ca	4.43 ± 0.62 Ca	4.90 ± 0.28 Ca
	50	2.91 ± 0.30 Da	2.79 ± 0.53 Da	3.19 ± 0.30 Da	2.89 ± 0.49 Da
	60	ND	ND	ND	ND
Nylon	0	6.88 ± 0.39 Aa	6.58 ± 0.49 Aa	7.14 ± 0.17 Aa	6.92 ± 0.31 Aa
	40	5.60 ± 0.23 Ba	5.49 ± 0.33 Ba	5.82 ± 0.27 Ba	5.43 ± 0.44 Ba
	50	4.58 ± 0.53 Ca	4.31 ± 0.38 Ca	4.58 ± 0.30 Ca	4.19 ± 0.46 Ca
	60	3.36 ± 0.35 Da	3.06 ± 0.47 Da	3.60 ± 0.60 Da	3.22 ± 0.22 Da
	70	ND	ND	ND	ND
PEI	0	6.78 ± 0.72 Aa	6.47 ± 0.21 Aa	6.94 ± 0.23 Aa	6.58 ± 0.45 Aa
	10	5.32 ± 0.58 Ba	5.03 ± 0.64 Ba	5.68 ± 0.18 Ba	5.27 ± 0.59 Ba
	20	4.46 ± 0.31 Ca	4.07 ± 0.47 Ca	4.52 ± 0.15 Ca	4.32 ± 0.48 Ca
	30	3.12 ± 0.10 Da	2.98 ± 0.56 Da	3.36 ± 0.41 Da	3.06 ± 0.31 Da
	40	ND	ND	ND	ND

[a] Means ± standard deviations from three replications. Means with the same capital letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] SMAC, Sorbitol MacConkey agar; SPRAB; Phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate; OV-XLD, overlay XLD agar on TSA.
[c] ND, below detection limit (1.0 log CFU/g).

Effect of RF heating on product quality during post-packaging pasteurization. The color and volatile flavor components (capsaicinoids for red pepper and piperine for black pepper) of spice samples surrounded with various plastic films after RF heating for time intervals required to reduce *S.* Typhimurium and *E. coli* O157:H7 to below the detection limit (1 log CFU/g) are summarized in Tables II-9 and II-10. Within all tested packaging materials, L^*, a^*, and b^* values of RF-treated samples were not significantly ($P > 0.05$) different from those of untreated samples. There were also no significant ($P > 0.05$) differences in volatile flavor components, capsaicinoids and piperine, between untreated and treated red and black pepper powder. Although they varied slightly in accordance with RF heating treatment with several plastic films, statistically significant differences were not detected between any of the tested samples. Thus, RF heating did not affect the color or amount of volatile flavor components of powdered red and black pepper spice packaged in different packaging materials ($P > 0.05$).

Table II-9. Color values and capsaicinoids of treated and untreated red peppers surrounded with various plastic films subjected to RF heating[a]

Packaging materials	Treatment time (s)	Color[b]			Capsaicinoids (mg/100g)		
		L^*	a^*	b^*	CAP[c]	DHC[c]	Total[d]
PP	0	33.54 ± 0.44 a	21.19 ± 0.56 a	17.34 ± 0.47 a	35.41 ± 1.44 a	36.15 ± 0.46 a	71.56 ± 1.53 a
	50	33.95 ± 0.44 a	20.93 ± 0.47 a	17.71 ± 0.29 a	36.88 ± 0.77 a	35.80 ± 0.57 a	72.68 ± 0.98 a
PE	0	31.58 ± 0.29 a	21.00 ± 0.31 a	17.94 ± 0.44 a	34.46 ± 0.60 a	37.66 ± 0.75 a	72.12 ± 1.20 a
	50	31.72 ± 0.42 a	21.25 ± 0.55 a	18.73 ± 0.54 a	35.19 ± 0.91 a	37.53 ± 0.74 a	72.72 ± 1.26 a
Nylon	0	30.25 ± 0.39 a	22.43 ± 0.30 a	18.38 ± 0.46 a	33.44 ± 0.42 a	37.09 ± 0.40 a	70.53 ± 0.65 a
	70	30.61 ± 0.61 a	22.47 ± 0.46 a	18.16 ± 0.33 a	33.91 ± 0.66 a	37.26 ± 0.51 a	71.17 ± 0.16 a
PEI	0	32.62 ± 0.39 a	23.10 ± 0.32 a	18.18 ± 0.50 a	34.87 ± 0.86 a	37.22 ± 0.80 a	72.09 ± 1.20 a
	40	32.60 ± 0.35 a	23.07 ± 0.20 a	18.60 ± 0.68 a	34.01 ± 0.67 a	37.91 ± 1.33 a	71.92 ± 0.56 a

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column per packaging material are not significantly different ($P > 0.05$).
[b] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).
[c] CAP, capsaicin; DHC, dihydrocapsaicin.
[d] Total capsaicinoids; capsaicin + dihydrocapsaicin.

Table II-10. Color values and piperines of treated and untreated black pepper surrounded with various plastic films subjected to RF heating[a]

Packaging materials	Treatment time (s)	Color[b]			Piperine (mg/100g)
		L^*	a^*	b^*	
PP	0	44.57 ± 1.20 a	2.20 ± 0.11 a	13.34 ± 0.55 a	29.68 ± 0.78 a
	60	43.40 ± 1.36 a	2.30 ± 0.19 a	12.56 ± 0.87 a	29.71 ± 1.90 a
PE	0	43.63 ± 0.72 a	2.61 ± 0.16 a	12.71 ± 0.50 a	28.39 ± 0.61 a
	60	44.19 ± 0.60 a	2.73 ± 0.07 a	13.31 ± 0.64 a	29.09 ± 1.37 a
Nylon	0	43.77 ± 0.86 a	2.99 ± 0.18 a	12.79 ± 0.84 a	29.34 ± 2.02 a
	70	44.58 ± 0.91 a	2.76 ± 0.20 a	12.01 ± 0.84 a	29.13 ± 1.31 a
PEI	0	44.77 ± 0.45 a	2.67 ± 0.14 a	12.51 ± 0.10 a	29.49 ± 1.22 a
	50	43.58 ± 1.11 a	2.91 ± 0.13 a	12.25 ± 0.28 a	30.09 ± 0.85 a

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column per packaging material are not significantly different ($P > 0.05$).
[b] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).

II-2.4. Discussion

With the direct interaction between RF energy and the food material, heat is generated within the material and throughout its mass. This can significantly increase heating rates and reduce treatment time (Tang and Wang, 2007). When red and black pepper spices were treated with RF heating, increased heating rates correlated with increased reduction level of foodborne pathogens in sample. RF treatment causes microbial inactivation predominantly through thermal effect such as denaturation of enzymes, proteins, nucleic acids, or other vital components as well as disruption of cell membranes (Datta and Davidson, 2000; Heddleson and Doores, 1994). Geveke et al. (2002) applied RF energy at 18 MHz and an electric field strength of 0.5 kV/cm to the liquid foods containing *Listeria innocua, E. coli* K-12, or yeast while heat was simultaneously minimized to control temperature. They concluded that there was no non-thermal effects of RF energy on microbial inactivation.

Although RF heating is an indirect heating process resulting in great potential as post-packaging pasteurization technique, there are limited studies of effectiveness of RF heating on pathogenic microorganisms in packaged foods. In the present study, after packaging with various plastic films such as PP, PE, nylon, and PEI, RF treatment rapidly increased the temperatures of powdered red and black pepper spices high enough to control foodborne pathogens. RF heating could meet the requirements to achieve 5-log reduction of *S.* Typhimurium and *E. coli* O157:H7 without affecting

the product quality. Among tested packaging materials, PEI film reduced the treatment time required to reduce both pathogens below the detection limit.

Recently there have been some research efforts to apply PEI assistance in RF heating for treatment of low moisture foods (Jiao et al., 2014; Jiao et al., 2015). However, these studies were limited to improvement of RF heating uniformity and did not assess microbial inactivation. Comparing other plastic films, PEI has the closest dielectric constant to that of red and black pepper spices and a lower dielectric loss factor. The dielectric constant is dominating the electric field distribution and the dielectric loss factor determines permeability of electromagnetic energy. If the dielectric constant is too high or low, electric fields converge at sample edges or corners. In the case of the dielectric loss factor, if it is too high, electromagnetic energy cannot permeate packaging materials (Metaxas, 1996). Therefore, in order to improve the effectiveness of RF heating during post-packaging pasteurization, dielectric properties of packaging materials should be considered.

Following heating treatment, sub-lethally injured foodborne pathogens are potentially as dangerous as their uninjured counterparts (Lee and Kang, 2001; McCleery and Rowe, 1995). This is because heat-injured cells could undergo recovery and become normal. Therefore, the cell numbers enumerated on selective agar is not good enough to represent the total surviving populations in the samples. After RF heating with different packaging materials, there were no significant ($P > 0.05$) differences between injured and uninjured cells in powdered red and black pepper

spice. This suggests that RF heating effectively inactivated *S.* Typhimurium and *E. coli* O157:H7 in red and black pepper powder without generating heat-injured cells which could recover.

In addition, after the maximum treatment applied for inactivation of foodborne pathogens, color values (L^*, a^*, and b^*) and volatile flavor component values of samples surrounded with various packaging materials were not significantly ($P > 0.05$) different from those of the control. Other researchers also reported that RF heating treatment resulted in food products of superior quality. Ha et al. (2013) found that the RF heated peanut butter crackers maintained color, flavor, texture, and overall acceptability. Geveke et al. (2007) reported that the RF heating method produced no loss in ascorbic acid content and caused little enzymatic browning in orange juice. Based on my results, no significant quality differences were observed between untreated and RF-treated red and black pepper powder surrounded with various packaging materials, but treatment time required to reduce both pathogens to below the detection limit was different according to packaging materials.

This results indicate that RF heating leads to effective inactivation of *S.* Typhimurium and *E. coli* O157:H7 in powdered red and black pepper packaged in various plastic films, as well as producing spices of superior quality. The results of this study are fundamental in order to understand and model the response of spices to the RF electromagnetic field at certain packaging materials, and by extension, to apply commercial RF sterilization. With a fuller understanding of the influence of packaging

materials on heating rates in red and black pepper, RF heating could be a very promising alternative technology to control microbiological contamination in spices after packaging.

II-3. Effect of electrode gap on inactivation of *Cronobacter sakazakii* in powdered infant formula by RF heating

II-3.1. Introduction

Cronobacter is a newly defined genus composed of six species and was previously known as *Enterobacter sakazakii* (Food and Agriculture Organisation/World Health Organisation, 2008). *C. sakazakii* is an emerging foodborne pathogen, often transmitted through powdered infant formula and is responsible for a series of neonatal infections (Iversen and Forsythe, 2003). The first two cases of neonatal infections considered to have been caused by *C. sakazakii* were reported in 1961 (Urmenyi and Franklin, 1961). Numerous cases have been subsequently occurred (Strydom et al., 2012). Necrotizing meningitis and enterocolitis caused by *C. sakazakii* have high mortality rates (Lucas and Cole, 1990). *C. sakazakii* has been reported as frequently isolated from powdered infant formula and the environment of milk powder factories (Kandhai et al., 2004).

In a survey study conducted in the United Kingdom, Turkey, South Africa, and Egypt, the prevalence of *C. sakazakii* in milk powder samples was determined to be 5% (Cawthorn et al., 2008; El-Sharoud et al., 2009; Gökmen et al., 2010; Iversen and Forsythe, 2004). Arku et al. (2008) concluded the viability of *C. sakazakii* in spray dried skimmed-milk samples for 12 weeks of storage. This finding verified that *C. sakazakii* can survive for long periods in the low water activity environment of milk powder (Breeuwer et al., 2003).

Radio-frequency (RF) heating involves the use of electromagnetic energy at frequencies between 1 and 300 MHz to generate heat in dielectric material. Dielectric heating can be more uniform than conventional heating because of the direct interaction between food materials and electromagnetic waves (Zhao et al., 2000). Compared to microwave heating, RF heating offers the advantages of providing more uniform heating due to deep penetration and simple uniform field patterns (Marra et al., 2009). Since most factors such as frequency, electric field strength, and plate surface were fixed in a RF heater, distance between electrodes could be industrially important factor in the efficacy of RF heating (Piyasena et al., 2003). However, there are no studies considering the effect of electrode gap on the inactivation efficacy of RF heating.

The inactivation of microorganisms by heat processing methods has been traditionally assumed to follow first-order kinetics. All cells or spores in a population are assumed to have equal resistance to lethal treatments, and therefore a linear relationship between the declines in the logarithm of the number of survivors over treatment time would be expected (Schaffner and Labuza, 1997). Three kinds of deviations from linearity have frequently been observed in many thermal treatments: curves with a shoulder, curves with tailing, and sigmoidal curves (Cerf, 1977; Peleg and Cole, 1998; van Boekel, 2002; Xiong et al., 1999). The survival curves of foodborne pathogens on powdered red and black pepper spices were not log linear and were clearly concave in my previous study (Jeong and Kang, 2014).

The objective of this study were to investigate the effect of electrode gap on inactivation of *C. sakazakii* in powdered infant formula and product quality such as color, moisture content, and sulfhydryl activities. Also, predictive model equations were derived in order to calculate certain treatment time for accomplishing expected inactivation levels by RF heating.

II-3.2. Materials and Methods

Bacterial strains. Three strains of *C. sakazakii* (ATCC 12868, ATCC 29544, and ATCC 51329) were obtained from the Bacterial Culture Collection of Seoul National University (Seoul, South Korea) and were used in the experiments. Stock cultures were kept frozen at –80 °C in 0.7 ml of tryptic soy broth (TSB; Difco Becton, Dickinson, Sparks, MD, USA) and 0.3 ml of 50% (vol/vol) glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37 °C for 24 h, and stored at 4 °C.

Preparation of pathogen inocula. All strains of *C. sakazakii* were cultured individually in 25 ml of Enterobacteriaceae enrichment broth (EEB; Mossel formula, LAB, United Kingdom) at 37 °C for 24 h, harvested by centrifugation (4,000 × g for 20 min at 4 °C), and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in BPW, corresponding to approximately 10^6 to 10^7 CFU/ml. Subsequently, the suspended pellets of each strain of the *Cronobacter* spp. were combined to produce mixed culture cocktails (three strain). These cell suspensions, consisting of a final concentration of ca. 10^7 CFU/ml, were used in the inactivation study.

Sample preparation and inoculation. Commercial powdered infant formula (Namyang Co., Gongju, South Korea) was purchased at a local grocery store (Seoul, South Korea). For inoculation, 5 ml of culture cocktail was added dropwise to 250 g of samples inside sterile high-density polyethylene (HDPE) bags (300 ×450 mm). The inoculated samples were thoroughly mixed by hand massaging for 10 min to produce a homogeneous dispersal of inoculum throughout the powered infant formula and dried for 2 h inside a biosafety hood (22 ± 2 °C) with the fan running until the water activity (a_w) of the sample equaled that of an noninoculated sample (ca. 0.42). The water activities of noninoculated and inoculated samples were measured using the AquaLab model 4TE water activity meter (Decagon Devices, Pullman, WA, USA). The final cell concentration was 10^5 to 10^6 CFU/g. The inoculated infant formula powder samples were then immediately used in each experimental trial.

RF heating treatment. RF heating were carried out in a previously described apparatus (Jeong and Kang, 2014). A RF heating system consisted of a RF heater (Seoul National University, Seoul, South Korea; Dong Young Engineering Co. Ltd., Gyeongnam, South Korea), a temperature signal conditioner (TMI-4; FISO Technologies Inc., Quebec, Canada), and a computer (Fig. II-7). The RF heater generated a RF electric field at a frequency of 27.12 MHz and a maximum power of 9 kW. Its cavity was composed of two parallel-plate electrodes (30.0 × 35.0 cm; 0.6 cm thick) and three electrode gaps (8, 10, and 12 cm) were selected. A sample filled

in glass beaker, 5.0 cm in diameter and 7.2 cm deep, was placed on the center of the bottom electrode. RF heating was applied to each prepared sample and heated to 90 °C in order to maximize the efficacy of pasteurization while maintaining product quality.

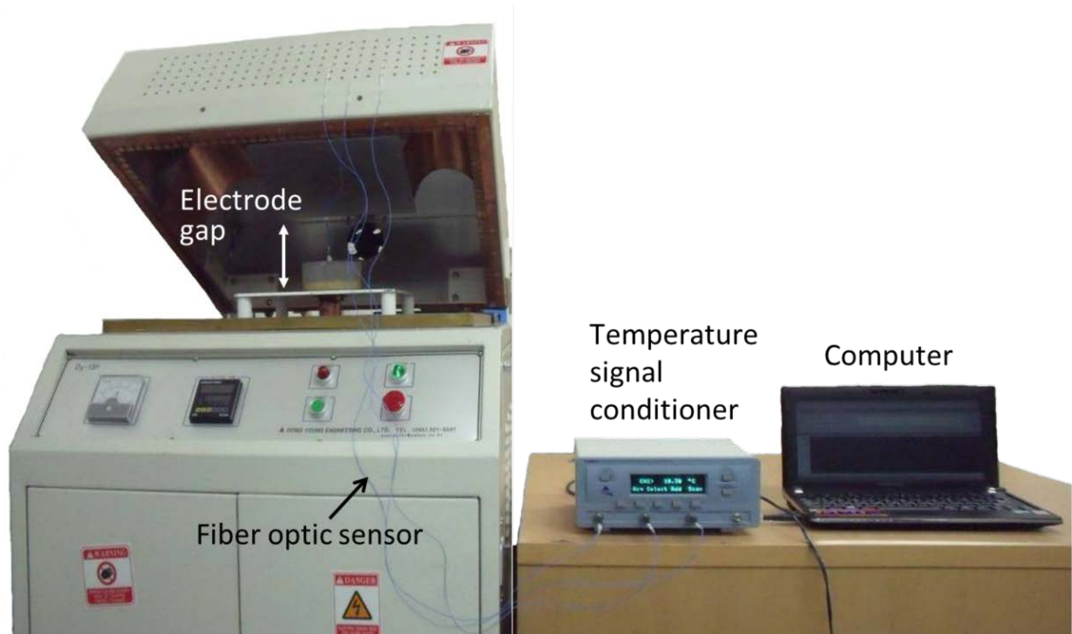


Fig. II-7. RF heating system comprised of a RF heater and a temperature signal conditioner.

Temperature measurement. A fiber optic temperature sensor (FOT-L; FISO Technologies Inc., Quebec, Canada) connected to a temperature signal conditioner was used to measure real-time temperatures in samples during RF heating. The sensor was directly inserted at the center of the treated infant formula powder and the temperature was recorded at 1 s intervals. Since the fiber optic sensor was coated with electric insulating material, it did not interfere with the temperature profile of the treated sample (Wang et al., 2003). All experiments were repeated three times, and averages and standard deviations of RF treated sample temperatures were compared to determine the heating rate of samples according to electrode gap.

Bacterial enumeration. At selected time intervals, 25 g of treated samples were removed and immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of BPW (detection limit, 1 log CFU/g) and homogenized for 2 min with a stomacher (Easy Mix; AES Chemunex, Rennes, France). After homogenization, 1-ml aliquots of sample were 10-fold serially diluted in 9 ml blanks of BPW, and 0.1 ml of sample or diluent was spread-plated onto selective medium, chromogenic *Enterobacter sakazakii* agar (ESA) (Brilliance, DFI formulation; Oxoid), for the enumeration of *C. sakazakii* cells. The agar plates were incubated at 37 °C for 24 h, and then the cells were enumerated by counting blue-green colonies.

Enumeration of injured cells. The liquid repair method was used to enumerate injured cells of *C. sakazakii*. One-milliliter aliquots of treated sample were 10-fold serially diluted in 9 ml of EEB, and the diluted medium was incubated at 37 °C for 2 h to allow injured cells to be recovered. After the recovery step, 0.1 ml of diluent was spread-plated onto chromogenic selective medium. All agar plates were incubated for 22 h at 37 °C, and the typical blue-green colonies were counted. It has been reported that the optimal temperature range for growth of *Cronobacter* strains is 37 °C (Iversen et al., 2004). Injured cells are easily recovered on nonselective broth or liquid medium in less than 2 h, and the liquid medium recovery method is simpler and faster than solid agar repair methods, such as the overlay method (Cole et al., 1993). By performing preliminary experiments, we confirmed that the 2 h of incubation period in liquid broth did not cause multiplication of uninjured cells in control samples and that the recovery level of injured *C. sakazakii* cells in liquid broth was not significantly different from that in the agar overlay method.

Quality measurement. To measure the effect of RF heating on the color of powdered infant formula using various electrode gaps, a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan) was used to measure the color changes of RF-treated samples. The values of L^*, a^*, and b^* were used to quantify color attributes and measurements were taken from treated and untreated noninoculated infant formula powder taken at random locations. L^*, a^*, and b^* values indicate color lightness,

redness, and yellowness of the sample, respectively. After RF heating treatment, the post-treatment moisture content was measured immediately with the halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). Reactive sulfhydryl groups were measured according to the method outlined by Kalab (1970) using a 20% solution of powdered infant formula.

Modeling of survival curves. All experiments were conducted with duplicate samples and repeated three times and survival curves were fitted with the first-order model (equation 1), Weibull model (equation 2), and log-logistic model (equation 3) by using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA).

The first-order model (Schaffner and Labuza, 1997) is given by:

$$\log\frac{N}{N_0} = -\frac{t}{D} \qquad (1)$$

where N_0 is the initial number of cells (CFU/g), N is the number of survivors after treatment time (CFU/g), D is the time required to destroy 90% of the microorganisms (min), and t is the treatment time (min).

The Weibull model (Bialka et al., 2008) is described as:

$$\log\frac{N}{N_0} = -\left(\frac{1}{2.303}\right)\left(\frac{t}{\alpha}\right)^{\beta} \qquad (2)$$

where α value represents the time necessary to inactivate the first 0.434 log cycles of the population and β value represents the shape of the line, such as upward

concavity of a curve when $\beta < 1$, downward concavity when $\beta > 1$, and linear curve when $\beta = 1$.

The log-logistic model (Chen and Hoover, 2003) is given by:

$$\log\left(\frac{N}{N_0}\right) = \frac{A}{1+e^{4\sigma(\tau-\log t)/A}} - \frac{A}{1+e^{4\sigma(\tau+6)/A}} \qquad (3)$$

where σ is the maximum rate of inactivation (log CFU/ml), τ is the log time to the maximum rate of inactivation (log minutes), t is the thermal treatment time (s), and A is the lower asymptote minus the upper asymptote (log CFU/ml). The regression coefficient (R^2) and mean square error (MSE) were used to evaluate the goodness of fit of the three models.

Statistical analysis. All experiments were repeated three times with duplicate samples. Data were analyzed by the analysis of variance procedure of Statistical Analysis System (SAS Institute, Cary, NC), and mean values were separated using Duncan's multiple-range test. $P < 0.05$ was used to determine significant differences in the processing treatment.

II-3.3. Results

Average temperature-time histories of powdered infant formula with different electrode gaps. Average temperatures of powdered infant formula during RF heating with various electrode gaps are shown in Fig. II-8. The temperature rose immediately in response to RF energy when the samples were treated with RF heating, and the heating rate of infant formula powder was inversely proportional to the electrode gap. The temperature increase under the electrode gap of 8 cm (1.21 °C/s) was higher than that of 10 cm (0.93 °C/s) and 12 cm (0.77 °C/s). After 54 s of RF heating with the electrode gap of 8, 10, and 12 cm, the temperature of powdered infant formula reached ca. 91, 72, and 64 °C, respectively. For the electrode gap of 10 and 12 cm, the maximum heating times to reach 90 °C from room temperature were 70 and 85 s, respectively.

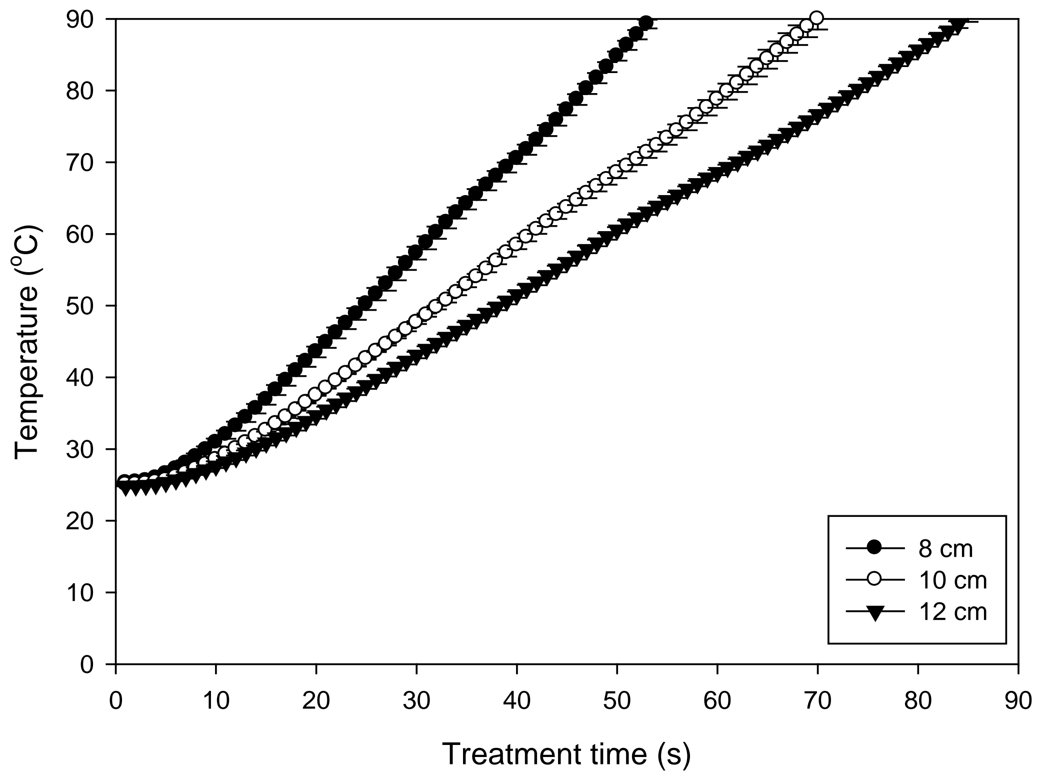


Fig. II-8. Average temperature-time histories of powdered infant formula during RF heating as influenced by electrode gaps. The results are means from three experiments, and error bars indicate standard deviations.

Inactivation of pathogenic bacteria by RF heating with various electrode gaps. The survival curves corresponding to the inactivation of *C. sakazakii* by RF heating with different electrode gaps in powdered infant formula are shown in Fig. II-9. A decrease in the applied electrode gap resulted in a faster heating rate and therefore a gradual increase in inactivation of *C. sakazakii*. RF Treatment with the electrode gap of 12 cm for 60 s inactivated populations of *C. sakazakii* by about 0.23 log CFU/g. For the electrode gap of 8 cm, treatment for 60 s reduced this pathogen by an additional 2.62 log CFU/g more than did the treatment with 12 cm of electrode gap. The survival curves of the pathogen exhibited shoulder behavior at all electrode gaps.

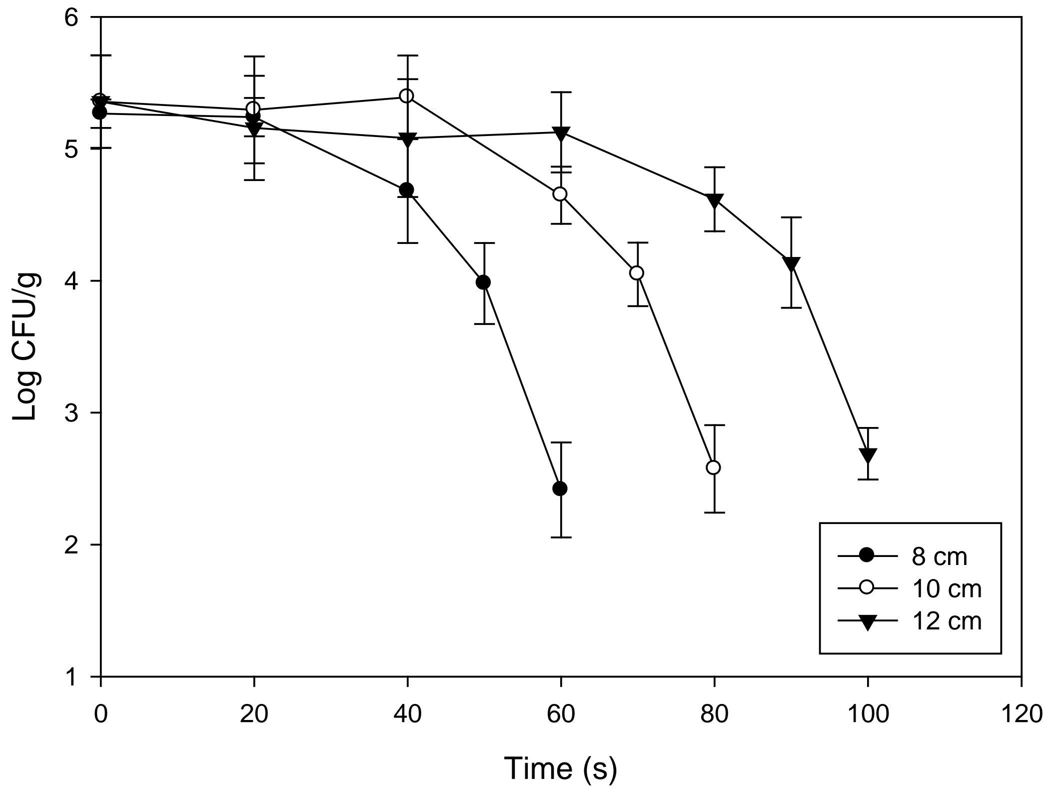


Fig. II-9. Survival curves for *Cronobacter sakazakii* in powdered infant formula treated with RF heating using electrode gaps of 8 cm (●), 10 cm (○), and 12 cm (▼). The results are means from three experiments, and error bars indicate standard deviations.

Recovery of heat-injured cells. Surviving cells and heat-injured cells of *C. sakazakii* from powdered infant formula following RF heating were compared (Table II-11). When inoculated infant formula powder were treated with RF heating, slightly higher numbers of both pathogens were detected by the procedure involving the recovery step (liquid broth recovery method) than by direct plating on selective agar (ESA). However, during the entire treatment time with different electrode gaps, no significant ($P > 0.05$) differences between levels of surviving cells, including sublethally injured *C. sakazakii* cells, were observed in powdered infant formula.

Table II-11. Comparison of pathogen populations between surviving cells and cells including heat-injured cells in inoculated powdered infant formula following RF heating[a]

Electrode gap (cm)	Treatment time (s)	Population (log_{10} CFU/g) by selection medium	
		SA[b]	SAR
8	0	5.27 ± 0.11 Aa	5.20 ± 0.01 Aa
	20	5.24 ± 0.15 Aa	5.31 ± 0.46 Aa
	40	4.68 ± 0.39 Ba	4.95 ± 0.45 Aa
	50	3.98 ± 0.31 Ca	4.08 ± 0.46 Ba
	60	2.42 ± 0.36 Da	2.45 ± 0.45 Ca
10	0	5.36 ± 0.35 Aa	5.63 ± 0.41 Aa
	20	5.30 ± 0.40 Aa	5.66 ± 0.26 Aa
	40	5.39 ± 0.32 Aa	5.54 ± 0.08 Aa
	60	4.65 ± 0.22 Ba	5.04 ± 0.22 Ba
	70	4.05 ± 0.24 Ca	4.45 ± 0.26 Ca
	80	3.19 ± 0.33 Da	3.20 ± 0.32 Da
12	0	5.36 ± 0.35 Aa	5.63 ± 0.41 Aa
	20	5.16 ± 0.40 ABa	5.46 ± 0.17 Aa
	40	5.08 ± 0.45 ABa	5.52 ± 0.20 Aa
	60	5.13 ± 0.30 ABa	5.46 ± 0.13 Aa
	80	4.62 ± 0.24 BCa	4.91 ± 0.30 Ba
	90	4.14 ± 0.34 Ca	4.47 ± 0.25 Ba
	100	3.27 ± 0.20 Da	3.51 ± 0.46 Ca

[a] Means ± standard deviations from three replications. Means with the same capital letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] SA, plating directly on selective agar; SAR, plating on selective agar preceded by a recovery step.

Effect of RF heating with different electrode gaps on product quality. Color values of powdered infant formula after RF heating with various electrode gaps for time intervals required to reduce *C. sakazakii* by ca. 3 log CFU/g are summarized in Table II-12. Within all tested electrode gaps, L^*, a^*, and b^* values of RF-treated samples were not significantly ($P > 0.05$) different from those of untreated samples. Table II-13 show the moisture content and sulfhydryl activity of infant formula powder following RF treatment. As the electrode gap increased, those values of RF treated samples were significantly different ($P < 0.05$) resulting from prolonged treatment time. Especially the activation of sulfhydryl groups, index of milk serum protein denaturation, greatly increased.

Table II-12. Color values of powdered infant formula following RF heating using varying electrode gaps[a]

Electrode gap (cm)	Treatment time (s)	Parameter[b]		
		L*	a*	b*
	0	93.92 ± 0.12 a	– 4.52 ± 0.02 a	21.89 ± 0.29 a
8	60	93.59 ± 0.24 ab	– 4.64 ± 0.16 a	21.94 ± 0.03 a
10	80	93.35 ± 0.16 b	– 4.65 ± 0.19 a	21.80 ± 0.15 a
12	100	93.43 ± 0.16 b	– 4.62 ± 0.16 a	22.09 ± 0.63 a

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column are not significantly different ($P > 0.05$).
[b] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).

Table II-13. Moisture content and sulfhydryl activity of powdered infant formula following RF heating using varying electrode gaps[a]

Electrode gap (cm)	Treatment time (s)	Moisture content (%)[b]	Reactive sulfhydryl (μmol/L)
	0	1.99 ± 0.11 a	36.62 ± 5.84 a
8	60	1.95 ± 0.19 a	40.52 ± 7.04 a
10	80	1.64 ± 0.06 b	79.62 ± 7.23 b
12	100	1.62 ± 0.07 b	93.52 ± 2.78 c

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column are not significantly different ($P > 0.05$).
[b] All moisture contents are expressed on a dry basis.

Suitable model of survival curves. The traditional linear and non-log-linear model for the inactivation of *C. sakazakii* in powdered infant formula during RF heating with different electrode gaps is represented in Table II-14. For *C. sakazakii,* mean MSE values with the log-linear, Weibull, and log-logistic model were 0.24, 0.01, and 0.003, respectively. Mean R^2 values of Weibull and log-logistic model were 0.98 and 0.99, respectively, which were better than the values of 0.68 of the log-linear model. Table II-14 also show the effect of electrode gap on inactivation of *C. sakazakii* in infant formula powder. As the electrode gap decreased, time required to reach 5 log reduction decreased.

Table II-14. Comparison of goodness of fit of log-linear, Weibull, and log-logistic model for the survival curves of *Cronobacter sakazakii* in powdered infant formula treated with RF heating[a]

Model	Electrode gap (cm)	MSE	R^2	t_{5d} (s)[b]
Log-linear	8	0.36	0.68	166.67
	10	0.22	0.67	250.01
	12	0.15	0.68	500.04
Weibull	8	0.00	0.99	68.85
	10	0.01	0.99	97.80
	12	0.02	0.97	122.04
Log-logistic	8	0.00	0.99	68.15
	10	0.00	0.99	99.24
	12	0.01	0.98	118.78

[a] MSE, mean square error; R^2, regression coefficient.
[b] t_{5d}, time required to reach 5 log reduction.

II-3.4. Discussion

The heating rate of powdered infant formula increased with decreasing electrode gap during RF heating. To clarify explain the effect of electrode gap, the heat generating RF energy absorbed by the powdered infant formula is given by the equation $P = 2\pi f E^2 (S/d) \varepsilon_0 \varepsilon''$, where P is the electrical power transferred to the food as heat, f is the frequency, E is the electric field strength, S is the plate surface, d is the distance between electrodes, ε_0 is the dielectric constant of a vacuum considered equal to 8.85×10^{-12} F/m, and ε'' is the dielectric loss factor of the sample. From this equation, the heat generated is proportional to the frequency, the electric field strength, the plate surface, and the dielectric loss factor, which were fixed in the present study leaving only the impact of electrode gap to be examined. Our results agree with the equation; more heat was generated as a result of a smaller electrode gap during RF heating. Similar results were observed in in-shell almonds and macadamia nuts (Gao et al., 2011; Wang et al., 2014).

RF heating for the treatment time required to reach 90 °C achieved 2.85-, 2.17-, and 2.09-log reductions of *C. sakazakii* without generating heat-injured cells at the electrode gaps of 8 cm, 10 cm, and 12 cm, respectively. In the first moments of treatment with all electrode gaps, the change in the level of *C. sakazakii* was very small (shoulder of the survival curve) and then the microbial populations declined following a concave downwards curve when the temperature increased into the >

70 °C range. In my previous RF heating study (Jeong and Kang, 2014), survival curves for *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on red and black pepper powder with various moisture contents exhibited characteristic concave downwards curves. Various models have been proposed to describe non-log-linear survival curves (Baranyi and Roberts, 1994; Bhaduri et al., 1991; Cole et al., 1993). Non-log-linear models assume that bacterial cells in a population do not have identical heat resistances, and a survival curve is the cumulative form of a distribution of lethal agents (Chen and Hoover, 2004). In the present study, among the non-log-linear inactivation models the Weibull distribution (equation 2) and the log-logistic model (equation 3) were selected to describe the experimental data obtained under nonisothermal conditions.

The goodness of fit of the linear and non-log-linear models were compared by calculating the MSE and R^2 values. The MSE is a measure of the variability remaining in the predictive models, and a lower MSE indicates that the model describes the data adequately (Adair et al., 1989). The R^2 value is often used as an overall measure of predictive models, and a higher R^2 value indicates a better prediction attained by a particular model (Grau and Vanderlinde, 1993). In the present study, log-linear, Weibull, and log-logistic model equations were used to fit the pathogen survival curves on infant formula powder treated with RF heating. Weibull and log-logistic models fit well with R^2 values approximately equal to 0.97–0.99. Therefore, for powdered infant formula pasteurization by RF heating, the Weibull and log-logistic

model more accurately described the survival curves for *C. sakazakii* than did the first-order kinetics with various electrode gaps.

The color values, moisture contents, and sulfhydryl activities of infant formula powder subjected to RF treatment with the electrode gap of 8 cm were not significantly ($P > 0.05$) different from those of nontreated samples. However, these qualities significantly ($P < 0.05$) changed at prolonged treatment time as the electrode gap increased. It has been proposed that low-medium heat treatment causes milk serum protein to be denatured (Cluskey et al., 1997; Jenness, 1954). Therefore, our results suggest 8 cm as an ideal electrode gap for RF heating, since it led to very similar pathogen reduction compared to 10 and 12 cm, but without degrading the sensory properties of powdered infant formula.

In conclusion, our results indicated that RF heating under minimized electrode gap could reduce *C. sakazakii* in powdered infant formula without affecting color quality changes. The non-log-linear model produced better fit to the data than the traditional linear model for all tested treatments. The Weibull and log-logistic model, which had been mostly used for describing inactivation of the bacterial cells by heat treatment, could be successfully used to predict survival curves of *C. sakazakii* in infant formula powder by RF heating. Furthermore, the results of this study can be used to define the optimum operational conditions for RF heating to achieve desired levels of inactivation of foodborne pathogens on powdered foods while minimizing the production costs.

Chapter III.

Combination treatments of RF heating with various sanitizing technologies

III-1. Enhanced inactivation of *Cronobacter sakazakii* in powdered infant formula by RF heating combined with UV radiation and mechanism of the synergistic bactericidal action

III-1.1. Introduction

Cronobacter sakazakii was first described as yellow-pigmented *Enterobacter cloacae* in 1958 and then was reclassified as the novel genus which contains five species (Iversen et al., 2008; Urmenyi and Franklin, 1961). Although illnesses due to *C. sakazakii* have been reported in all age groups, it appears that infants less than 2 months old are at the highest risk for infection. *C. sakazakii* has been associated with foodborne outbreaks such as neonatal meningitis, septicemia and necrotizing enterocolitis (Iversen and Forsythe, 2003). Dried infant formula has been recognized as the only food vehicle of transmission in *C. sakazakii* infections (Centers for Disease Control and Prevention, 2002; Iversen and Forsythe, 2003; Kandhai et al., 2004).

Most of the research on thermal processing for controlling *C. sakazakii* in infant formula has focused on the rehydrated or reconstituted state, not the end product in powdered form. These studies have shown no unusual heat-resistance of *C. sakazakii* in reconstituted or rehydrated milk powder (Edelson-Mammel and Buchanan, 2004; Nazarowec-White et al., 1999; Osaili et al., 2009). However, the milk powder manufacturing process involves two heat treatment steps; pasteurization of standardized raw milk and drying, which can be accomplished by spray drying or roller drying (Westergaard, 2004). Drying can allow *C. sakazakii* to survive and persist in the powder bed. *C. sakazakii* has been known to be more resistant in low water activity food such as powdered infant formula (Edelson-Mammel et al., 2005;

Lehner and Stephan, 2004). Therefore, the inactivation of *C. sakazakii* in the final dehydrated milk powder is of great concern to the dairy industry.

In order to reduce levels of *C. sakazakii* in powdered infant formula, several decontamination methods including gamma and electron beam irradiation, supercritical carbon dioxide treatment, and gaseous ozone treatment have been proposed (Hong et al., 2008; Kim et al., 2010; Lee et al., 2006a; Osaili et al., 2007; Torlak and Sert, 2013). However, due to poor consumer acceptance, high installation cost, and difficulty of scaling up for large volume, these methods have limitations for commercial application. For these reasons, it is necessary to develop an alternative method for inactivation of *C. sakazakii* that can be applied to the final dehydrated infant formula.

Radio-frequency (RF) heating has emerged as a promising thermal sanitizing technology for food in recent years (Houben et al., 1991; Zhao et al., 2000). RF heating can be more rapid and uniform than conventional heating because of molecular friction resulting from the direct interaction between electromagnetic waves and food materials (Marra et al., 2009; Piyasena et al., 2003). In my previous study, the effectiveness of RF heating processing for pasteurization of powdered infant formula was less than that of powdered red and black pepper spices. Combinations with different technologies, known as hurdle technology, could be an alternative to overtreatment of RF heating. Combined treatments can improve food

safety and maintain organoleptic qualities of foods, while reducing the intensity of each hurdle (Khan et al., 2017).

Ultraviolet (UV) radiation were selected as a non-thermal technology to investigate antimicrobial effects in combination with RF heating. UV treatment has been approved has been approved for use as a disinfectant for surface treatment of foods (Food and Drug Administration, 2002). Since it can cause cumulative damage to microbial DNA, UV radiation was recommended for use in combination with other techniques such as near-infrared heating, hydrogen peroxide, ozone, and sodium hypochlorite (Ha and Ha, 2011; Ha and Kang, 2014; Hadjok et al., 2008; Selma et al., 2008). However, none of the studies examined huddle effect of RF heating in combination with UV radiation.

The objective of this study was to evaluate the antimicrobial effects of RF heating combined with UV radiation against *C. sakazakii* in powdered infant formula. The mechanism of inactivation was investigated by measuring leakage of UV-absorbing substances and propidium iodide (PI) uptake values, and analyzing transmission electron microscopy. Also, any changes in color, moisture content, and sulfhydryl activity of powdered infant formula were assessed.

III-1.2. Materials and Methods

Bacterial strains. Three strains of *C. sakazakii* (ATCC 12868, ATCC 29544, and ATCC 51329) were obtained from the Bacterial Culture Collection of Seoul National University (Seoul, South Korea) and were used in the experiments. Stock cultures were kept frozen at –80 °C in 0.7 ml of tryptic soy broth (TSB; Difco Becton, Dickinson, Sparks, MD, USA) and 0.3 ml of 50% (vol/vol) glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37 °C for 24 h, and stored at 4 °C.

Preparation of pathogen inocula. All strains of *C. sakazakii* were cultured individually in 25 ml of Enterobacteriaceae enrichment broth (EEB; Mossel formula, LAB, United Kingdom) at 37 °C for 24 h, harvested by centrifugation (4,000 × g for 20 min at 4 °C), and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in BPW, corresponding to approximately 10^6 to 10^7 CFU/ml. Subsequently, the suspended pellets of each strain of the *Cronobacter* spp. were combined to produce mixed culture cocktails (three strain). These cell suspensions, consisting of a final concentration of ca. 10^7 CFU/ml, were used in the inactivation study.

Sample preparation and inoculation. Commercial powdered infant formula (Namyang Co., Gongju, South Korea) was purchased at a local grocery store (Seoul, South Korea). For inoculation, 5 ml of culture cocktail was added dropwise to 250 g of samples inside sterile high-density polyethylene (HDPE) bags (300 × 450 mm). The inoculated samples were thoroughly mixed by hand massaging for 10 min to produce a homogeneous dispersal of inoculum throughout the powered infant formula and dried for 2 h inside a biosafety hood (22 ± 2 °C) with the fan running until the water activity (a_w) of the sample equaled that of an noninoculated sample (ca. 0.42). The water activities of noninoculated and inoculated samples were measured using the AquaLab model 4TE water activity meter (Decagon Devices, Pullman, WA, USA). The final cell concentration was 10^5 to 10^6 CFU/g. The inoculated infant formula powder samples were then immediately used in each experimental trial.

Combined treatment of RF heating and UV radiation. The combined treatment of RF heating and UV radiation was sequentially conducted in previously described apparatus (Jeong and Kang, 2014; Kim et al., 2013). Firstly, UV experiments were carried out in a UV radiation apparatus consisted of two banks of germicidal emitting lamp (254 nm, G6T5, Sankyo Denki, Japan). The UV lamps were located in the ceiling and bottom of the radiation vessel and was allowed to stabilize by turning them on for at least 15 min. The reaction time for UV treatment was 60 s, and the corresponding UV doses were 1.22 kJ/m^2 calculating by multiplying the irradiation

time for the intensity of UV lamp. The UV intensity was measured by using a spectrometer at 253.7 nm wavelength (AvaSpec-ULS2048-USB2-UA-50, Avantes, Netherlands). Secondly, a sample filled in glass beaker, 5.0 cm in diameter and 7.2 cm deep, was placed on the center of the bottom electrode of a RF heater generated a RF electric field at a frequency of 27.12 MHz and a maximum power of 9 kW. Treatments consisted of 20, 30, 40, 50, and 60 s RF exposure of powdered infant formula in order to maximize the efficacy of pasteurization while maintaining product quality. Combined UV and RF treatment was also applied in reverse order. For the inactivation mechanism study, 5 ml of cell suspensions kept in glass beaker were treated with RF, UV, and RF-UV under identical conditions: previously confirmed that inactivation patterns of three pathogens on glass petri dishes were similar with those in powdered infant formula.

Bacterial enumeration. Treated samples (25 g) were immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of BPW (detection limit, 1 log CFU/g) and homogenized for 2 min with a stomacher (Easy Mix; AES Chemunex, Rennes, France). After homogenization, 1-ml aliquots of sample were 10-fold serially diluted in 9 ml blanks of BPW, and 0.1 ml of sample or diluent was spread-plated onto selective medium, chromogenic *Enterobacter sakazakii* agar (ESA) (Brilliance, DFI formulation; Oxoid), for the

enumeration of *C. sakazakii* cells. The agar plates were incubated at 37 °C for 24 h, and then the cells were enumerated by counting blue-green colonies.

Enumeration of injured cells. The liquid repair method was used to enumerate injured cells of *C. sakazakii*. One-milliliter aliquots of treated sample were 10-fold serially diluted in 9 ml of EEB, and the diluted medium was incubated at 37 °C for 2 h to allow injured cells to be recovered. After the recovery step, 0.1 ml of diluent was spread-plated onto chromogenic selective medium. All agar plates were incubated for 22 h at 37 °C, and the typical blue-green colonies were counted. It has been reported that the optimal temperature range for growth of *Cronobacter* strains is 37 °C (Iversen et al., 2004). Injured cells are easily recovered on nonselective broth or liquid medium in less than 2 h, and the liquid medium recovery method is simpler and faster than solid agar repair methods, such as the overlay method (Cole et al., 1993). By performing preliminary experiments, we confirmed that the 2 h of incubation period in liquid broth did not cause multiplication of uninjured cells in control samples and that the recovery level of injured *C. sakazakii* cells in liquid broth was not significantly different from that in the agar overlay method.

Transmission electron microscopy analysis. Transmission electron microscopy (TEM) analysis was conducted after RF, UV, and UV-RF treatment to investigate structural damages of pathogen cells. Treated *C. sakazakii* cells in glass beaker

described above were resuspended using 10 ml of phosphate-buffered saline (PBS) collected by centrifugation at 4,000 × g at 4 °C for 10 min. The cells were fixed at 4 °C for 4 h in modified Karnovsky's fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After primary fixation, each sample was centrifuged and washed three times with 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C for 10 min. Cells were postfixed with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C for 2 h and briefly washed twice with distilled water at room temperature. The washed cells were stained overnight with 0.5% uranyl acetate at 4 °C. The cells were then dehydrated at room temperature using a graded ethanol series (10 min each in 30, 50, 60, 70, 95, and 100%), finishing with three consecutive 100% ethanol washes. The transition was performed with 100% propylene oxide at room temperature for 15 min. The cells were then infiltrated for 2 h with a 1:1 solution of propylene oxide and Spurr's resin, and then placed in Spurr's resin overnight. In order to get specimen blocks, the polymerization of the resin was conducted in an oven at 70 °C for 24 h. Specimens were sectioned (70-nm thick) by means of an ultramicrotome (MT-X; RMC, Tucson, AZ, USA) and then stained with 2% uranyl acetate for 7 min, followed by Reynolds' lead citrate for 7 min. The sections were then observed with a transmission electron microscope (Libra 120; Carl Zeiss, Heidenheim, Germany).

Measurement of extracellular UV-absorbing substances and propidium iodine uptake. Cell membrane damage induced by each treatment was quantitatively assessed by determining the release of UV-absorbing materials and propidium iodine uptake from injured cells. Untreated and treated *C. sakazakii* were resuspended using 10 ml of PBS and centrifuged at 10,000 × g at 4 °C for 10 min. The upper 1 ml of the supernatant was removed, and the UV absorbance was measured at a wavelength of 260 and 280 nm with a spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA). The cell pellets were resuspended and diluted in PBS to an optical density at 680 nm (OD_{680}) of approximately 0.2 and then mixed with propidium iodine (PI; Sigma Chemical Co.) solution to a final concentration of 2.9 μM. After incubation for 10 min, the samples were centrifuged at 10,000 × g for 10 min and washed twice in PBS to remove excess dye. The final cell pellets were resuspended in PBS, and fluorescence was measured with a spectrofluorophotometer at an excitation wavelength of 493 nm and an emission wavelength of 630 nm. Fluorescence values for each sample were normalized with the OD_{680} of the cell suspensions.

Quality measurement. To measure the effect of RF heating on the color of powdered infant formula using various electrode gaps, a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan) was used to measure the color changes of RF-treated samples. The values of L^*, a^*, and b^* were used to quantify color attributes and measurements were taken from treated and untreated noninoculated infant formula

powder taken at random locations. L^*, a^*, and b^* values indicate color lightness, redness, and yellowness of the sample, respectively. After RF heating treatment, the post-treatment moisture content was measured immediately with the halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH). Reactive sulfhydryl groups were measured according to the method outlined by Kalab (1970) using a 20% solution of powdered infant formula.

Statistical analysis. All experiments were repeated three times with duplicate samples. Data were analyzed by the analysis of variance procedure of Statistical Analysis System (SAS Institute, Cary, NC), and mean values were separated using Duncan's multiple-range test. $P < 0.05$ was used to determine significant differences in the processing treatment.

III-1.3. Results

Synergistic bactericidal effect of combined UV-RF treatment. The survival numbers of *C. sakazakii* cells in powdered infant formula during RF heating, UV radiation, and sequential application of both technologies is presented in Fig. III-1. Reductions of 0.86, 1.94, and 4.062 log units were observed in *C. sakazakii* after sequential UV-RF combined treatment for 40, 50, 60 s, respectively. The sums of results for RF and UV inactivation were lower than values obtained by the combined application of both technologies. In other words, synergistic effects were observed for all treatment times against *C. sakazakii*. However, statistically significant ($P < 0.05$) differences between the sums of RF and UV inactivation and values for inactivation achieved with combination treatment were observed only after treatment times of 50 s or more. Log reductions resulting from the synergistic effect after 50 s of treatment, calculated by subtracting the sums of RF and UV reductions from the values obtained during sequential UV-RF combined treatment, were 0.78 and 1.34 log units at 50 and 60 s of treatment, respectively. There were no significant ($P > 0.05$) differences in microbial reduction between the orders of combined treatment.

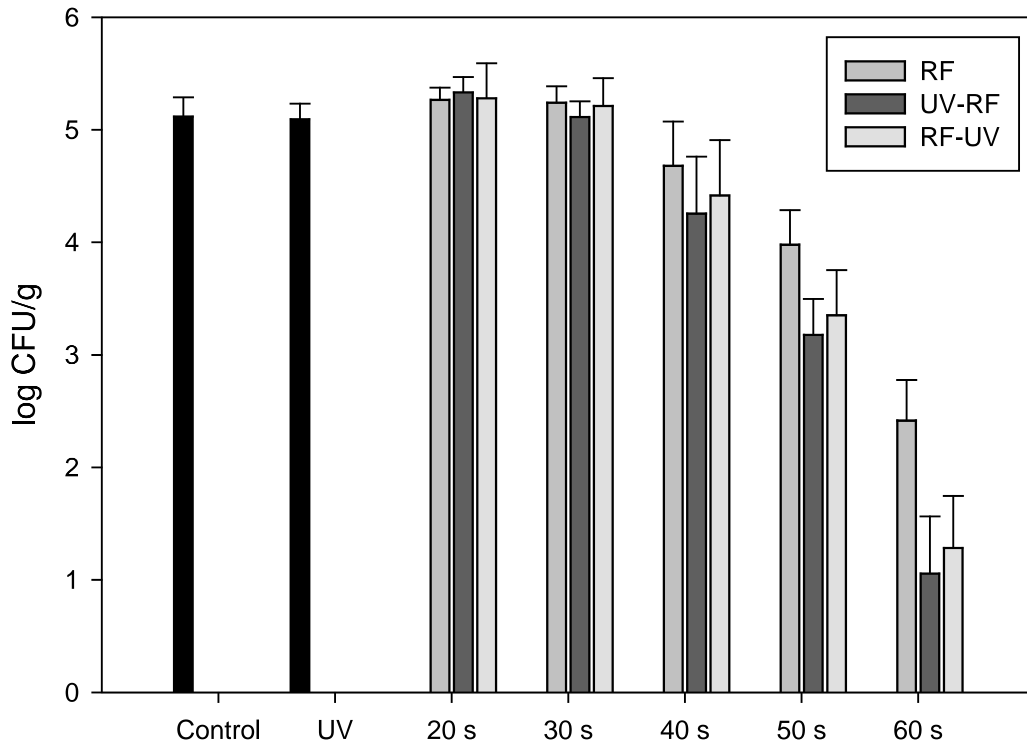


Fig. III-1. Survival (log CFU/g) of *Cronobacter sakazakii* in powdered infant formula during RF heating, UV radiation, and combined application of both technologies. The results are means from three experiments, and error bars indicate standard deviations.

Recovery of UV-RF-injured cells. Levels of sublethally injured *C. sakazakii* cells in powdered infant formula following UV-RF combined treatment are shown in Table III-1. Determining the difference between inactivation of samples subjected to the injured-cell recovery method and that of samples plated directly on selective media revealed the presence of 0.37, 0.49, and 0.44 log units of injured *C. sakazakii* cells after 40, 50, and 60 s treatments, respectively. Slightly smaller reductions of *C. sakazakii* numbers were observed at the final stages of the treatment (40, 50, and 60 s) by the procedure involving the recovery step (liquid broth recovery method) than by direct plating on selective agar (ESA). However, no statistically significant ($P > 0.05$) differences between levels of surviving cells, including sublethally injured *C. sakazakii* cells, were observed during the entire treatment time.

Table III-1. Comparison of pathogen populations between surviving cells and cells including heat-injured cells in inoculated powdered infant formula treated with UV radiation (UV), RF heating (RF), and application of both technologies sequentially (UV-RF)[a]

Treatment	Treatment time (s)	Population (log10 CFU/g)	
		SA[b]	SAR[b]
Control		5.12 ± 0.11 Aa	5.87 ± 0.71 Aa
UV	60	5.09 ± 0.14 Aa	5.51 ± 0.41 Aa
RF	20	5.27 ± 0.11 Aa	5.39 ± 0.31 Aa
	30	5.24 ± 0.15 Aa	5.54 ± 0.15 Aa
	40	4.68 ± 0.39 Aba	5.05 ± 0.69 Aba
	50	3.98 ± 0.31 Ca	4.22 ± 0.42 Ca
	60	2.42 ± 0.36 Da	2.83 ± 0.52 Da
UV-RF	20	5.33 ± 0.14 Aa	5.71 ± 0.43 Aa
	30	5.11 ± 0.14 Aa	5.46 ± 0.42 Aa
	40	4.25 ± 0.51 Ba	4.62 ± 0.57 Ba
	50	3.18 ± 0.32 CDa	3.67 ± 0.68 CDa
	60	1.06 ± 0.51 Da	1.50 ± 0.64 Da

[a] Means ± standard deviations from three replications. Means with the same capital letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] SA, plating directly on selective agar; SAR, plating on selective agar preceded by a recovery step.

Microscopic evaluation of damages. Selected TEM images of ultrastructural changes in *C. sakazakii* cells induced by RF, UV, and UV-RF treatments are shown in Fig. III-2. Microscopic analyses at the cellular level verified that there was cytoplasmic and membrane structural damage during RF heating (Fig. III-2C) and UV-RF combined treatment (Fig. III-2D). More specifically, cytoplasmic shrinkage and aggregation were observed in both RF- and UV-RF-treated cells, in contrast to untreated and UV-treated cells. Furthermore, UV-RF-treated cells experienced significant cell wall damage, leading to a leakage of cellular contents from the cytoplasm. In the case of UV-treated cells, morphological changes as well as collapse of internal cellular structures were not observed compared to control cells (Fig. III-2B).

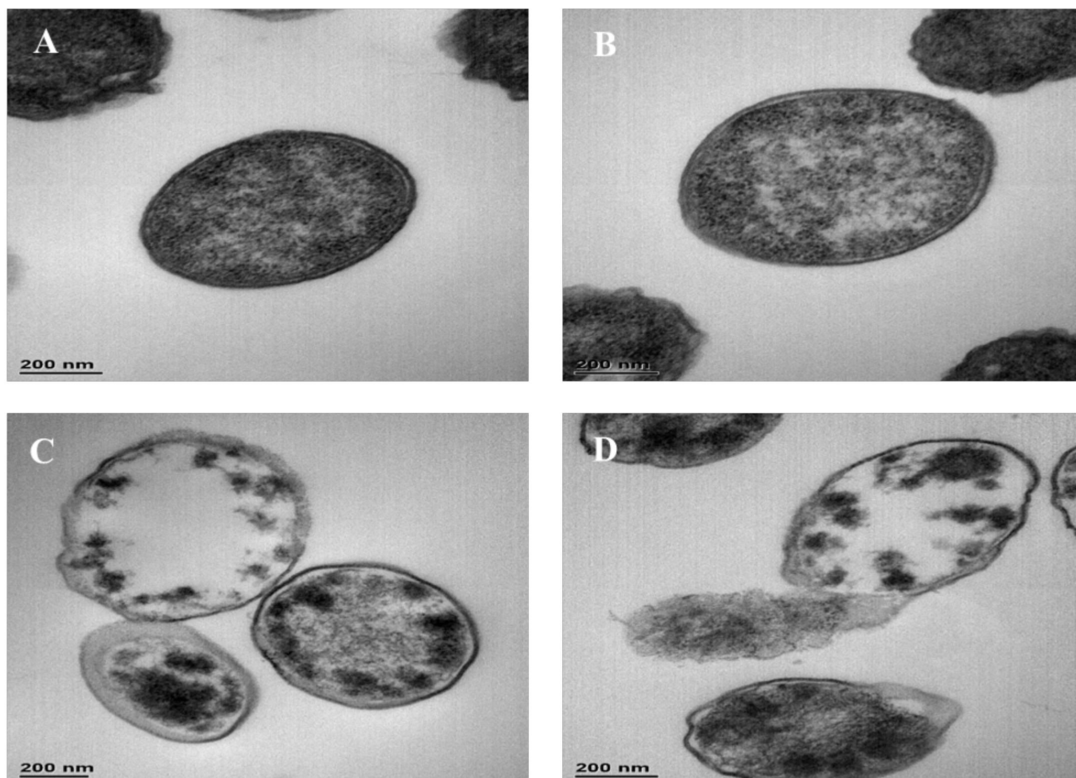


Fig. III-2. Comparison of damage induced by UV radiation, RF heating, and their combination in *Cronobacter sakazakii* cells, observed by TEM. (A) Control sample; (B) UV-treated sample; (C) RF-treated sample; (D) UV-RF-treated sample.

Determination of cell membrane damage by leakage of bacterial intracellular substances and PI uptake. As a further quantitative test of cell membrane damage, leakage of UV-absorbing substances from *C. sakazakii* cells measured at 260 and 280 nm is shown in Table III-2. A disruption of the cell membrane causes an increase in the amount of intracellular substances found outside of the cell. Spectrophotometric observation can detect these substances at 260 nm for nucleic acids and 280 nm for proteinaceous materials (Ukuku and Geveke, 2010). The overall pattern for the leakage of nucleic acids at 260 nm was similar to that of proteins at 280 nm. Also, RF-, UV-, and UV-RF-treated cells were stained with the fluorescent dye PI, which is excluded from cells with intact membranes (Aeschbacher et al., 1986). Table III-2 shows the PI uptake values of *C. sakazakii* after each treatment. Based on trends of leaked intracellular components and PI uptake values, there was no significant ($P > 0.05$) damage to cellular membranes following UV treatment. Leakage of UV-absorbing substances of *C. sakazakii* and the degree of PI uptake in RF- and UV-RF treated cells were much greater than those in UV-treated cells. Among them, the cells subjected to UV-RF treatments showed significantly ($P < 0.05$) higher leakage of intracellular components and PI uptake values than did cells subjected to the other treatments.

Table III-2. Levels of membrane damage of UV, RF, and UV-RF sequentially treated cells obtained from the leakage of intracellular UV-absorbing materials and propidium iodine (PI) uptake test[a]

Treatment	Absorbance[b]		PI uptake value[c]
	260 nm	280 nm	
Control	0.052 ± 0.012 a	0.229 ± 0.008 a	0.00 ± 0.00 a
UV	0.048 ± 0.009 a	0.257 ± 0.013 a	0.44 ± 0.09 a
RF	0.128 ± 0.010 b	0.891 ± 0.011 b	13.97 ± 1.22 b
UV-RF	0.262 ± 0.018 c	1.749 ± 0.069 c	29.00 ± 2.15 c

[a] Mean of three replications ± standard deviation. Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 260 nm : nucleic acids; 280 nm : proteins.
[c] The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} (PI value = fluorescence value after treatment – fluorescence value of non-treated/ OD_{680}).

Effect of UV-RF combined treatment on product quality. The color values of powdered infant formula after the maximum treatment time of RF-, UV-, and combined UV-RF treatment are summarized in Table III-3. The L*, a*, and b* values of UV-RF-treated (60 s) infant formula powder were not significantly ($P > 0.05$) different from those of nontreated samples. Table III-4 shows the moisture content and sulfhydryl activity, index of milk serum protein denaturation, of powdered infant formula following each treatment. There were no significant ($P > 0.05$) differences between untreated and treated samples among all tested samples. Thus, combined application of RF and UV treatment did not affect the quality of infant formula powder product.

Table III-3. Color values of UV, RF, and UV-RF sequentially treated infant formula powder[a]

Treatment	Color[b]		
	L*	a*	b*
Control	92.99 ± 0.49 a	– 4.23 ± 0.33 a	22.13 ± 0.35 a
UV	93.36 ± 0.34 a	– 4.29 ± 0.10 a	22.21 ± 0.31 a
RF	93.45 ± 0.38 a	– 4.36 ± 0.13 a	22.01 ± 0.54 a
UV-RF	93.22 ± 0.62 a	– 4.22 ± 0.23 a	21.94 ± 0.32 a

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column are not significantly different ($P > 0.05$).
[b] Color parameters are L* (lightness), a* (redness), b* (yellowness).

Table III-4. Moisture content and sulfhydryl activity of powdered infant formula following RF, UV, and UV-RF combined treatment[a]

Treatment	Moisture content (%, db)[b]	Reactive sulfhydryl (μmol/L)
Control	1.82 ± 0.28 a	38.36 ± 5.29 a
UV	1.91 ± 0.13 a	39.69 ± 5.19 a
RF	1.80 ± 0.26 a	37.88 ± 4.09 a
UV-RF	1.78 ± 0.21 a	35.57 ± 4.73 a

[a] Means ± standard deviations from three replications. Values followed by the same letters within the column are not significantly different ($P > 0.05$).
[b] All moisture contents are expressed on a dry basis.

III-1.4. Discussion

Although thermal resistance among *C. sakazakii* strains varied as much as 20-fold, *C. sakazakii* has been known to be more thermotolerant than any other member of the *Enterobacteriaceae* (Edelson-Mammel and Buchanan, 2004; Nazarowec-White and Farber, 1997). Thermal treatments cannot be efficiently applied to powdered infant formula because of their low thermal conductivity. Kim et al. (2010) demonstrated that heating at 63, 68, and 73 °C for 30 min could not ensure a 3-log reduction of *C. sakazakii* in dehydrated powdered infant formula. Ha and Kang (2014) concluded that less than a 2 log reduction of *C. sakazakii* can be achieved by near-infrared heating. In this study, RF treatment alone was also insufficient to inactivate *C. sakazakii* by the required amount about 3-log reduction in infant formula powder.

Hurdle combinations for controlling *C. sakazakii* has been reported in powdered infant formula. Microwave heating followed by refrigerated storage at 5 °C resulted in the progressive death of *C. sakazakii* in the reconstituted form (Pina-Pérez et al., 2014). In a study performed by Pina-Pérez et al. (2009), 2.2-log reductions in numbers of *C. sakazakii* in rehydrated infant formula were obtained by the sequential combination of pulsed-electrical-field treatment and refrigerated storage at 8 °C. Liu et al. (2012) obtained either additive or synergistic effects for inactivation of *C. sakazakii* by rehydrating infant formula powder with hot water and UV radiation. The application of hurdle technology is becoming more attractive in terms of maintaining

product quality, since individual treatments such as heating can be used at lower intensity. The use of overheating for reducing foodborne pathogens results in denaturation of milk serum proteins, as well as changes in optical, physical, and mechanical properties of powdered infant formula.

To date, there are no published data dealing with the effectiveness of RF and UV combination treatment against foodborne pathogens. For this reason, my results can only be compared with the results obtained by other thermal processing. Hamanaka et al. (2011) treated fresh fig fruit with sequential UV and infrared treatment and achieved 3 log reductions in the counts of fungi after 30 s infrared heating followed by 30 s UV radiation. Gayán et al. (2012) reported that the sequential combination of mild heat and UV treatment showed an additive effect in *Salmonella enterica* inactivation. Therefore, in order to improve antimicrobial effect of RF heating in powdered infant formula, a sequential combination of RF heating and UV radiation was evaluated. In the present study, combined RF and UV treatment resulted in greater reductions in cell numbers of *C. sakazakii* than did either treatment alone as a result of synergism. Additionally, due to the shorter RF processing time, combined UV-RF treatments did not affect product quality including color values, moisture contents, and degree of milk serum protein denaturation.

Even though UV-RF treatment was highly effective, the significance of sublethally injured pathogens in food samples should not be ignored. Injured cells are potentially as dangerous as their normal counterparts because they can recover and

become functionally normal under suitable conditions (Wu, 2008). Thus, cell populations enumerated directly on selective media following treatment cannot representative of the total surviving cells in powdered infant formula powder. In this study, there were no significant ($P > 0.05$) differences in reduction levels determined by plating on selective agars with and without a recovery step at all treatment time intervals. This suggests that combined UV-RF treatment effectively inactivated *C. sakazakii* in infant formula powder without generating many injured cells that potentially could recover.

To clarify the mechanism of the synergistic antimicrobial effect of UV-RF combined treatment, membrane damage to *C. sakazakii* cells caused by RF, UV, and UV-RF combined treatment was evaluated by qualitative and quantitative methods. The UV-RF combined treatment significantly damaged cell envelopes of *C. sakazakii*, as detected in TEM images, leaked intracellular components values, and PI uptake values. Qualitative observations obtained by TEM analysis were consistent with quantitative results of cell membrane damage measured by leakage of intracellular substances and PI uptake. As a result, we concluded that damage to the cell membrane was the main factor related to the synergistic antimicrobial effect of combined RF and UV treatment. Although it is well established that UV radiation inactivates foodborne pathogens by damaging their nucleic acids, it has also been suggested that photons can interact with the cell wall and membrane (Sawai et al., 1995). More studies on damage of intracellular substance such as protein and nucleic acids are needed to

further understand the mechanism of the synergistic antimicrobial effect of UV-RF combined treatment.

In conclusion, performing UV radiation with RF heating to temperatures lower than those used for pasteurization was found to be suitable for controlling *C. sakazakii* in powdered infant formula without affecting product quality. UV-RF treatment at a moderate intensity would virtually ensure that a serving would not contain this enteric pathogen and could be applied practically by the dairy industry. Although the pilot instrument used in this study was batch type and the capacity was comparatively small, RF and UV combined processing for powdered foods could easily be expanded to practical industrial scale by utilizing it in the form of continuous line processing.

III-2. Combination treatment of RF heating and organic acid spray for inactivation foodborne pathogens on raw shelled almonds

III-2.1. Introduction

There has been an increasing consumption of nut products from 2000 to 2011, as consumers have taken interest in health and nutrition (Statista, 2014). Among them, almonds are one of the most popular nuts with a 28% nut market share in 2011 (Almond Board of California, 2012). Consumption of raw almonds from California was associated with outbreaks of salmonellosis from 2000 to 2001, 2003 to 2004, and 2005 to 2006 (Centers for Disease Control and Prevention, 2004; Isaacs et al., 2005; Ledet et al., 2007). *Salmonella enterica* serovar Enteritidis phage type 30 (PT 30) was identified as the outbreak strains. No outbreaks involving serovar Typhimurium on almonds have been reported; however, nut-associated outbreaks were caused by this foodborne pathogen. Of the nut-associated outbreaks reported to the Centers for Disease Control and Prevention between 1998 and 2008, 20% were caused by *S.* Typhimurium (Jackson et al., 2013).

Since 2007, in response to these outbreaks, almonds grown in California and sold in North America must be processed to achieve a minimum 4-log reduction of *Salmonella* (U.S. Department of Agriculture, 2007). Several processes for inactivate *Salmonella* on almonds have been investigated, including propylene oxide (PPO) fumigation, chlorine dioxide gas, and various heat processes such as roasting and steam (Abd et al., 2012; Chang et al., 2010; Danyluk et al., 2005; Lee et al., 2006b; Wihodo et al., 2005). However, due to limitations of these interventions, few

treatments are available for decontamination of almonds that are consumed raw. The major drawback of PPO fumigation is the potential presence of residues and their negative impact on consumer perception and export marketing (Brandl et al., 2008). Chlorine dioxide is an effective gaseous alternative to PPO for controlling *Salmonella* on raw almonds, but it can result in discoloration of the kernel at high concentration (Wihodo et al., 2005). With conventional thermal treatments such as roasting, externally generated heat is slowly transferred to almonds because of low thermal conductivities, necessitating lengthy treatments, which may lead to thermal damage (Doores, 2002). Although steam is more effective than roasting, prolonged exposure causes quality deterioration (Lee et al., 2006b). Therefore, the development of new technologies that can effectively inactivate *Salmonella* on raw shelled almonds without affecting product quality.

In recent years, radio-frequency (RF) heating has been wider acceptance due to more rapid and uniform heating compared with conventional heating. In my previous studies, RF energy at 27.12 MHz was shown to be effective for inactivating foodborne pathogens on powdered foods (Jeong and Kang, 2014). On the other hand, chemical compounds has been developed for minimizing microbial contamination on surfaces of agricultural commodities. Pao et al. (2006) achieved 5-log reduction of *Salmonella* on raw almonds using 10 % citric acid. However, considering product quality, acidic solutions should be applied at lower concentrations. Since no published data exist on the survival of *Salmonella* on raw shelled almonds during combined treatment with

organic acid sprays and RF heating, I chose to combine these chemical and physical interventions. Among various organic acids, lactic acid was selected for this study due to its high antimicrobial effect (Park et al., 2011).

The objectives of this study were to evaluate the efficacy of RF heating along with lactic acid spray for reducing *S.* Enteritidis and *S.* Typhimurium on almond kernels, and to determine the effect of the combined treatment on product quality. Also, I investigated the mechanism of inactivation.

III-2.2. Materials and Methods

Bacterial strains. All bacterial strains, namely, *S*. Enteritidis PT 30 (ATCC BAA-1045) and *S*. Typhimurium (ATCC 700408) were obtained from the Department of Food and Animal Biotechnology bacterial culture collection of Seoul National University (Seoul, South Korea). These strains were isolated from human or animal. Stock cultures were kept frozen at 80 °C in 0.7 ml of tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD) containing 0.3 ml of 50% glycerol. For this study, working cultures were prepared by streaking for isolation onto tryptic soy agar (TSA; Dicfo), incubating at 37 °C for 24 h, and storing at 4 °C.

Preparation of pathogen inocula. For each experiment, inoculum was prepared individually for each strain using the method described by (Danyluk et al., 2005). A loopful (ca. 10 μl) from a single isolated colony of each strain of *S*. Enteritidis and *S*. Typhimurium was cultured in 30 ml of TSB at 37 °C for 24 h, then a loopful was transferred into 30 ml of TSB, and incubated at 37 °C for 18 h. For production of a bacterial lawn, 1 ml of the overnight culture was spread onto each of three TSA plates and followed by incubation at 37 °C for 24 h. The bacterial lawn was dislodged by adding 9 ml of 0.2% peptone water (PW; Difco) to each plate and rubbing with a sterile cotton swab. Cell suspensions were collected from the three plates and pooled,

corresponding to approximately 10^9 CFU/ml. These final suspensions of both pathogenic serovars were used in this study.

Sample preparation and inoculation. Raw shelled almonds of the variety Nonpareil were purchased from a local grocery store (Seoul, South Korea) and sorted to remove any damaged kernels before being used for experiments. For inoculation, 25 ml of prepared inoculum (*S*. Enteritidis and *S*. Typhimurium) was added to 400-g samples inside sterile stomacher bags (Labphas, Inc., Sainte-Juilie, Quebec, Canada), and then mixed by hand for 1 min. The inoculated samples were dried for 24 h inside a biosafety hood (24 ± 2 °C) with the fan running until the moisture content and water activity of the samples equaled those of a noninoculated samples (ca. 4.20%, dry basis and 0.42, respectively). The final cell concentration was 10^6 to 10^7 CFU/g.

Preparation of lactic acid solution. Lactic acid (LA, above 90.0%; Daejung Chemical Co., Siheung-si, South Korea) was mixed with enough sterile distilled water (DW) to make a 2% (vol/vol) solution, and the solution was prepared within 1 h before experiments. The pH for DW and 2% LA solution was 6.86 and 2.03, respectively. The concentration and volume of LA applied to the sample were chosen after preliminary experiments were performed.

Combined treatment of RF heating and LA sprays. RF heating and spraying with LA were carried out in a previously described apparatus (Jeong and Kang, 2014). Firstly, 25 g of inoculated almonds were placed in a polypropylene jar, 4.5 cm in diameter and 4.0 cm deep (NALGENE 2118-0002; Thermo Scientific, Hudson, NH), was placed on the center of the bottom electrode of a RF heater generated a RF electric field at a frequency of 27.12 MHz and a maximum power of 9 kW. Treatments consisted of 10, 20, 30, and 40 s RF exposure of raw shelled almonds in order to maximize the efficacy of pasteurization while maintaining product quality. Secondly, 2% LA sprays were carried out using a hand-operated sprayer (650 ml, Apollo, Siheung, South Korea). An approximate volume of 1.4 ml of 2% LA was sprayed evenly over RF-treated almonds. Combined RF and LA treatment could not applied in reverse order, since a RF heater was turned off after 30 s due to arc discharge resulting from increased dielectric loss factor of almonds (Fig. III-3). For the inactivation mechanism study, 5 ml of cell suspensions kept in glass beaker were treated with RF, LA, and RF-LA under identical conditions: previously confirmed that inactivation patterns of three pathogens on glass petri dishes were similar with those in raw shelled almonds.

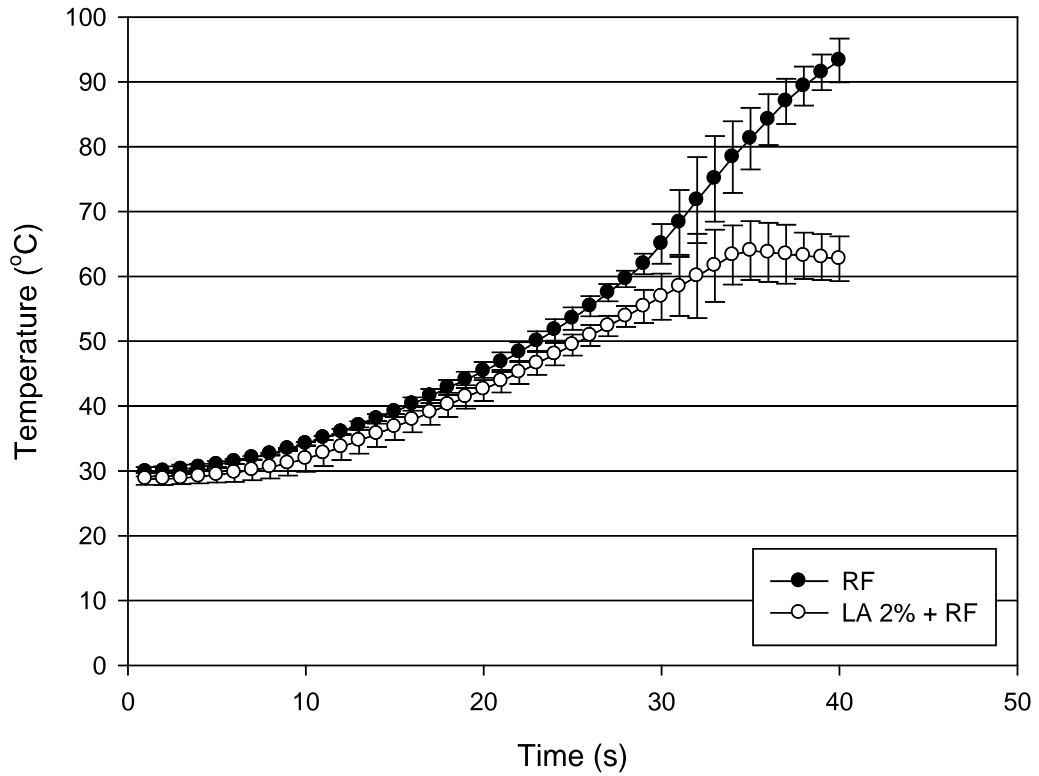


Fig. III-3. Average temperature-time histories of raw shelled almond surfaces during RF heating and 2% lactic acid sprays. The results are means from three experiments, and error bars indicate standard deviations.

Bacterial enumeration. For enumeration of pathogens, 25 g of treated almonds were transferred immediately into sterile stomacher bags containing 100 ml of 0.2% PW pre-chilled in a 4 °C refrigerator (detection limit, 0.7 log CFU/g), and homogenized for 2 min with a stomacher (Easy Mix; AES Chemunex, Rennes, France). After homogenization, 1-ml aliquots of sample were serially diluted in 9-ml blanks of 0.2% PW, and 0.1 ml of sample or diluent was spread-plated onto a selective medium, xylose lysine desoxycholate agar (XLD; Oxoid, Ogdensberg, NY), for enumeration of *S. enterica*. Where low levels of surviving cells were anticipated, 1 ml of undiluted homogenate was equally divided onto four plates of medium and spread-plated. All agar plates were incubated at 37 °C for 24 h, and typical black colonies were counted. To confirm identity of the pathogens, colonies randomly selected from the enumeration plates were subjected to the *Salmonella* latex agglutination assay (Oxoid).

Enumeration of heat-injured cells. The overlay (OV) method was used to enumerate heat-injured cells of *S. enterica* using TSA as a nonselective medium for recovery of injured cells (Lee and Kang, 2001). Appropriate dilutions were spread-plated onto TSA medium and incubated at 37 °C for 2 h to allow heat-injured microorganisms to repair, and then 7 to 8 ml of XLD selective medium was overlaid on the plates. After solidification, plates were further incubated for an additional 22 h at 37 °C and black colonies were enumerated. Preliminary experiments verified that

the 2-h incubation recovery period on TSA did not result in multiplication of uninjured cells in the control samples (data not shown).

Measurement of extracellular UV-absorbing substances and propidium iodine uptake. Cell membrane damage induced by each treatment was quantitatively assessed by determining the release of UV-absorbing materials and propidium iodine uptake from injured cells. Untreated and treated *S.* Enteritidis PT 30 cells were resuspended using 10 ml of phosphate-buffered saline (PBS) and centrifuged at 10,000 × g at 4 °C for 10 min. The upper 1 ml of the supernatant was removed, and the UV absorbance was measured at a wavelength of 260 and 280 nm with a spectrophotometer (SpectraMax M2e, Molecular Devices, CA, USA). The cell pellets were resuspended and diluted in PBS to an optical density at 680 nm (OD_{680}) of approximately 0.2 and then mixed with propidium iodine (PI; Sigma Chemical Co.) solution to a final concentration of 2.9 μM. After incubation for 10 min, the samples were centrifuged at 10,000 × g for 10 min and washed twice in PBS to remove excess dye. The final cell pellets were resuspended in PBS, and fluorescence was measured with a spectrofluorophotometer at an excitation wavelength of 493 nm and an emission wavelength of 630 nm. Fluorescence values for each sample were normalized with the OD_{680} of the cell suspensions.

Quality measurement. To evaluate the effect of RF heating on quality during storage, changes in color, peroxide value, and acid value were measured. The accelerated shelf life tests were conducted in a temperature and humidity chamber (TH-TG-300; Jeio tech. Co., Ltd.) at 35 °C and 30% relative humidity for 10 and 20 days. The storage time was simulated for commercial storage at 4 °C for 1 and 2 years using a Q_{10} value of 3.4 for lipid oxidation and validated by real-time storage experiments (Taoukis et al., 1997; Wang et al., 2006). Kernel skin and core color of RF-treated and untreated noninoculated almonds were measured at random locations using a Minolta colorimeter (CR400; Minolta Co., Osaka, Japan). The values of L*, a*, and b* were used to quantify color attributes and indicate lightness, redness, and yellowness of the sample, respectively.

The peroxide value and acid value were determined by the oil extracted from the almond samples using a solvent recovery extractor (4002842; JP Selecta S.A., Barcelona, Spain) and tested according the AOCS official method Cd 8-53 and Cd 3a-63, respectively. After titration of the almond oil in acetic acid/chloroform solutions (3:2 [v/v]) with 0.1 N sodium thiosulfate, the peroxide value was calculated by the following equation (1):

$$\mathrm{PV} = \frac{(S-B)\times N_1\times 1000}{W} \qquad (1)$$

where PV is the peroxide value (meq/kg), S and B is consumption of 0.1 N sodium thiosulfate (ml) at the end point for the sample and the blank, respectively, N_1 is the normality of sodium thiosulfate, and W is the weight of the almond oil (g).

The acid value was calculated by equation 2, based on the titration of the almond oil in ether/ethanol solutions (1:1 [v/v]) with 0.1 N potassium hydroxide.

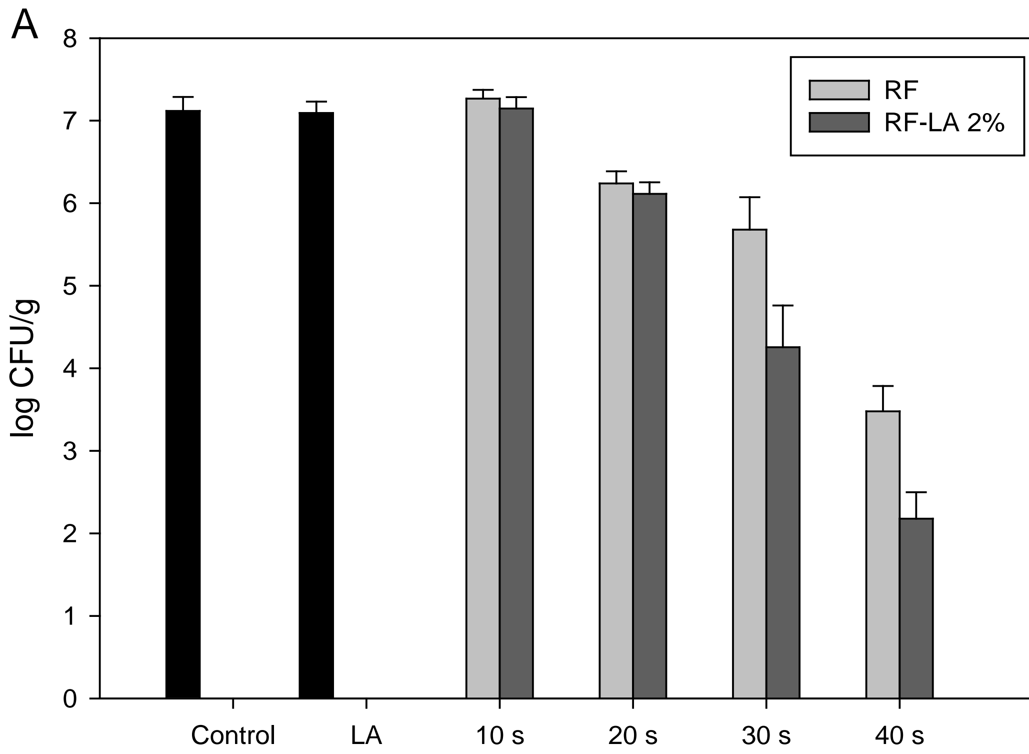
$$\mathrm{AV} = \frac{V \times N_2 \times 56.11}{W} \qquad (2)$$

where AV is the acid value (%), V is consumption of 0.1 N potassium hydroxide (ml) at the end point for the sample, and N_2 is the normality of potassium hydroxide.

Statistical analysis. All experiments were performed in triplicate. Data were analyzed by the analysis of variance procedure and Duncan's multiple-range test of the Statistical Analysis System (SAS Institute, Cary, NC). A P value of < 0.05 was used to determine significant differences.

III-2.3. Results

Survival curves of foodborne pathogens. The survival numbers of *S.* Enteritidis PT 30 and *S.* Typhimurium on raw shelled almonds during single RF or 2% lactic acid (LA) spray treatment and sequential application of both technologies is presented in Fig. III-4. The overall reduction patterns of *S.* Enteritidis PT 30 were similar to those of the *S.* Typhimurium on almond kernels. Significant ($P < 0.05$) log reductions of both pathogens were observed after 20 s of RF-LA combined treatment. RF-LA combined treatment for 40 s achieved 4.94- and 5.48- log reductions in *S.* Enteritidis PT 30 and *S.* Typhimurium, respectively. RF heating alone for 40 s reduced cell numbers of *S.* Enteritidis PT 30 and *S.* Typhimurium by 3.64 and 4.25 log CFU/g, respectively. There was no additional reduction of both pathogens was yielded by combined sequential treatment of RF heating and distilled water spray (data not shown). After 40 s of LA spray treatment alone, there were no significant ($P > 0.05$) differences in microbial population between untreated and treated raw shelled almonds.


A
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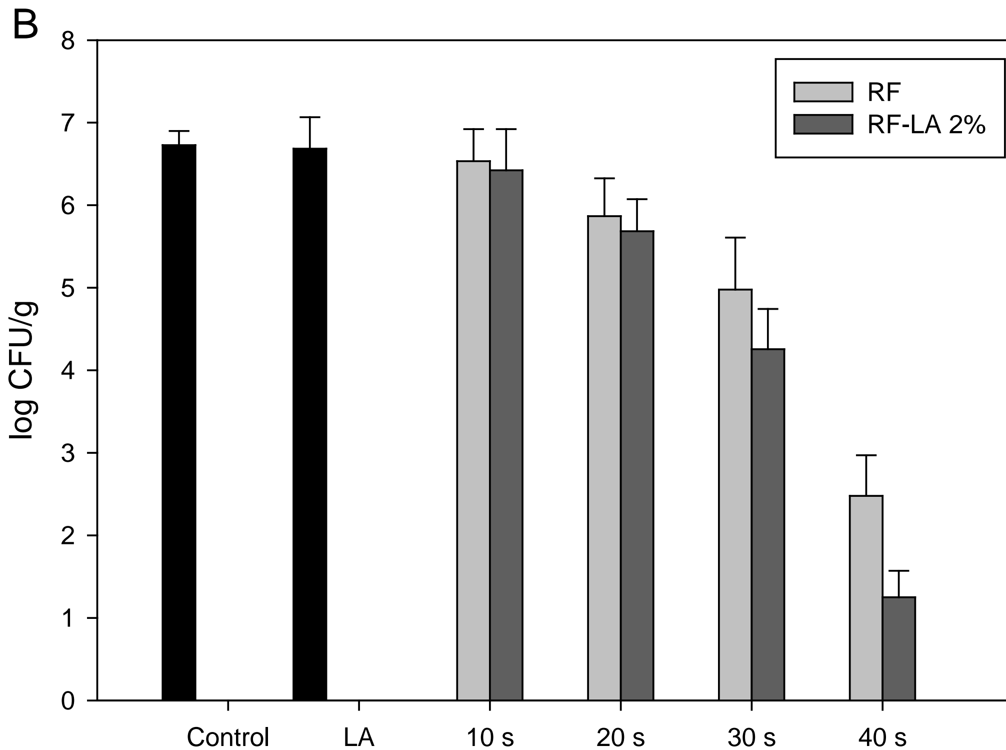


Fig. III-4. Survival curves for *Salmonella* Enteritidis PT 30 (A) and *Salmonella* Typhimurium (B) on raw shelled almond kernels treated with single RF heating or 2% lactic acid sprays and RF heating combined with 2% lactic acid sprays. The results are means from three experiments, and error bars indicate standard deviations.

Recovery of injured cells. Levels of sublethally injured *S.* Enteritidis PT 30 and *S.* Typhimurium on raw shelled almonds following single RF or 2% LA spray and RF-LA combined treatment are shown in Table III-5. When surface-inoculated almonds were subjected to RF-LA combined treatment, smaller reductions of both pathogens were observed by the agar for recovery than by direct plating on selective agar. However, during the entire treatment time, no significant ($P > 0.05$) differences in the levels of cells enumerated between the selective agar (XLD) and the agar for recovery (OV-XLD) were observed on raw shelled almonds.

Table III-5. Comparison of pathogen populations between surviving cells and cells including heat-injured cells on raw shelled almond kernels following single RF heating or 2% lactic acid sprays and RF heating combined with 2% lactic acid spray[a]

Treatment	Treatment time (s)	Population (log CFU/g) by organism and selection medium			
		S. Enteritidis PT30		*S.* Typhimurium	
		XLD[b]	OV-XLD	XLD	OV-XLD
None		7.12 ± 0.17 Aa	7.54 ± 0.65 Aa	6.73 ± 0.17 Aa	6.63 ± 0.25 Aa
2 % LA		7.09 ± 0.14 Aa	7.43 ± 0.44 Aa	6.69 ± 0.38 Aa	6.93 ± 0.44 Aa
RF	20	6.24 ± 0.15 Ba	6.67 ± 0.86 Ba	5.87 ± 0.46 Ba	5.76 ± 0.68 Ba
	30	5.68 ± 0.39 Ca	5.52 ± 0.82 Ca	4.98 ± 0.63 Ca	5.25 ± 0.82 Ca
	40	3.48 ± 0.31 Da	3.62 ± 0.42 Da	2.48 ± 0.49 Da	2.62 ± 0.49 Da
RF-2% LA	20	6.11 ± 0.14 Ba	6.60 ± 0.53 Ba	5.68 ± 0.39 Ba	5.60 ± 0.43 Ba
	30	4.25 ± 0.51 Ca	4.46 ± 0.19 Ca	4.25 ± 0.49 Ca	4.46 ± 0.54 Ca
	40	2.18 ± 0.32 Da	2.51 ± 0.45 Da	1.25 ± 0.32 Da	1.51 ± 0.51 Da

[a] Means ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[b] XLD, xylose lysine desoxycholate; OV-XLD, overlay XLD agar on TSA.

Determination of cell membrane damage by leakage of bacterial intracellular substances and PI uptake. As a quantitative test of cell membrane damage, leakage of UV-absorbing substances from *S.* Enteritidis PT 30 and *S.* Typhimurium cells measured at 260 and 280 nm is shown in Table III-6. Spectrophotometric observation can detect intracellular substances at 260 nm for nucleic acids and 280 nm for proteinaceous materials (Ukuku and Geveke, 2010). Leakage of UV-absorbing substances of *S.* Enteritidis PT 30 and *S.* Typhimurium cells treated with RF-LA was significantly ($P < 0.05$) higher than the sum of levels of UV-absorbing substances with single RF or LA treatment. Also, RF-, LA-, and RF-LA-treated cells were stained with the fluorescent dye PI, which is excluded from cells with intact membranes (Aeschbacher et al., 1986). Table III-6 shows the PI uptake values of *S.* Enteritidis PT 30 and *S.* Typhimurium after each treatment. The overall pattern of PI uptake was similar to that of leakage of intracellular components. The cells subjected to RF-LA treatments showed significantly ($P < 0.05$) higher PI uptake values than did cells subjected to the other treatments.

Table III-6. Levels of membrane damage of LA-, RF-, and RF-LA- sequentially treated cells obtained from the leakage of intracellular UV-absorbing materials and propidium iodine (PI) uptake test[a]

Treatment	Absorbance[b]		PI uptake value[c]
	260 nm	280 nm	
None	0.545 ± 0.030 a	0.314 ± 0.017 a	0.00 ± 0.00 a
2 % lactic acid	0.537 ± 0.024 a	0.314 ± 0.024 a	0.21 ± 0.06 a
RF	1.486 ± 0.035 b	0.723 ± 0.024 b	15.79 ± 1.46 b
RF-2% lactic acid	1.502 ± 0.042 b	0.716 ± 0.030 b	17.64 ± 2.15 b

[a] Mean of three replications ± standard deviation. Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 260 nm : nucleic acids; 280 nm : proteins.
[c] The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} (PI value = fluorescence value after treatment – fluorescence value of non-treated/ OD_{680}).

Effect of RF-LA combined treatment on product quality. Color values of kernel skin and almond cores after RF-LA combined treatment for 40 s required to achieve maximum reduction of *S.* Enteritidis PT 30 and *S.* Typhimurium are summarized in Table III-7. The L^*, a^*, and b^* values of RF-LA treated almonds were not significantly ($P > 0.05$) different from those of untreated samples during the entire storage time. Table III-8 shows the lipid oxidation parameters of almonds following RF-LA combined treatment. There were no significant ($P > 0.05$) differences in PV and AV between untreated and treated samples. Although they varied slightly in accordance with RF-LA treatment at several storage times, no statistically significant differences ($P > 0.05$) were detected between any of the tested samples. Therefore, RF heating combined with 2% LA spray for 40 s did not degrade the quality of raw shelled almonds.

Table III-7. Kernel skin and core color values of RF-LA-treated and untreated almonds stored at 35 °C and 30% relative humidity for 20 days[a]

Parameter	Storage time (days) at 35 °C and 30% relative humidity[b]		
	0	10	20
Kernel skin color[c]			
L^*			
Control	49.58 ± 0.92 a	48.02 ± 1.07 a	47.09 ± 0.64 a
RF treated	50.25 ± 0.63 a	49.32 ± 1.29 a	47.32 ± 0.57 a
a^*			
Control	13.07 ± 0.91 a	13.75 ± 0.72 a	13.24 ± 0.81 a
RF treated	13.88 ± 0.66 a	13.23 ± 0.51 a	13.92 ± 0.73 a
b^*			
Control	34.08 ± 0.86 a	34.53 ± 0.80 a	34.20 ± 0.52 a
RF treated	34.70 ± 0.39 a	34.83 ± 0.53 a	34.60 ± 1.42 a
Kernel core color (L^*)[d]			
Control	77.34 ± 1.49 a	77.04 ± 0.73 a	77.35 ± 0.45 a
RF treated	77.87 ± 0.93 a	76.40 ± 1.79 a	77.82 ± 1.54 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 10 and 20 days at 35 °C and 30% relative humidity to simulate 1 and 2 years storage at 4 °C, respectively.
[c] Color parameters are L^* (lightness), a^* (redness), b^* (yellowness).
[d] Accepted L^* values for good quality are more than 40.

Table III-8. Peroxide values and acid values of RF-LA-treated and untreated almonds stored at 35 °C and 30% relative humidity for 20 days[a]

Parameter	Storage time (days) at 35 °C and 30% relative humidity		
	0	10	20
Peroxide value (meq/kg)[c]			
Control	0.54 ± 0.00 a	1.21 ± 0.12 a	1.35 ± 0.85 a
RF treated	0.62 ± 0.01 a	1.64 ± 0.46 a	1.65 ± 0.75 a
Acid value (%)[c]			
Control	0.76 ± 0.10 a	0.64 ± 0.02 a	0.76 ± 0.02 a
RF treated	0.79 ± 0.08 a	0.68 ± 0.25 a	0.80 ± 0.05 a

[a] Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] 10 and 20 days at 35 °C and 30% relative humidity to simulate 1 and 2 years storage at 4 °C, respectively.
[c] Accepted PV and AV for good quality are less than 5 meq/kg and 1.5%, respectively.

III-2.4. Discussion

Strains of *Salmonella* may survive high temperatures within low-moisture foods such as almonds, peanuts, and peanut butter (Doyle and Mazzotta, 2000). An increase in heat tolerance of *Salmonella* after exposure to low water activity environments has been well known (Mattick et al., 2000). Among them, *S*. Enteritidis PT 30 implicated in the outbreak was found to be more resistant to dry heat compared to other strains. Some typical industry roasting processes did not achieve a minimum 4-log reduction of *S*. Enteritidis PT 30 (Danyluk et al., 2006). Therefore, the Almond Board of California has initiated several research projects addressing the effect of dry heat processes on inactivating *S*. Enteritidis PT 30 on almonds (Almond Board of California, 2007). In this study, RF treatment alone was insufficient to reduce *S*. Enteritidis PT 30 by 4-log reduction in raw shelled almonds.

Hurdle combinations for controlling *S*. Enteritidis has been reported in raw almonds. Near-infrared heating combined with sequential hot air resulted in the progressive death of *S*. Enteritidis in almonds (Yang et al., 2010). Jeong et al. (2009) used convection heating and moist-air impingement to enhance the inactivation rate of *S*. Enteritidis on almonds. In a study performed by Brandl et al. (2008), an additional reduction of *S*. Enteritidis of 0.43 log was yielded by combined treatment of hot water and infrared heating. Since individual treatments such as heating can be used at lower intensity, the application of hurdle technology is becoming more

attractive in terms of maintaining product quality. The use of overheating for controlling foodborne pathogens results in lipid oxidation, as well as changes in optical, mechanical, and physical properties of powdered infant formula.

To date, there are no published research dealing with the effectiveness of RF and organic acid treatment against foodborne pathogens in raw shelled almonds. Therefore, I employed spraying with LA solution on almonds for improving antimicrobial effect of RF heating. In the present study, RF-LA treatment yielded grater reductions in cell numbers of *S*. Enteritidis PT 30 and *S*. Typhimurium than did RF heating alone. Log reductions resulting from the synergistic effect after 40 s of treatment, calculated by subtracting the sums of RF and LA reductions from the values obtained during sequential RF-LA combined treatment, were 1.30 and 1.23 log units of *S*. Enteritidis PT 30 and *S*. Typhimurium, respectively. This tendency was also observed in levels of membrane damage to each treated cell, inferred from leakage of intracellular UV-absorbing substances and PI uptake.

Even though RF-LA treatment was highly effective in controlling foodborne pathogens in almonds, the occurrence of sub-lethally injured cells in food samples should not be ignored. Sub-lethally injured cells are potentially as dangerous as their uninjured counterparts because they can recover and become functionally normal under favorable conditions (Lee and Kang, 2001; McCleery and Rowe, 1995). Therefore, the cell numbers were assessed by plating on selective agar with and without a recovery step. There were no significant ($P > 0.05$) differences between

injured cells and uninjured cells in surface inoculated almonds after the maximum RF-LA treatment. This suggests that RF-LA combination treatment effectively inactivated *S*. Enteritidis PT 30 and *S*. Typhimurium in raw almonds without generating heat-injured cells which could recover.

The synergistic bactericidal effect of RF-LA combination treatment may be attributed to the interaction of lactic acid with RF heating. Low-pH exposure can cause sublethal injury to cell membranes and resultant disruption of the proton motive force across cell membranes, due to loss of H-ATPase (Lin et al., 2004). This could accelerate the entry of sprayed LA solution into the cell. However, in this study, the level of membrane damage that can be inferred as a result of the leakage of intracellular substances and PI uptake of RF-LA-treated cells was not significantly different from those of RF-treated cells. Although it is not a fully proven hypothesis, RF heating of *S*. Enteritidis PT 30 and *S*. Typhimurium cells might induce a disturbance in cell membrane, allowing the cells to become permeable to the sprayed LA solution rather than damaging the cell membrane per se. In addition, temperature is a primary factor influencing the activity of organic acids, with increasing temperature enhancing the effectiveness of organic acids (Presser et al., 1998; Uljas et al., 2001). Therefore, the use of LA sprays with RF heating could be used as a novel technique to reduce intensity of RF heat and to increase the efficacy of existing thermal treatment. Despite high antimicrobial effect, combined RF-LA treatments did not affect product quality due to lower levels of RF and applied LA.

In conclusion, about a 5-log reduction of *S*. Enteritidis PT 30 and *S*. Typhimurium can be achieved on almonds by incorporating a simple LA spraying following RF heat treatment without causing any quality loss. Although organic acid sprays have been widely used in the meat industry for decontaminating livestock carcasses, this is not currently applied to nut processing (Berry and Cutter, 2000; Castillo et al., 2001). Given the results of this study, the potential utilization of LA sprays during RF heating could be considered as an alternative to other interventions. The effectiveness of this RF-LA combined treatment could be improved by refining the procedure, such as readjusting the maximum power of RF heater and spray volume or concentration of LA. Also, further study is needed to clarify synergistic effect on the microbial inactivation by RF-LA combination treatment. Through inactivation mechanism study, it may be possible to enhance the antimicrobial efficacy of combined RF-LA treatment.

Chapter IV.

Computer simulation model development

and prediction for RF heating

of dry food materials

IV-1. Introduction

Dry products such as spices, grains, nuts, herbs, bakery products, and infant formulas are generally regarded as shelf stable foods and can be stored for a long time due to their low moisture contents (Schweiggert et al., 2007). Nevertheless, presence of pathogens may cause considerable qualitative and quantitative losses in these products (Banerjee and Sarkar, 2003; Barak et al., 2011; Breeuwer et al., 2003; Friedemann, 2007; Ghosh et al., 2007; Sanchez-Marinez et al., 1997; Wang et al., 2007b; Wang et al., 2007c). In particular, use of contaminated spices can lead to severe foodborne illnesses, since they are utilized in ready-to-eat foods which are not subjected to further cooking (Little et al., 2003).

Radio frequency (RF) energy is an electromagnetic wave with a frequency between 1 to 300 MHz, which provides rapid and volumetric heating (Marra et al., 2009; Piyasena et al., 2003). This technology has been studied as a non-chemical alternative for pasteurization in dry products such as powdered pepper spices and peanut butter cracker (Ha et al., 2013; Jeong and Kang, 2014; Kim et al., 2012). A better understanding of the complex mechanism of RF heating will lead to better processing performance and energy savings (Ghani et al., 1999). The most common reference parameter in the thermal process is the decimal reduction time (D-value). This has been utilized to quantify the effect of an elevated temperature on microbial populations (Hartel and Heldman, 2012). However, it is necessary to keep the food

under that condition for a specified period to ensure an efficient reduction of foodborne pathogens (Ghani et al., 1999).

Computer simulation has been effectively used to help understand RF heating process. Neophytou and Metaxas (1999) used finite element method to simulate electric field inside RF applicators by solving both wave and Laplace equations. Yang et al. (2003) modeled RF heating of alfalfa and radish seeds packed inside rectangular polypropylene boxes in a 1 kW RF system using commercial software TLM-FOOD HEATING based on transmission line and finite different time domain method. Ting Chan et al. (2004) solved wave equations to simulate electric field patterns in 1% carboxymethyl cellulose (CMC) solution, placed in a 6 kW, 27.12 MHz RF system using finite element method. Marra et al. (2007) successfully simulated the temperature distribution and heating uniformity inside a cylindrical meat roll subjected to a 600W RF heating using a commercially available finite element based software FEMLAB. A computer simulation using FEMLAB was also successfully performed to study various factors causing heating non uniformity in fresh fruits, when subjected to RF heating inside a 12 kW, 27.12 MHz RF system (Birla et al., 2008b; Birla et al., 2008a). Romano and Marra (2008) studied the effect of regular sample shapes and their orientations on RF heating behavior in meat samples using a computer model and predicted that cubes should have better heating uniformity than cylinders and spheres. Wang et al. (2007a) simulated and validated a computer model to study the influence of dielectric properties of mashed potato and circulating water

on the electric field distribution, heating rate, and temperature distribution in a 6 kW RF system.

However, to date, there are a few studies related to prediction about effects of various parameters on the inactivation of foodborne pathogens in dry foods during RF heating. Therefore, it is necessary to systematically study the RF heating characteristics and evaluate treatment parameters to improve the antimicrobial effect of RF heating in dry foods based on the validated computer simulation model. The overall objective of this study was to develop a computer simulation model for powdered red pepper when subjected to a RF system using commercial finite element software COMSOL and validate the computer simulation model by comparing with the experimental temperature profiles of red peppers after 50 s RF heating. Also, the validated computer simulation model was applied to predict the effects of frequency, electrode area, and dielectric properties of packaging material on inactivating foodborne pathogens in powdered red pepper.

IV-2. Materials and Methods

Sample preparation. Commercially dried red pepper powders were purchased from a local grocery store (Seoul, South Korea) and stored at the room temperature of 22 ± 2 °C. The initial moisture content of red pepper powder was 10.6 ± 0.1% on dry basis. The bulk density of red peppers at room temperature was measured by a basic volume method using a $30 \times 22 \times 6$ cm^3 plastic rectangular container and obtained to be 759.6 kgm^{-3}.

Dielectric and thermal properties measurement. Dielectric properties of red pepper samples were measured with a liquid test fixture (16452A; Agilent Technologies, Palo Alto, CA) connected to a precision impedance analyzer (4294A; Agilent Technologies). Thermal properties such as thermal conductivity and specific heat were measured by a dual needle probe method (KD2 Pro, Decagon Devices, Pullman, WA, USA) at every 10 °C interval from 20 to 90 °C.

Physical model. A 9 kW, 27.12 MHz parallel plate RF heating system (Seoul National University, Seoul, South Korea; Dong Young Engineering Co. Ltd., Gyeongnam, Korea) was used in this study. The RF system included metallic enclosure, generator, and RF applicator with a pair of RF electrodes. A schematic diagram of the RF applicator is shown in Fig. IV-1. The bottom electrode is the

integral part of metallic enclosure. Top electrode position could be changed with the help of adjustable screws. RF power from the generator was fed in the middle of top electrode. A red pepper powder in a polypropylene jar, 6.4 cm in diameter and 10.8 cm deep, (NALGENE 2118-0008; Thermo Scientific, Hudson, NH) was placed on the bottom electrode.

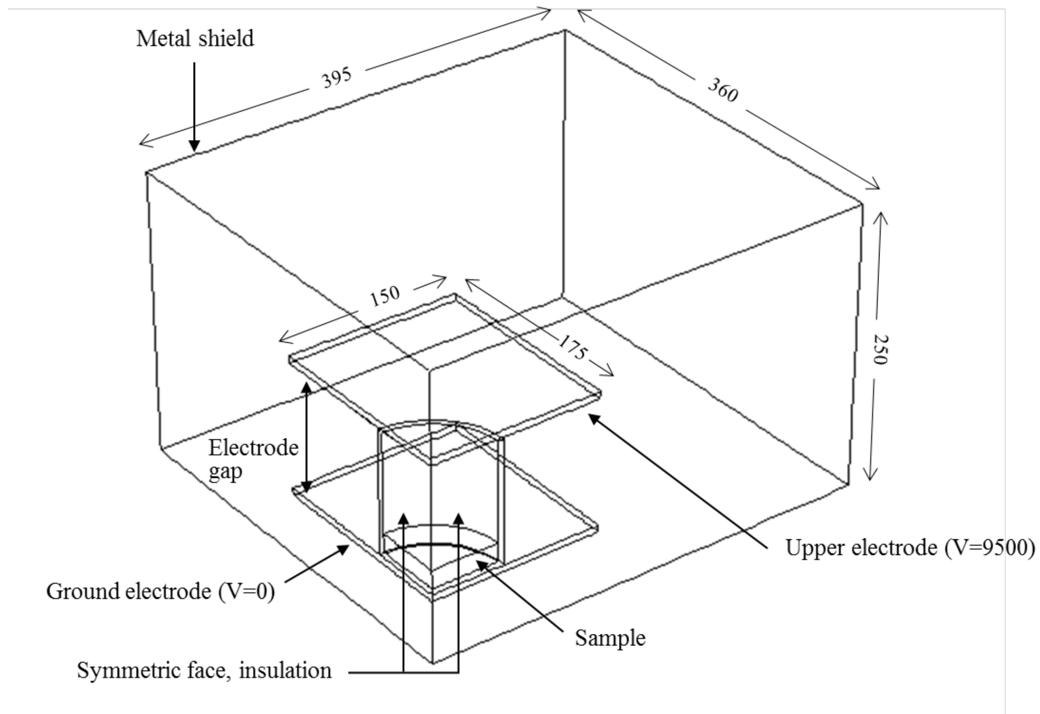


Fig. IV-1. Geometry and boundary conditions of one quadrant of 9 kW, 27.12 MHz radio frequency systems used in simulation (dimensions are in mm).

Governing equations. The electric field intensity in the electromagnetic field was solved by the Maxwell's electromagnetic field equations. Since the wavelength (11 m) of electromagnetic wave in the 27.12 MHz RF system is usually much larger than the RF cavity size ($0.40 \times 0.36 \times 0.25$ m^3), Maxwell's equations can be simplified to Laplace equations with a quasi-static assumption (Birla et al., 2008a; Choi and Konrad, 1991):

$$-\nabla \cdot \left((\sigma + j2\pi f \varepsilon_0 \varepsilon') \nabla V \right) = 0$$

where σ is the electric conductivity of food material (S m^{-1}), f is the frequency (Hz), ε_0 is the permittivity of electromagnetic wave in free space (8.854×10^{-12} F m^{-1}), ε' is the dielectric constant of food material, and V is the electric potential between the two electrodes (V).

The amount of power conversion from electromagnetic energy to thermal energy is related to the dielectric properties of treated materials. RF power conversion in the food is governed by (Metaxas, 1996):

$$Q = 2\pi f \varepsilon_0 \varepsilon'' |E|^2$$

where Q is the power conversion to thermal energy in foods (W m^{-3}), ε'' is the loss factor of food material, and $E = -\nabla V$ is the electric field intensity in the food material (V m^{-1}).

By considering heat convection at the material's surface and the heat conduction within the food material, the heat transfer inside the food material is described by Fourier's equation:

$$\frac{\partial T}{\partial t} = \nabla \alpha \nabla T + \frac{Q}{\rho C_p}$$

where $\partial T / \partial t$ is the heating rate in food material (°C s^{-1}), α is the thermal diffusivity (m^2 s^{-1}), ρ and C_p are the density (kg m^{-3}) and heat capacity (J kg^{-1} K^{-1}), respectively.

Initial and boundary conditions. Fig. IV-1 show the geometrical and electrical boundary conditions of the 9 kW, 27.12 MHz RF system used in the simulation. The initial temperature was set as room temperature at 25 °C. Only the top surface of food sample was uncovered and exposed to air, and the sides and bottom were covered by the plastic container. The convective heat transfer coefficient of the top surface was set as 20 W m^{-2} K^{-1} for nature convection (Romano and Marra, 2008). The metal enclosure boundary of RF machine was considered as thermal insulation ($\nabla T = 0$). The top electrode was set as the electromagnetic source since it introduced the high frequency electromagnetic energy from the generator to the heating cavity and the bottom electrode was set as ground ($V = 0$ V). It was difficult to directly measure the high voltage during processing without disturbing the electric field (Marshall and Metaxas, 1998), so the voltage on the top electrode was assumed to be constant during the RF treatment and estimated by the following equation (Birla et al., 2008a):

$$V = \left(d_{air}\sqrt{(\varepsilon')^2 + (\varepsilon')^2} + d_{mat}\right)\left(\frac{\rho C_p}{\pi f \varepsilon_0 \varepsilon''}\frac{dT}{dt}\right)$$

where d_{air} is the air gap between top electrode and food sample (m), and d_{mat} is the height of the food material (m).

The estimated voltages were 9600 V for simulation based on the heating rate in preliminary tests. All the metal shielding parts except for the top electrode were grounded, and considered as electrical insulation $\nabla \cdot E = 0$.

Solving methodology. A finite element method (FEM) based software COMSOL Multiphysics (V4.2 COMSOL Multiphysics, CnTech Co., LTD., Wuhan, China) was used to solve the coupled electric currents and heat transfer equations in solids. The software was run on a Dell workstation with two Dual Core 3.10 GHz Xeon processors, 8 GB RAM on a Windows 7 64 bit operating system. Fig. IV-2 illustrates the steps taken in the simulation (Alfaifi et al., 2014). Extremely fine tetrahedral mesh was generated in the food sample to guarantee the accuracy of temperature distribution results. Other parts of the system were meshed with normal size tetrahedral meshes. Mesh size was chosen based on the convergence study when the difference in the resulted temperatures between successive calculations was less than 0.1%. The initial and maximum time steps used in this study were set as 0.001 and 1 s, respectively. Each simulation task took around 18 min to finish.

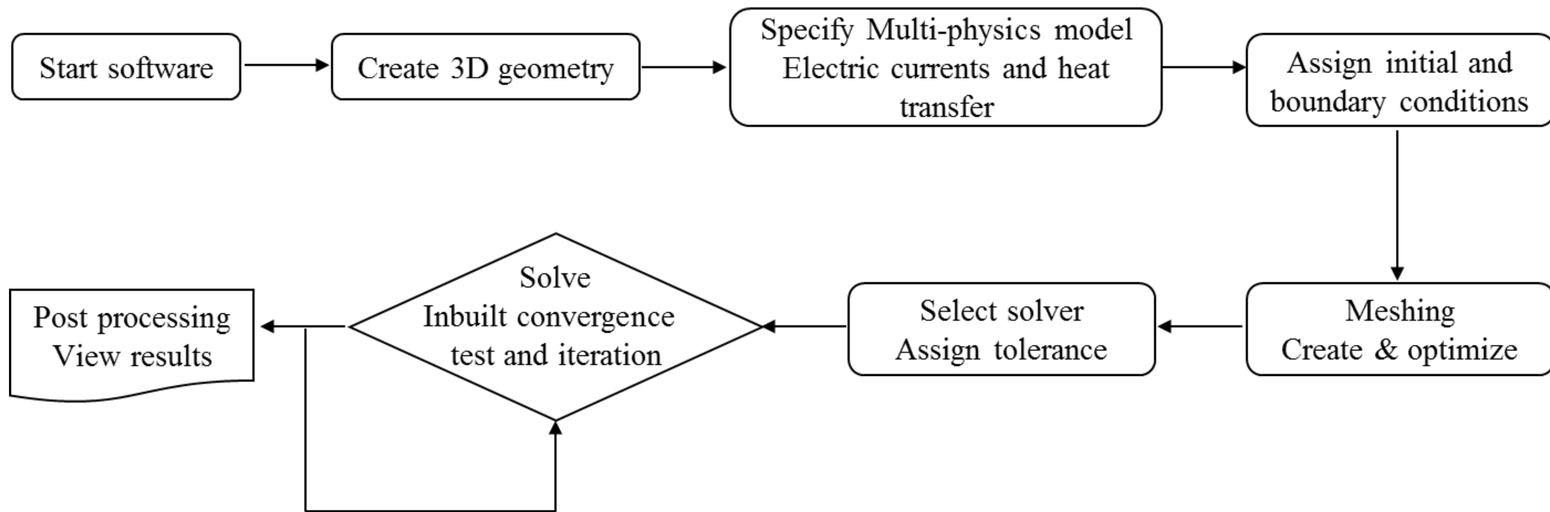


Fig. IV-2. Flow chart of modelling steps using COMSOL Multiphysics.

Model parameters. The dielectric, thermal, and physical properties of the product and the surrounding materials are essential in modelling the RF heating process. The properties of powdered red pepper, polypropylene, and air at room temperature were listed in Table IV-1 for computer simulation. Dielectric properties of red pepper powder had a non-linear relationship with temperature and were measured in this study.

Table IV-1. Electrical and thermo-physical properties of materials used for computer simulation

Material properties	Red pepper powder	Air[a]	Polypropylene[a]
Density (ρ, kg m^{-3})	759.6	1.2	900
Thermal conductivity (k, W m^{-1} K^{-1})	$0.0002T^2 + 0.0004T + 0.1125$	0.025	0.26
Heat capacity (C_p, J Kg^{-1} K^{-1})	$-0.00001T^3 + 0.0017T^2 - 0.00437T + 2.5802$	1200	1800
Dielectric constant (ε')	$0.00004T^3 - 0.0058T^2 + 0.3657T - 1.974$	1	2.0
Dielectric loss factor (ε'')	$0.00003T^3 - 0.0039T^2 + 0.1939T - 2.578$	0	0.0023

[a] COMSOL material library, V4.2.

Model validation. The RF system was used in experiments to heat powdered red pepper and then validate the computer simulation model. About 25 g of red pepper powder were placed into the polypropylene jar positioned at the center of the bottom electrode. A fiber optic temperature sensor (FOT-L; FISO Technologies Inc., Quebec, Canada) connected to a temperature signal conditioner was used to measure the temperature profile in samples during RF heating. The sensor was directly inserted at the center of the treated red pepper powder and the temperature was recorded at 5 s intervals. The experiments were replicated three times. The measured temperature profile of powdered red pepper in the container center was compared with the simulated result.

Model applications. After validation, the computer simulation model was used to predict the effect of different parameters on the antimicrobial effect of RF heating in powdered red pepper. The rate of bacteria inactivation was evaluated by the first order kinetics. The reaction rate constant (k_T) is function of temperature and is usually described by Arrhenius equation:

$$k_T = Ae^{-E/RT}$$

where A is the reaction frequency factor (s^{-1}), t is the exposure time (s), E is the activation energy (kJ kg^{-1} mol^{-1}), R is the universal gas constant (kJ kg^{-1} mol^{-1} K^{-1}), and T is the temperature (°C).

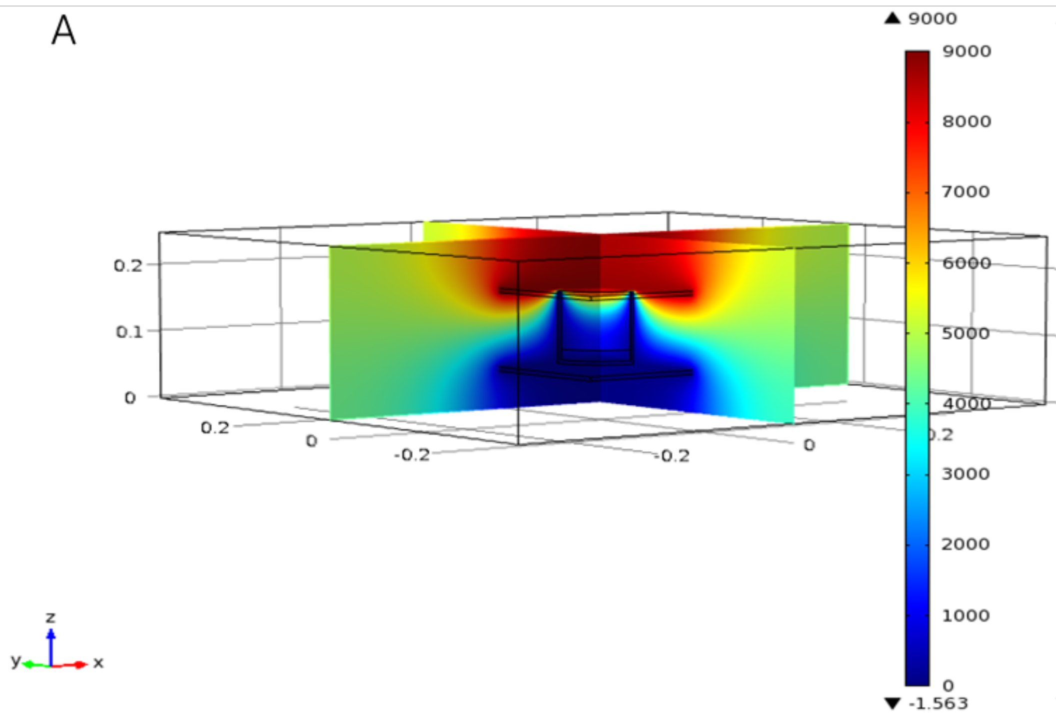
The decimal reduction time (*D-value*) is often utilized to describe the same relationship. The relationship between the reaction rate constant and the decimal reduction time is (Hartel and Heldman, 2012):

$$k_T = \frac{2.303}{D - value}$$

A series of simulations were run by changing frequencies (13.56, 27.12, and 40.68 MHz) and electrode areas (150 × 175, 300 × 350, 350 × 400, and 430 × 500 mm^2) progressively when red pepper powder samples shaped in cylindrical were placed on the ground electrode. Also, in order to investigate the effect of dielectric properties of packaging materials on the bactericidal effect of RF heating, simulations were run with the dielectric constant ranging from 2 to 5, and dielectric loss factors of packaging materials varying from 0.0002 to 0.2. Sample dielectric properties were fixed as $4.26 - j \cdot 0.63$, while physical and thermal properties of packaging materials were kept the same as those of the polypropylene container.

IV-3. Results

Simulated electric potential and electric field distribution for powdered red pepper. The physical phenomena during RF heating were visualized by the computer simulation using COMSOL Multiphysics. Fig. IV-3 shows simulated electric potential and electric field distribution of red pepper powder after RF heating at an electrode gap of 12 cm. The electric potential of top was high and that of bottom was low, since the electromagnetic energy from the generator was introduced by the top electrode. As a result, direction of the electric field was downward to the bottom electrode.


A
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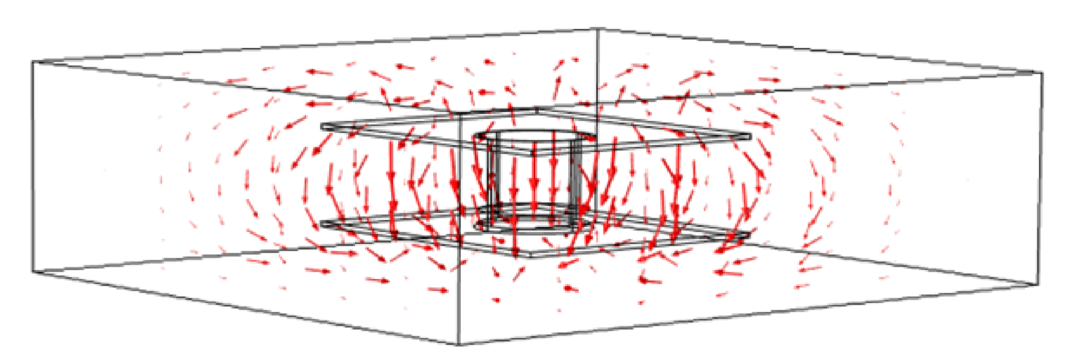
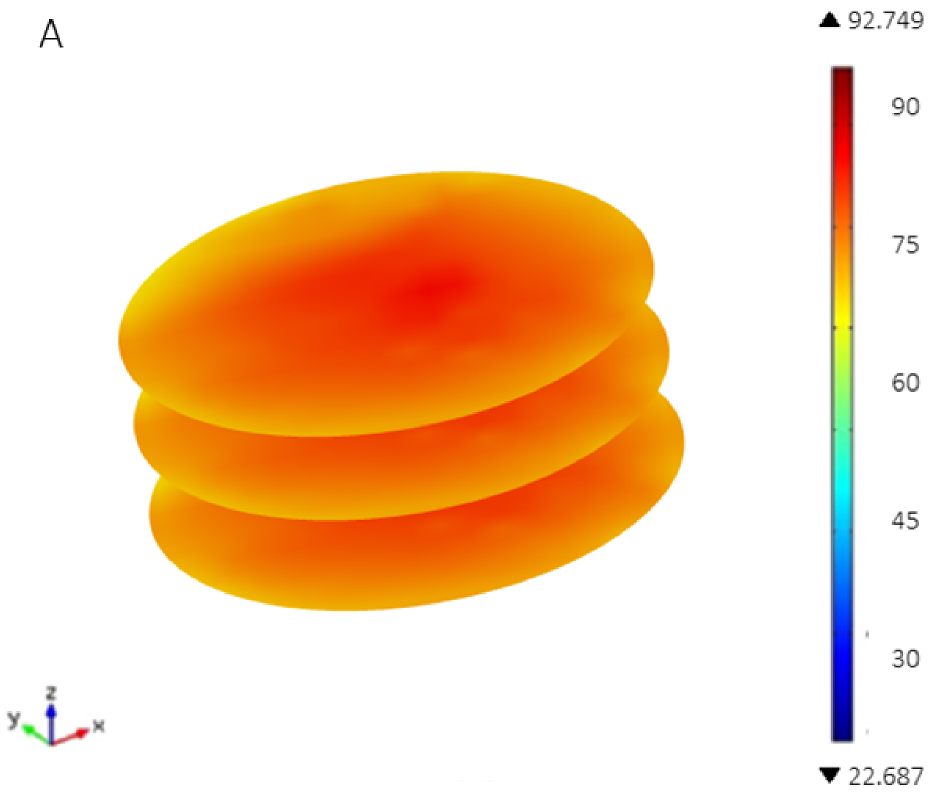


Fig. IV-3. Simulated electric potential (A) and electric field (B) distribution of red pepper powder sample after 50 s RF heating with an electrode gap of 12 cm.

Simulated temperature profiles for powdered red pepper. Fig. IV-4 shows the simulated temperature profiles of RF treated red pepper powder in three horizontal (20, 40, and 60 mm) and three vertical (0, 150, and 300 mm) layers with an initial temperature of 25 °C after 50 s RF heating at an electrode gap of 12 cm. In horizontal layers, the temperature values were higher in the middle layer of powdered red pepper. Because of the heat loss to the contacted container bottom, the temperatures (86.1–88.5 °C) in the bottom layer were lower than those (88.2–92.8 °C) in the middle layer. The top layer temperatures (74.5–84.7 °C) were the lowest in three layers, which might be attributed to evaporative cooling in the space between the top electrode and the sample surface. In vertical layers, the sample temperature increased from the outer layers to central layer of powdered red pepper, and was lower at the most outer layer, which was in contact with the container side walls. The highest temperature values were observed at the center of middle layers.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75
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45
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y
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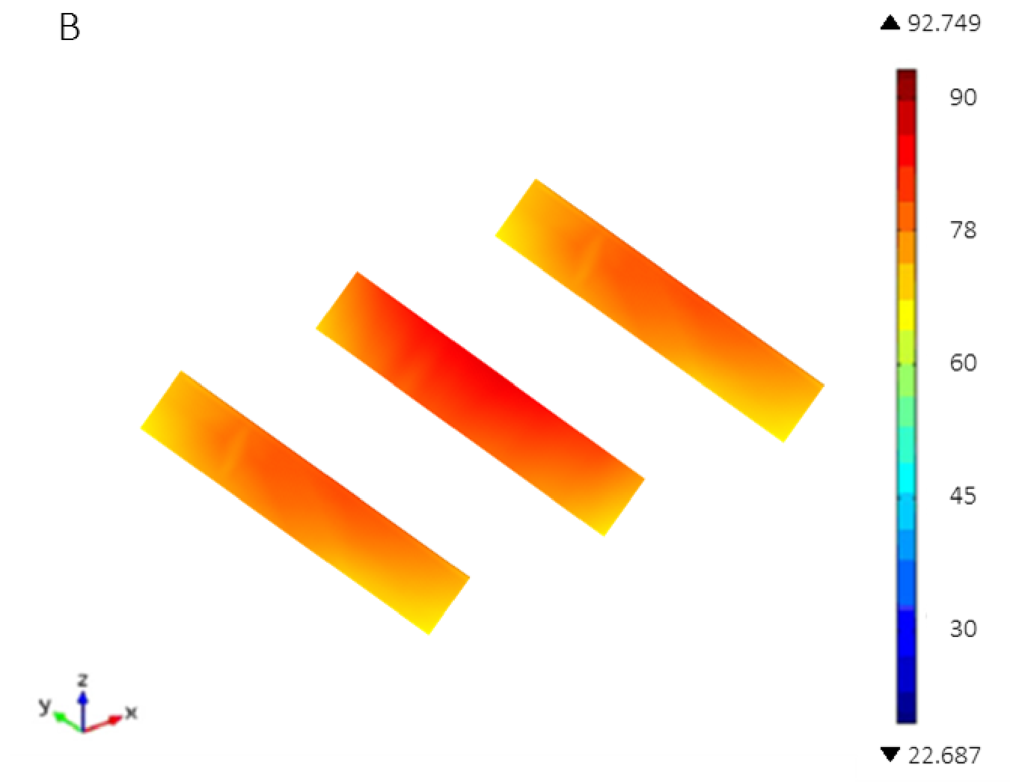


Fig. IV-4. Simulated temperature (°C) profiles of red pepper powder sample at three different horizontal layers from the bottom and three different vertical layers from the vertical center plane of sample after 50 s RF heating with an electrode gap of 12 cm.

Model validation. The simulated temperature-time histories of powdered red pepper at center was compared with experimental results as shown in Fig. IV-5. This comparison indicated that simulated temperature profile measured at the center was in good agreement with experimental data. Both patterns showed that the temperature increased with increasing treatment times and red pepper powder increased from ca. 25 °C to 90 °C when exposed to RF energy for 50 s. A similar heating rate of 1.32 °C/s and a small RMSE of 0.5 °C were obtained between simulation and experiment over the heating period. This validated model were used in subsequent predictions for antimicrobial effect of RF heating in powdered red pepper.

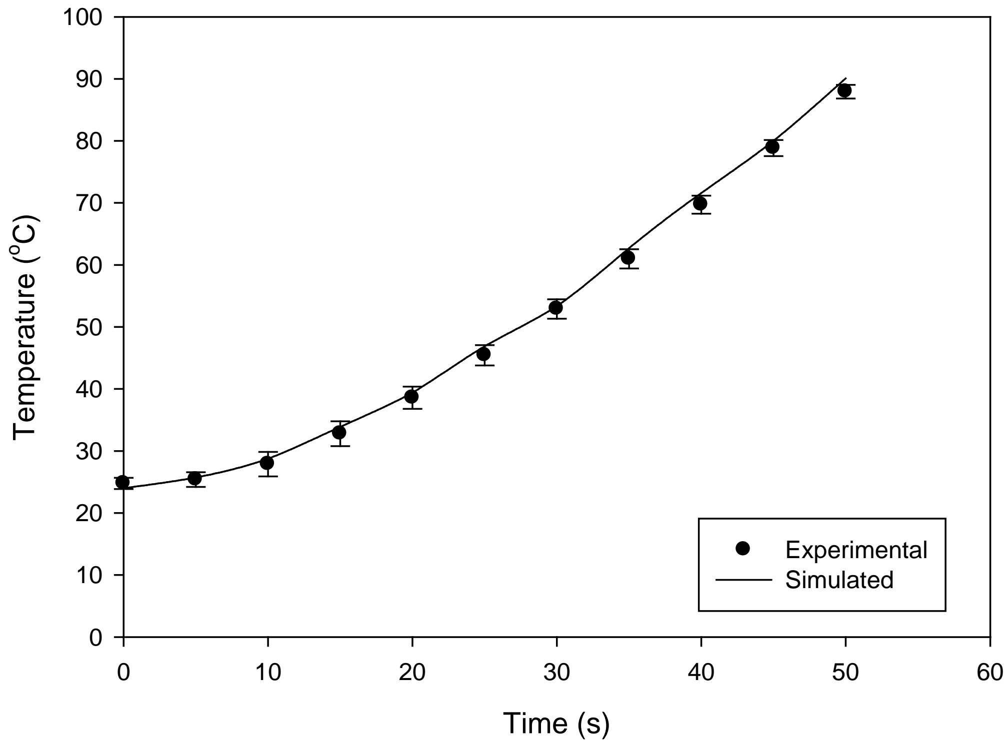


Fig. IV-5. Experimental and simulated temperature-time histories of red pepper powder at the center, placed in a polypropylene container on the grounded electrode during 50 s RF heating with an electrode gap of 12 cm.

Effect of processing parameters on inactivation of foodborne pathogen in powdered red pepper. Fig. IV-6 illustrates the effect of frequency on antimicrobial effect of RF heating in red pepper powder. Simulated results demonstrated that increasing frequency of RF heater caused surviving populations of *Salmonella* Typhimurium to decrease more effectively. The levels of surviving cells of this enteric pathogen were reduced to below the detection limit within 30 s of RF treatment with 40.68 MHz. With 27.12 MHz of RF electric field, levels of *S.* Typhimurium were reduced to below the detection limit after 50 s of treatment. The numbers of *S.* Typhimurium in red pepper were reduced by < 1 log CFU/g after 50 s RF treatment with 13.56 MHz. The effect of electrode area on the inactivation of *S.* Typhimurium in powdered red pepper during RF heating was assessed using the simulation model as shown in Fig. IV-7. The results showed that the electrode areas (lengths and widths) greatly affected the antimicrobial effect of RF heating. Decreasing electrode areas with four different values caused the increase of treatment time. RF heating with the electrode area of 430×500 mm^2 reduced *S.* Typhimurium to below the detection limit after 35 s. With 350×400 and 300×350 mm^2, this pathogen was reduced to below detectable levels after 40 s and 40 s of treatment, respectively. With the lowest electrode area, populations of *S.* Typhimurium decreased by 7 log CFU/g after 80 s.

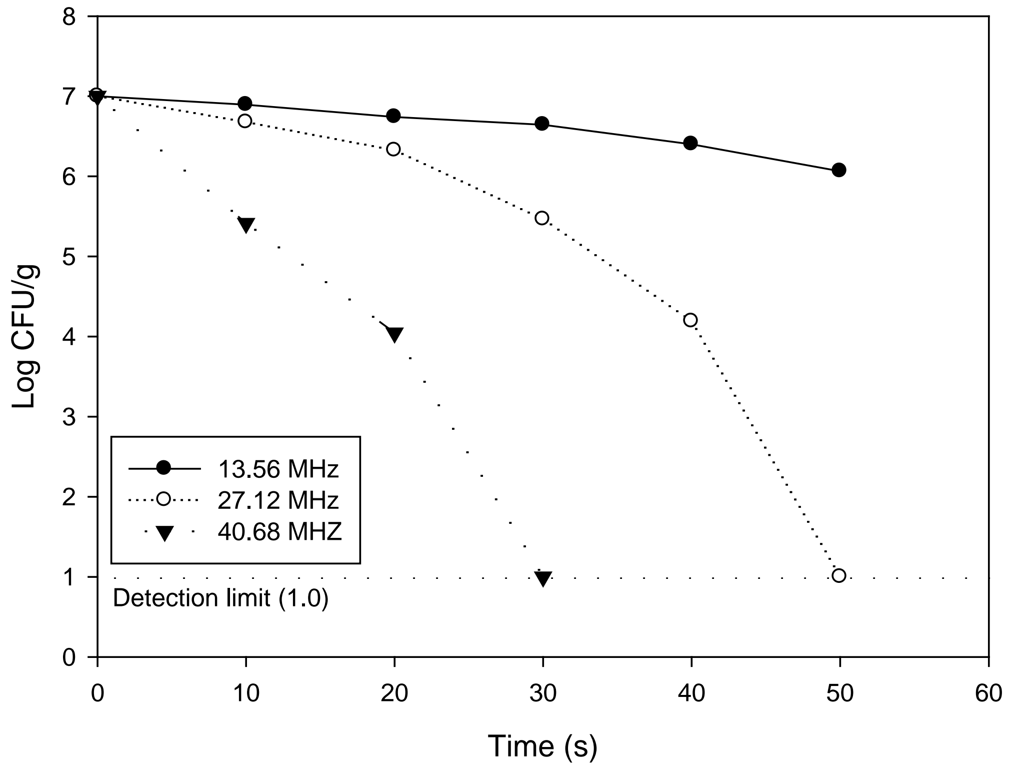


Fig. IV-6. Predicted survival curves for *Salmonella* Typhimurium on red pepper powder during RF heating with varying frequencies.

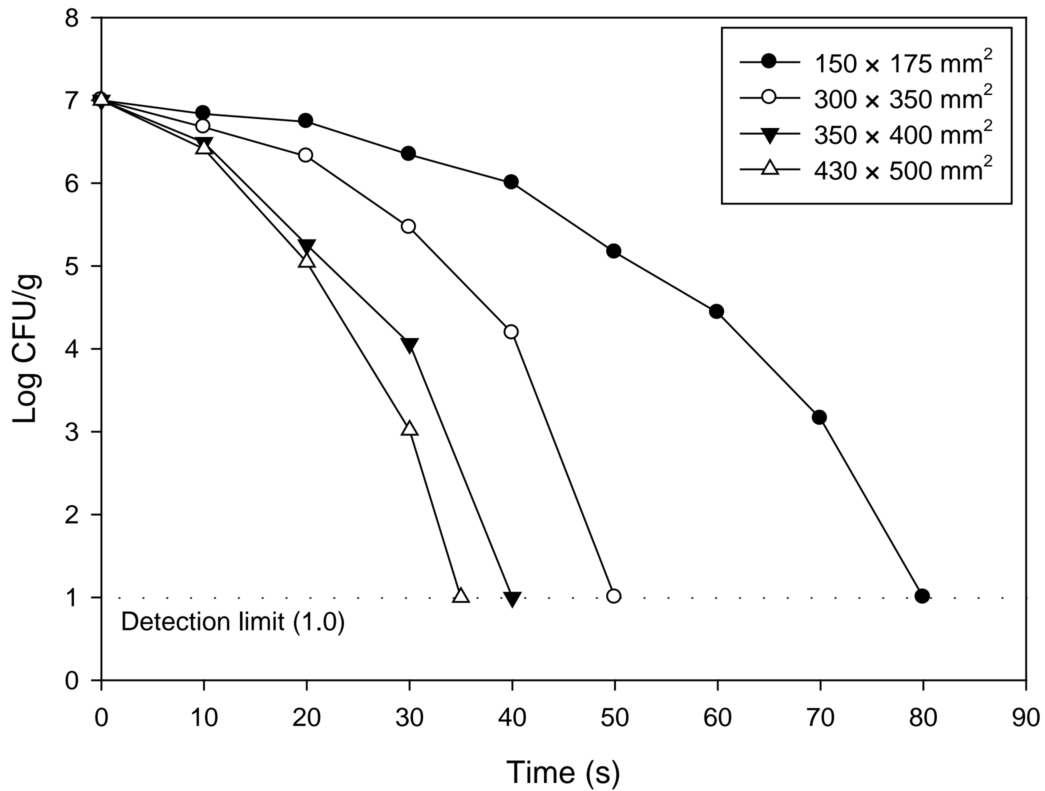
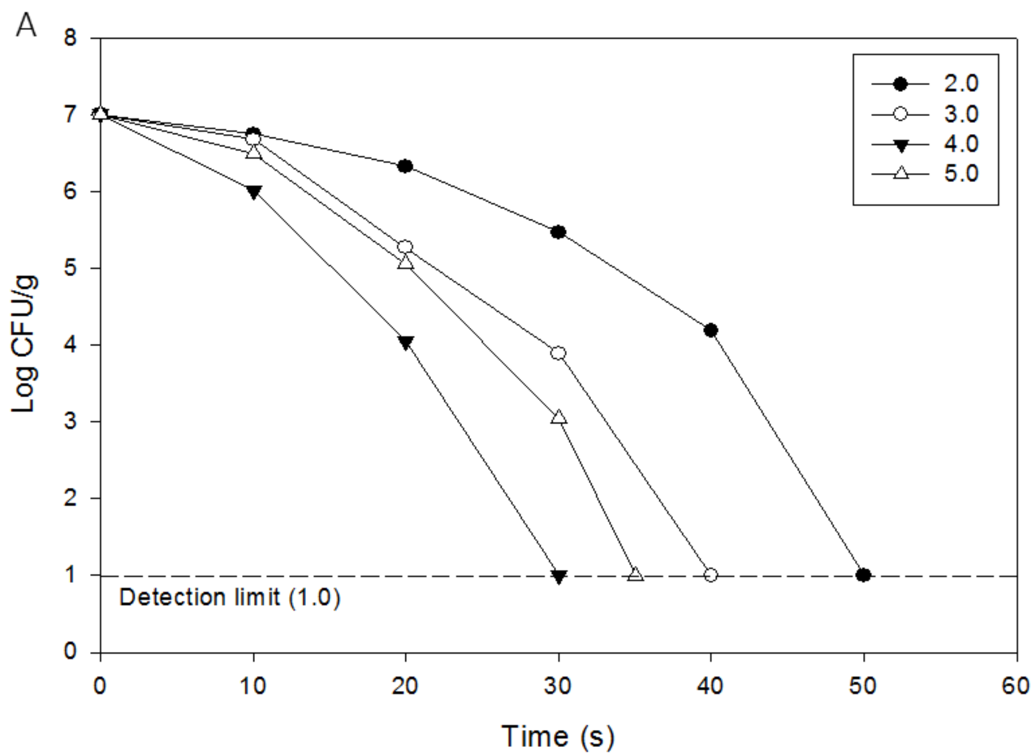


Fig. IV-7. Predicted survival curves for *Salmonella* Typhimurium on red pepper powder during RF heating with varying electrode areas.

Effect of packaging materials around powdered red pepper on inactivation of foodborne pathogen. Fig. IV-8 show the effect of the packaging material dielectric constant with four different dielectric loss factors on inactivation of *S.* Typhimurium in powdered red pepper with the given sampled dielectric properties of $4.26 - j \cdot 0.63$. The treatment time required to reduce *S.* Typhimurium to below the detection limit in red pepper was the shortest when the packaging material dielectric constant was 4.0. It suggested that high antimicrobial effect of RF heating could be achieved when the packaging material dielectric constant was approaching to the sample one. In the case of the dielectric loss factor, decreasing this value caused *S.* Typhimurium cells to decrease more effectively.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Log CFU/g
Time (s)
Detection limit (1.0)
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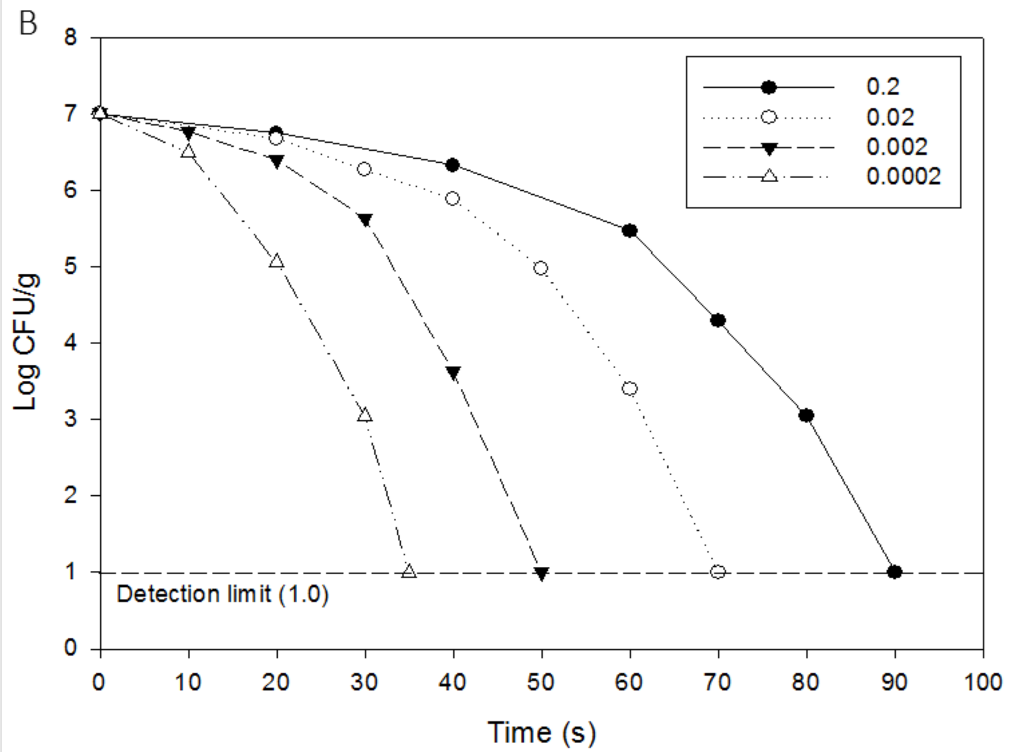

Fig. IV-8. Predicted survival curves for *Salmonella* Typhimurium on red pepper powder during RF heating with various packaging material at four dielectric constants (A) and loss factors (B).

IV-4. Discussion

A computer simulation was studied to predict the influence of processing parameters and packaging material on the inactivation of foodborne pathogens on powdered red pepper spice by RF heating. A finite element-based COMSOL Multiphysics were used to predict electric potential, electric field distribution, and temperature distribution of red pepper powder during RF heating. Similar results for electric potential and electric field distribution were simulated and validated in wheat flour subjected to 12 kW, 27.12 MHz RF heating (Tiwari et al., 2011b). The higher temperature distribution at the middle layer of horizontal and vertical layers could be attributed to an internal heating process resulting from the direct interaction between electromagnetic waves and foods (Zhao et al., 2000). Similar heating patterns have been observed for peanut butter cracker sandwiches subjected to RF treatments (Ha et al., 2013)

The simulated results demonstrated that the efficacy of RF heating in inactivating foodborne pathogens could be improved using a higher frequency and a bigger electrode area. With increases of frequency and electrode area, the electrical power transferred to food as heat (P) also changed, which is given by the equation $P = 2\pi f E^2 (S/d) \varepsilon_0 \varepsilon''$, where f is the frequency, E is the electric field strength, S is the electrode area, d is the distance between electrodes, ε_0 is the dielectric constant of a vacuum considered equal to 8.85×10^{-12} F/m, and ε'' is the dielectric loss factor of the

sample (Piyasena et al., 2003). From this equation, the heat generated is proportional to the frequency, the electric field strength, the electrode area, and the dielectric loss factor, but is inversely proportional to the distance between electrodes. In the present study, electric field strength and electrode gap were fixed at 0.3 kV/cm and 12 cm, respectively, leaving only the impact of the frequency and the electrode area to be examined. Our results agree with the equation; more heat was generated as a result of a higher frequency and electrode area, resultant more reduction of foodborne pathogen.

Also, if a proper material with a similar dielectric constant to that of powdered red pepper and a low dielectric loss factor can be added to surround the red pepper sample, a more uniform electric field and permeable electromagnetic energy can be produced, which resulted in a better antimicrobial effect of RF heating. These results were consistent with my previous study of chapter II-2. To date, there are no published data dealing with the effect of packaging materials around food samples on antimicrobial effect of RF heating. For this reason, my results can only be compared with the results on RF power distribution. Tiwari et al. (2011a) reported that RF uniformity can be achieved when the surrounding material dielectric constant is in a comparable range of the sample one, and the dielectric loss factor is relatively low.

In the present study, a computer simulation model using a finite element based commercial software COMSOL was used to evaluate the antimicrobial effect of RF heating. Experiments were conducted with powdered red pepper in a cylindrical plastic container to validate the simulation model. The experimental results were in

good agreement with the simulation ones, and both showed higher temperature values in the middle compared with those of the top and bottom layers. Centers were heated more than corner and edge areas in all layers. The validated computer simulation model was applied to simulate effects of various factors, such as frequency, electrode area, and dielectric properties of packaging material on the simulated results. Simulated results showed that the higher frequency, the bigger electrode area, and packaging material with a similar dielectric constant to sample one and low dielectric loss factor provided better antimicrobial effect of RF heating. The developed simulation model is an effective tool to understand and analyze the complexity of RF heating behavior and to improve bactericidal effect in other dry products and applications.

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국문초록

본 연구의 세부적인 목표는, 1) 기존 대류 가열 방식과 비교하여 고주파가열의 살균 효율 및 품질 영향 평가, 2) 염분함량, 포장재, 전극 간격이 고주파가열의 식중독균 제어 효과에 미치는 영향 규명, 3) 고주파가열과 자외선, 유기산 처리의 병행을 통한 식중독균 제어 효과 규명, 4) 식품 내 고주파가열의 양상을 예측하기 위한 컴퓨터 시뮬레이션 모델 개발이다.

고주파가열 시스템은 아몬드 내부 및 외부에 존재하는 병원균을 신속하고 효율적으로 제어할 수 있기 때문에 기존 가열 방식을 대체할 수 있음을 확인하였다. 피스타치오의 염분함량이 증가함에 따라 *S. enterica* 를 4 log 저감화 하는 데 필요로 하는 시간이 감소하다가 100 mg 이상에서는 더 이상 감소하지 않고 유지되었다. 시료의 가열 속도는 폴리에테르이미드로 포장했을 경우 가장 빠르게 증가 하였으며, *S.* Typhimurium 과 *E. coli* O157:H7 을 검출 한계 (0.7 log CFU/g) 이하로 저감화하는 시간을 감소시켰다. 폴리에테르이미드의 유전 상수는 고춧가루 및 후춧가루와 비슷하였고, 유전 손실율은 상대적으로 낮았다.

고주파가열 시스템의 전극 간격이 감소할수록 *C. sakazakii* 의 저감화 효과가 유의적으로 높게 나타났다 ($P < 0.05$).

고주파가열의 살균 효율 증진을 위해 자외선 조사와의 병행 처리 연구를 수행한 결과 분유의 품질 변화 없이 60 초 만에 *C. sakazakii* 를 4.06 log CFU/g 줄일 수 있었다. 또한 고주파가열과 자외선 조사의 개별 처리로 인한 저감화 효과의 합보다 병행 처리 시 저감화 정도가 컸기 때문에 시너지 효과를 관찰할 수 있었다. 이러한 시너지 효과의 원인을 규명하기 위해 병행 처리 후 식중독균으로부터 나온 내부 물질의 양과 프로피디움 요오드화물 흡착 정도를 측정하고, 투과전자현미경 이미지를 확인하였다. 그 결과 시너지 효과의 주된 원인은 식중독균의 세포막 손상이라는 것을 알 수 있었다. 고주파가열 및 자외선 조사 병행 처리에 따른 분유의 품질 변화를 확인하였을 때, 대조군 대비 유의적인 변화가 관찰되지 않았다 ($P > 0.05$). 다른 허들 기술로 고주파가열 및 젖산을 아몬드에 병행 처리했을 때, 60 초 만에 *S.* Enteritidis PT 30 과 *S.* Typhimurium 을 4.94−5.48 log CFU/g 저감화하였다. 또한 두 기술이 낮은 강도 및 농도로 적용되어 저장 기간 동안 견과류 품질에 유의적인 변화를 주지 않았다.

마지막으로 컴퓨터 시뮬레이션을 통해 다양한 요소가 식품 내 고주파가열의 살균 효과에 미치는 영향을 예측하는 연구를 수행하였다.

컴솔 다중 물리 소프트웨어를 이용하여 고주파가열 처리 후 고춧가루의 전위 분포, 전기장 분포, 온도 분포를 예측하였다. 고춧가루 중심 온도의 예측값과 실제 실험값을 비교하여 시뮬레이션 모델을 validation 한 후, 주파수, 전극 면적, 포장재에 따른 고주파가열의 살균 효과를 예측하였다. 그 결과, 주파수와 전극 면적이 증가함에 따라 고춧가루의 *S.* Typhimurium 을 검출 한계 (1 log CFU/g) 이하로 저감화하는 시간이 감소할 것이라는 예측 결과를 얻을 수 있었다. 또한, 포장재의 유전 상수가 고춧가루와 비슷할수록, 유전 손실율이 낮을수록 고주파가열의 살균 효과가 증가할 것이라고 예측되었다.

본 연구를 통해 도출된 결과는 실제 식품 산업에서 고주파가열 기술의 살균 효과를 최적화하는 데 유용하게 활용될 수 있을 것이며, 다른 기술과의 병행 처리를 통해 고주파가열의 효율을 증진시킴으로써 기존의 살균 기술을 대체할 수 있을 것이다. 따라서 본 연구는 새롭고 혁신적인 식품 살균 기술을 제시했다는 점에서 그 중요성이 매우 높다고 할 수 있다.

주제어 : 고주파가열, 염분함량, 유전율, 포장재, 전극 간격, 자외선 조사, 유기산, 젖산, 분무, 식중독균, 컴퓨터 시뮬레이션, 건조분말식품, 견과류

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