CONTROL OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS IN SHELL EGGS BY OZONE, ULTRAVIOLET RADIATION, AND HEAT

DISSERTATION

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Luis Alberto Rodríguez Romo, M.S.

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Dissertation Committee:

Approved by

Dr. Ahmed E. Yousef, Adviser

Dr. David B. Min

Dr. Polly D. Courtney

Dr. Edward R. Richter

_____

Adviser
Food Science and Nutrition
Graduate Program

ABSTRACT

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) is a food-transmitted pathogen responsible for salmonellosis. This gastrointestinal disease has a high incidence rate in the U.S., with 1.4 million cases, and approximate costs of $ 2.3 billion annually. Poultry products are the primary vehicles for the transmission of the microorganism to man, and it is estimated that 50% of the foodborne cases are related to the consumption of contaminated egg or egg products. Contamination of shell eggs by *Salmonella* Enteritidis constitutes a health hazard to consumers, an added liability to the food industry, and an extra burden on government agencies involved in regulations and surveillance. Although many procedures have been attempted, with variable success, to control *Salmonella* Enteritidis in shell eggs, available methods are partially effective, time consuming, or negatively impact egg quality. The objectives of this study were (a) to develop a procedure to inactivate *Salmonella* Enteritidis on the eggshell surface, at low temperatures, utilizing pressurized gaseous ozone in combination with ultraviolet radiation (UV); (b) to evaluate the feasibility of inactivating *Salmonella* within shell egg, with minimal impact on quality, using thermal pasteurization procedures, and (c) to develop a process to decontaminate shell eggs employing heat, vacuum, carbon dioxide, and ozone. Additionally, ozone penetration across the eggshell was investigated.

Shell eggs externally contaminated with *Salmonella* Enteritidis (~10^6 CFU/g eggshell) were treated with gaseous ozone (O_3) at 0-15 psig for 0-20 min, or with UV at 100-2500 $\mu W/cm^2$ for 0-5 min. Treatment combination consisted of exposing contaminated eggs to UV (1500-2500 $\mu W/cm^2$) for 1 min, and subsequent treatment with O_3 at 5 psig for 1 min. Results indicated that treating shell eggs with O_3 at 4-8°C and 15 psig for 10 min, or with UV (1500-2500 $\mu W/cm^2$) at 22-25°C for 5 min significantly ($p < 0.05$) reduced *Salmonella* on the surface of shell eggs by ≥ 5.9 or 4.3 $\log_{10}$, respectively. Treating contaminated eggs with UV, followed by O_3, synergistically inactivated the microorganism by ≥ 4.6 $\log_{10}$ in ~ 2 min total treatment time.

In a different study, feasibility of a thermal pasteurization procedure was tested to inactivate *Salmonella* Enteritidis, and the impact of the treatment on egg quality was evaluated. Shell eggs were internally contaminated (~ 10^6 CFU/g egg), using a newly developed inoculation protocol, to simulate natural microbial presence onto or around the egg vitelline membrane. Contaminated eggs were heated by immersion in water at 57, 58, or 59ºC for up to 40 min. Microbial inactivation resulted in sigmoid survivor's curves with shoulders and tails. Treatments at 57, 58, and 59ºC reduced *Salmonella* by 4.8, 5.1, and 5.4 $\log_{10}$, respectively, during 30-40 min heating. Haugh units significantly ($p < 0.05$) increased after thermal treatments. Albumen turbidity also increased at 58 and 59ºC, and was less affected at 57ºC. Treatments necessary to inactivate *Salmonella* by ≥ 5 $\log_{10}$ increased albumen turbidity or protein denaturation.

A procedure was developed to inactivate *Salmonella* Enteritidis within shell eggs using heat, vacuum, and pressurized gaseous ozone. Internally contaminated shell eggs (~

10^7 CFU/g egg) were heated at 57-59°C for up to 40 min, placed under vacuum (-7 to -10 psig), and subsequently treated with O_3 (12-14% wt/wt; ≤ 20 psig) or with mixtures of O_3 and CO_2. Results indicated that heat, pressurized O_3, and their combination significantly ($p < 0.05$) decreased *Salmonella* inside eggs. A response surface model was developed from data to optimize treatment conditions required to achieve 5-$\log_{10}$ microbial inactivation. For example, shell eggs heated at 57°C for 25 min, subsequently placed under vacuum (-7 to -10 psig), and treated with O_3 at 10 psig for 40 min, resulted in ≥ 6.3 $\log_{10}$ *Salmonella* reduction within shell eggs. Egg quality was not drastically affected after treatments with heat and O_3.

In addition, factors affecting ozone penetration across the eggshell were investigated using a colorimetric procedure. Clean eggshells, that were previously heated at 57°C for 30 min, were filled with a redox dye and subsequently treated with gaseous O_3 at 0-10 psig for ≤ 40 min. Results indicated that time of ozonation (at 10 psig) and heating prior to applying O_3 at 10 psig were significant ($p < 0.05$) for the penetration of the gas through the eggshell.

In conclusion, technology based on combinations of O_3 and UV, or O_3 and heat should be considered in the egg industry for future applications to produce *Salmonella*-free shell eggs.

Dedicated to my parents, my brothers, and family,
and to all the people that in one way or another have shared part
of their time and experience with me in this journey called life

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VITA

November 29, 1970…… Born - Monclova, Coahuila, Mexico

1994…….. B.S. Chemistry, Bacteriology, Parasitology, Autonomous University of Nuevo Leon, Mexico

1994…….. Visiting Researcher, Department of Food Science at University of Massachusetts Amherst

1994-1997…… Researcher, Microbiology Department, Autonomous University of Nuevo Leon, Mexico

1997…… M.S. Microbiology, Autonomous University of Nuevo Leon, Mexico

1997-present…… Graduate Teaching and Research Associate, The Ohio State University

PUBLICATIONS

1. Rodriguez-Romo, L.A., N.L. Heredia, R.G. Labbe, and J.S. Garcia-Alvarado. 1998. Detection of enterotoxigenic *Clostridium perfringens* in spices used in Mexico by dot blotting using a DNA probe. J. Food Prot. 61: 201-204.

2. Yousef, A.E., and L.A. Rodriguez-Romo. 2003. Decontamination of shell eggs by using ozone, UV light, and other procedures. U.S. Pat. Appl. Publ. Application No. US 2001-954923.

FIELDS OF STUDY

Major Field: Food Science and Nutrition Graduate Program

TABLE OF CONTENTS

Appendix

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Presence of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) in shell eggs constitutes a public health hazard, and poses a considerable economic impact on the poultry and egg industry. It is estimated that, in the U.S., *Salmonella* transmission through contaminated shell eggs or egg products results in 700,00 cases of salmonellosis and costs $1.1 billion annually (Frenzen *et al.*, 1999).

Sanitation and cleaning of shell eggs using washing procedures is a common practice required in plants operating under Federal Grading Service. Modern, commercial egg washers spray detergents and sanitizers on shell eggs that reduce microbial flora on the shell surface by 2-3 $\log_{10}$ under optimum conditions (Zeidler, 2001a). However, in spite of its broad commercial application, egg washing does not eliminate the risk of salmonellosis transmission to humans by contaminated eggs. Even though egg washing improves the appearance of shell eggs, and reduces the microbial load on the shell, improper washing practices could lead to penetration of *Salmonella* Enteritidis into the egg contents with subsequent potential microbial proliferation (Kim and Slavik, 1996).

Chemical and physical sanitation procedures have been tested, with variable success, to inactivate *Salmonella* spp. and natural flora on the surface of shell eggs. Some of these decontamination procedures include treatments with different sanitizers in wash water (Worley *et al.*, 1992; Knape *et al.*, 2001), hydrogen peroxide (Padron, 1995), ozone

(Koidis *et al.*, 2000), electrolyzed oxidative water (Russell, 2003), ultraviolet radiation (UV)(Kuo *et al.*, 1997a), pulsed light (Dunn, 1996), gamma radiation and X-rays (Tellez *et al.*, 1995; Serrano *et al.*, 1997), and gaseous plasma (Davies and Breslin, 2003).

Control of *Salmonella* Enteritidis within shell eggs has been attempted with a limited number of procedures that include the use of gamma and X-ray radiation, and thermal pasteurization (Tellez *et al.*, 1995; Serrano *et al.*, 1997; Schuman *et al.*, 1997). Irradiation effectively inactivates *Salmonella* Enteritidis within eggs, but the treatment causes a drastic reduction in egg quality (Lith *et al.*, 1995; Ma, 1996). Irradiation at ≤ 3 kGy is approved by the U.S. Food and Drug Administration (FDA) to reduce internal contamination in shell eggs. However, the approved dose is insufficient to reduce internal microbial load by 5 $\log_{10}$, and therefore the treatment cannot ensure egg safety (CFR, 2000b). Pasteurization of shell eggs, a technology approved by the U.S. Department of Agriculture, has been commercially introduced to inactivate internal *Salmonella* Enteritidis (USDA, 1997). The procedure is effective against *Salmonella* within eggs, however, prolonged heating results in protein denaturation with subsequent adverse effects on albumen clarity and egg quality (Hou *et al.*, 1996).

Ozone (O_3) is a strong antimicrobial agent that has been extensively studied for its potential applications in the food industry, and the FDA recently approved its use to inactivate microorganisms in food (CFR, 2001; Kim *et al.*, 2003). Additionally, ozone has the advantages of its spontaneous decomposition to a non-toxic product; *i.e.*, oxygen, and its potential use at low temperatures (Koidis *et al.*, 2000). Ozone has been tested in disinfecting poultry facilities, hatching eggs, poultry-processing chiller water, and carcass (Bailey *et al.*, 1996; Dave, 1999, Ito *et al.*, 1999). In addition, ozone inactivates

microorganisms that routinely contaminate the surface of shell eggs, setters, and hatchers (Whistler and Sheldon, 1988; Ito *et al.*, 1999; Koidis *et al.*, 2000). Although effectiveness of ozone against microorganisms could be diminished when applied on products with high-organic matter content, its use in combination with other treatments could result in enhanced antimicrobial activity (Labbe and Kinsley, 2001; Unal *et al.*, 2001). Combining ozone with UV radiation or heat has a synergistic effect on microbial inactivation (Diaz *et al.*, 2001; Novak and Yuan, 2003). Therefore, use of ozone in combination with other treatments should be evaluated for their feasibility against *Salmonella* in eggs.

The objectives of this study include, first, the development of a procedure to inactivate *Salmonella* on the surface of shell eggs, at low temperatures, using gaseous ozone under mild pressure, and in combination with UV radiation. The second objective involves evaluating the feasibility of thermally treating eggs to inactivate *Salmonella* Enteritidis without a negative impact on egg quality. Finally, the third objective is developing a procedure to decontaminate shell eggs using combinations of heat, vacuum, and gaseous ozone under pressure. In addition, penetration of ozone through the eggshell is evaluated.

CHAPTER 1

LITERATURE REVIEW

Characteristics of ozone

Ozone, an allotropic form of oxygen, is a triatomic molecule (O_3) that in its pure, concentrated, gaseous form has a pale blue color and a pungent characteristic odor (Weavers and Wickramanayake, 2001). In nature, small amounts of ozone (0.05 mg/liter) are formed in the stratosphere, at 15-35 km altitude, by the reaction of solar ultraviolet radiation (< 240 nm) with molecular oxygen (Horváth *et al.*, 1985; Kim *et al.*, 2003). The troposphere (< 15 km altitude) contains approximately 10% of atmospheric ozone, and only a slight proportion of it is present on the surface of Earth (Wojtowicz, 1996).

Ozone has a molecular weight of 48, a boiling point of –111.9°C, and a melting point of –192.7°C at atmospheric pressure (Merck Index, 1989). In addition, gaseous ozone has a higher density (2.14 g/liter) than air (1.28 g/liter)(Wojtowicz, 1996). The oxidation potential of ozone (2.07 V) is higher than that of hypochlorous acid (1.49 V) or chlorine (1.36 V)(Brady and Humiston, 1978). Ozone is partly soluble in water with a solubility ratio of 0.31-1.13 depending on water temperature (Horváth *et al.*, 1985). Solubility of gaseous ozone in water increases at high pressure, and decreases in the presence of ions, and at high pH (Kim *et al.*, 2003).

Gaseous ozone is more stable than aqueous ozone. Half-life of gaseous ozone is 12 h at atmospheric pressure, and its decomposition depends on reactivity with surfaces, temperature, concentration, and pressure (Koike *et al.*, 1998; Weavers and Wickramanayake, 2001). On the other hand, the half-life of ozone in aqueous phase varies from 2 to 65 min in distilled water at ~ 20ºC (Wynn *et al.*, 1973; Wickramanayake, 1984; Graham, 1997). Stability of aqueous ozone depends on the presence of ozone-demanding material in water, as well as ozone concentration, temperature, pH, UV light, and presence of metal ions and radical scavengers (Horváth *et al.*, 1985; Weavers and Wickramanayake, 2001, Kim *et al.*, 2003). Decomposition of aqueous ozone occurs in a step-wise mode, producing free radical species such as hydroperoxyl ($HO_2^{\cdot}$), hydroxyl ($^{\cdot}OH$), and superoxide ($^{\cdot}O_2$)(Hoigné and Bader, 1975; Grimes *et al.*, 1983). These free radicals have a strong oxidizing power and a half-life of microseconds, and are responsible for the high reactivity of ozone (Khadre *et al.*, 2001).

Generation and measurement of ozone

Ozone, for industrial applications, is generally produced at the point of use in closed systems by photochemical or electric discharge methods, but chemical, thermal, chemonuclear, electrolytic, and electrochemical procedures are also used (Horváth *et al.*, 1985). Low concentration of ozone (0.03 ppm) is produced by reaction of oxygen with 185 nm-wavelength radiation in high transmission ultraviolet lamps (Ewell, 1946). Electric discharge systems (corona discharge) are widely used for production of large amounts ozone in the industry (Horváth *et al.*, 1985). Production of ozone by corona discharge involves applying a high voltage between two electrodes separated by a

dilelectric material in the presence of oxygen or dry air. High-voltage alternating current excites oxygen electrons and induces splitting of oxygen molecules, which combine with other oxygen atoms to produce ozone. Concentration of ozone produced in this system depends on the voltage, current, frequency, dielectric material, discharge gap, absolute pressure within discharge gap, and the nature of gas passed through the electrodes (Horváth *et al.*, 1985). Efficiency of ozone production increases at low temperatures, and varies from 1-3% to 2-6% by weight when air and oxygen are used as feed gases, respectively (Weavers and Wickramanayake, 2001).

Recently, Andrews *et al.* (2002) indicated that ozone could be generated efficiently by an electrochemical procedure. Electrochemical ozone generation involves electrolysis of water into hydrogen and oxygen atoms. Hydrogen gas is vented from the gas-water mixture, and oxygen atoms are combined to produce ozone in an oxygen mix. Electrochemical generation is self-pressurized (≤ 20 psig), and ozone attains a concentration of 12-14% (w/w) in oxygen (Lynntech, 1998).

There are approximately 20 analytical procedures proposed to measure aqueous ozone using chemical, physical, and physicochemical methods (Horváth *et al.*, 1985; Weavers and Wickramanayake, 2001). Chemical methods are based on the quantification of products resulting from the reaction of ozone with an appropriate reagent. Oxidation of iodine solution by aqueous ozone has been used to analytically determine ozone concentration (Shechter, 1973). However, iodometric methods have variations in sensitivity and accuracy because they do not measure ozone alone, but total oxidants in solution (Adler and Hill, 1950). Bader and Hoigné (1988) developed the indigo method, a procedure based on the reaction of ozone with sulfonated indigo dye. In this method,

ozone reacts with the carbon-carbon double bond of the dye, resulting in its decolorization (Fig. 1.1). Change in color is determined spectrophotometrically, and the measured absorbance is used to calculate ozone concentration with a minimum detection limit of 0.005 μg/ml. The indigo method gives less interference, due to other oxidant compounds, than iodometric procedures, and it is recommended as standard to measure residual ozone (Gordon *et al.*, 1988; American Public Health Association *et al.*, 1995).

Several methods have been proposed to measure gaseous ozone using iodometry, ultraviolet absorption, and chemiluminescence procedures (Weavers and Wickramanayake, 2001). However, only the UV spectrophotometric method was recommended to measure ozone in gas phase accurately (Gordon *et al.*, 1988). In addition, instruments to measure ozone based on UV absorption, calorimetry, and amperometric methods are commercially available (Khadre *et al.*, 2001).

Antimicrobial activity of ozone

Ozone was introduced for the first time, as a chemical disinfectant, in the treatment of drinking water in 1893 at Oudshourn, Netherlands (Rice, 1986). Recently, in the United States, the Food and Drug Administration (FDA) approved the use of ozone in its gaseous and aqueous phase as an antimicrobial agent in food (CFR, 2001). Ozone has strong antimicrobial activity against bacteria, fungi, viruses, protozoa, and spores from bacteria and fungi (Khadre *et al.*, 2001). The mechanisms involved in microbial inactivation by ozone are complex, and some reports indicate that ozone acts against unsaturated lipids in the microbial cell envelope, lipopolysaccharides in Gram-negative bacteria, intracellular enzymes, and genetic material (Khadre *et al.*, 2001; Kim *et al.*,

2003). It appears that ozone reacts with the double bonds of unsaturated lipids in the cell envelope, causing leakage of cell contents and eventually microbial lysis (Scott and Lesher, 1963). Murray *et al.* (1965) indicated that, ozone initially targets lipoprotein and lipopolysaccharide layers of Gram-negative bacteria, changing membrane permeability and consequently leading to cell death. Komanapalli and Lau (1996) studied the effect of gaseous ozone at 600 mg/liter on *Escherichia coli* K-12 for up to 30 min, and observed decreased microbial viability over time, and progressive degradation of intracellular proteins. In addition to the damage to microbial cell envelopes, ozone may induce mutagenic effects on *Salmonella* Typhimurium, leading to cell injury or inactivation (Dillon *et al.*, 1992).

Ozone use, as an antimicrobial agent, has been extensively studied for its potential application in the food industry (Khadre *et al.*, 2001; Kim *et al.*, 1999; 2003). Aqueous and gaseous ozone have been tested and proven effective to ensure the microbiological safety of food products such as meat, poultry, fish, fruits and vegetables, and cheese (Kim *et al.*, 1999). Other potential uses of ozone in the food industry include the decontamination of food packaging material, food contact surfaces, and removal of residual pesticides on fruits (Kim *et al.*, 2003). In addition to its high effectiveness as an antimicrobial agent, ozone has the advantage of decomposing spontaneously to a non-toxic product *i.e.*, O_2 (Kim, 1998).

Salmonella and its significance as a foodborne pathogen

Salmonella spp. are widely distributed in nature, and cause infections in humans and animals. Salmonellae were first identified in 1888 as the cause of a disease outbreak

attributed to the ingestion of contaminated meat (D'Aoust, 1989). Currently, *Salmonella* spp. account for a high number of salmonellosis outbreaks caused by the consumption of contaminated water, eggs, poultry and beef, dairy products, vegetables, fresh fruits, and fruit juices, in both developed and developing countries (D'Aoust, 2001). *Salmonella* is one of the most prevalent foodborne pathogens in the United States, and it is estimated that 1.4 million infections and 600 deaths occur annually due to the consumption of foods contaminated with salmonellae (Mead *et al.*, 1999). In addition, salmonellosis results in annual economic costs of approximately $ 2.3 billion (Frenzen *et al.*, 1999).

The genus *Salmonella* is divided into two species, *Salmonella enterica*, which consists of six subspecies, and *Salmonella bongori*; currently the genus includes a total of 2449 serovars (Table 1.1)(Popoff *et al.*, 1998). *Salmonella enterica* subsp. *enterica*, the subspecies mostly involved in foodborne infections, constitutes > 99% of salmonellae isolated from man (Bell and Kyriakides, 2002). *Salmonella enterica* serovar Typhimurium LT2 is the type strain for the genus, and all serovars can potentially cause disease in humans (D'Aoust, 2001). Serovars of *Salmonella enterica* subsp. *enterica* could be divided into human host-adapted, and animal host-adapted based on their ability to specifically infect humans or animals (Poppe, 1999). Human host-adapted *Salmonella* include *Salmonella* Typhi, the causing agent of typhoid fever, and *Salmonella* Paratyphi A, B, and C, which are responsible for paratyphoid fever (D'Aoust, 2001). Examples of animal host-adapted *Salmonella* include *Salmonella* Choleraesuis, the causing agent of enterocolitis, pneumonia, and septicemia in pigs, and *Salmonella* Pullorum and *Salmonella* Gallinarum, which cause disease in turkeys and chickens (Poppe, 1999). It is estimated that almost 2400 non-host adapted serovars of salmonellae are responsible for

salmonellosis in both humans and animals (Bell and Kyriakides, 2002). Since the 1940's there has been a rapid increase in the isolation of non-host adapted serovars of salmonellae from humans and animals (Guthrie, 1992). In the United States, *Salmonella enterica* serovars Enteritidis, Heidelberg, Typhimurium, and Newport are the most frequently implicated in foodborne salmonellosis (Finke *et al.*, 2002). Human illness by *Salmonella enterica* serovar Enteritidis has increased worldwide in the last two decades, due to ingestion of contaminated eggs, and it is currently considered the primary cause of salmonellosis in the world (Guard-Petter, 2001).

General characteristics of *Salmonella*

Salmonella, a member of the family *Enterobacteriaceae*, is a Gram-negative, straight, non-sporing small rod (0.7-1.5 × 2.0-5.0 μm), which is usually motile with peritrichous flagella. The poultry-adapted *Salmonella* Pullorum and *Salmonella* Gallinarum are non-motile (D'Aoust, 1989; Bell and Kyriakides, 2002). Salmonellae are facultative anaerobes, produce gas from glucose, and are able to use citrate as their only carbon source (D'Aoust, 1989). In addition, they usually produce hydrogen disulfide, lysine and ornithine decarboxylases, but not urease or tryptophanase (Bell and Kyriakides, 2002). *Salmonella* spp. grow in the 2-47°C temperature range with rapid growth between 25 and 43°C (D'Aoust, 2001). Heat resistance of salmonellae increases at low water activity; however, under high water activity *Salmonella* Senftenberg strain 775 W is particularly heat-resistant (D'Aoust, 2001; Bell and Kyriakides, 2002). *Salmonella* spp. are able to proliferate at pH 3.6-9.5, but optimum growth occurs at pH 6.5-7.5 (D'Aoust, 2001).

Epidemiology of *Salmonella enterica* serovar Enteritidis

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) is one of the most widespread *Salmonella* serovars implicated in foodborne illness in the United States (Angulo and Swerdlow, 1999). In recent years, there has been a worldwide increase in salmonellosis caused by *Salmonella* Enteritidis, and current U.S. statistics show a rate of 1.9 cases per 100,000 people (CDC, 2003). In 1997, Gomez *et al.* reported that *Salmonella* Enteritidis was responsible for 85% of the total cases of human salmonellosis in Europe; Germany, Austria, and Poland had a morbidity rate of 100-200 cases per 100,000 people, while in England and France the rate was 40 and 10 cases for the same population proportion, respectively. Epidemiological investigations proved the strong association between foodborne-salmonellosis in the U.S. and Europe, and the consumption of egg en egg products (Angulo and Swerdlow, 1999; Wall and Ward, 1999; Grimont *et al.*, 1999; Tschäpe *et al.*, 1999). Gast and Beard (1992), suggested that human-salmonellosis outbreaks, related to consumption of eggs, occurred as a consequence of three independent events; (i) contamination of eggs with *Salmonella* Enteritidis by infected hens, (ii) improper handling of eggs or egg products allowing proliferation of the microorganism to infectious levels, and (iii) ingestion of raw or undercooked contaminated eggs. Frenzen *et al.* (1999) estimated that 700,000 cases of salmonellosis occur annually in the U.S., due to contaminated shell eggs, which represents 47% of the total foodborne infections by *Salmonella*. It is estimated that these cases cost $ 1.1 billion annually.

National surveillance for *Salmonella* infections is based on laboratory confirmation for specific serovars of microorganisms isolated from outbreaks, however,

only a small proportion of cases are reported to the Centers for Disease Control and Prevention (CDC)(Angulo and Swerdlow, 1999). Unreported cases occur mainly because many individuals suffer relatively mild salmonellosis symptoms, and only a small fraction of the affected population seeks medical care (Cohen and Tauxe, 1986). Surveillance data from CDC for 1987 indicated that 79% of the *Salmonella* Enteritidis outbreaks, reported for the year, were associated with Grade A shell eggs (CDC, 1988). In 1988, there were 147 cases of salmonellosis that were associated with consumption of homemade ice cream prepared with contaminated raw eggs, as well as by scrambled eggs and omelets (Table 1.2). Other egg-based foods involved in the transmission of *Salmonella* Enteritidis were mayonnaise, and Hollandaise and Béarnaise sauces, prepared with contaminated raw or undercooked eggs (Table 1.2). In 1994, a national outbreak of salmonellosis occurred due to *Salmonella* Enteritidis contamination of commercial pasteurized ice-cream mix, which had been shipped in trucks that had previously transported unpasteurized, contaminated raw egg; approximately 224,000 cases resulted from this incident (Hennessy *et al.*, 1996). In addition, contaminated baked eggs, egg-based salad dressings, homemade beverages, and salads have been implicated in salmonellosis outbreaks in recent years (Table 1.2). Epidemiological investigations indicate that the most commonly isolated *Salmonella* Enteritidis phage types, from outbreaks in the U.S., are PT8, PT13a, and PT13 (Angulo and Swerdlow, 1999).

Rates of foodborne salmonellosis, caused by *Salmonella* Enteritidis, have declined in the U.S. from 3.8 to 1.9 cases per 100,000 population from 1995 to 1999. However, no further decrease was observed through 2001, and it is estimated that *Salmonella* is responsible for approximately 10% of the total foodborne illnesses

occurring in the U.S. (Mead *et al.*, 1999; CDC, 2003). Furthermore, the emergence of *Salmonella* Enteritidis PT4 in the U.S., a persistent phage type that increased illness rate in Europe, could result in incremented levels of egg-related salmonellosis (Angulo and Swerdlow, 1999; Guard-Petter, 2001).

Clinical features of salmonellosis

Salmonella Enteritidis is the causing agent of human gastroenteritis, an infection that results in a clinical syndrome generally known as salmonellosis. Symptoms of gastroenteritic salmonellosis may include severe abdominal pain, non-bloody diarrhea, myalgia, chills, nausea, headache, fever, vomiting, and prostration. In addition, other medical conditions such as pericarditis, neurological and neuromuscular disease, and reactive arthritis may result in some individuals after the infection (D'Aoust, 1989).

Symptoms occur 12-72 h after consumption of *Salmonella* Enteritidis-contaminated food, and the infective microbial dose, necessary to cause foodborne illness, varies from ≤ 100 cells in high fat foods to 10^5 cells in lower lipid content foods (Bell and Kyriakides, 2002). The microorganism multiplies and colonizes the small intestine, produces an enterotoxin that causes inflammatory reaction and diarrhea, and in some cases it can invade the blood stream to cause more severe illness (Jay, 1996; Poppe, 1999; D'Aoust, 2001; Bell and Kyriakides, 2002). Duration of gastroenteritis syndrome generally varies from 4 to 10 days; during this time, microbial invasion of the small intestine and colon could affect absorption of nutrients in the patient (Poppe, 1999). Susceptibility of humans to *Salmonella* infections depends on a series of factors that include the dose of the pathogen, the type of contaminated food, and the age and immune

condition of the host (D'Aoust, 1989; Poppe, 1999). The newborn, elderly, and individuals with immune deficiencies are more susceptible than the rest of the population to infection by *Salmonella* Enteritidis. In these groups at risk, salmonellosis could result in serious systemic infections with sporadic cases of death (D'Aoust, 2001). Healthy individuals rarely die from salmonellosis, and they normally recover from the disease after treatment with fluid and electrolyte replacement; antibiotic therapy is not usually recommended in developed countries (Bell and Kyriakides, 2002). Nevertheless, some *Salmonella* strains are highly invasive and can cause serious systemic infections that require appropriate antibiotic treatment (D'Aoust, 2001).

After recovery from the disease, non-symptomatic patients may shed *Salmonella* in feces (10^1-10^8 cells/g feces), with decreasing number of excreted microorganisms over time, for up to 6 months (D'Aoust, 1989). However, it is estimated that approximately 5% of recovering patients become chronic carriers and intermittently excrete the microorganism over a long period of time, which constitutes a public health hazard (Jay, 1996; Poppe, 1999).

General characteristics of shell eggs

Chicken (*Gallus domesticus*) is the most important bird used to produce eggs for human consumption around the world, and eggs are a unique well-balanced source of nutrients in the human diet. Egg proteins have a high biological value, and are often used as the standard to compare the quality of other proteins in foods. In addition, eggs contain unsaturated fatty acids, iron, phosphorus, trace minerals, and vitamins (Stadelman,

1995a; Watkins, 1995). Shell eggs consist of 9.5% shell, 63% albumen, and 27.5% yolk (Li-Chan *et al.*, 1995). A schematic representation of the egg parts is presented in Fig.1.2.

Egg formation is a process that occurs in the ovary and the oviduct of the chicken's female reproductive system. Formation of the unfertilized egg starts with generation of the yolk (*ovum*) in the ovary, followed by its release to the upper part of the oviduct. Subsequently, yolk membrane, albumen, and shell are produced during the pass of the yolk through the different portions of the long tubular oviduct. Laying chickens produce a complete shell egg approximately every 24 h, which is the time required for the egg to reach its full size and shape (Romanoff and Romanoff, 1949; Stadelman, 1995b).

The yolk structure consists of the latebra, the germinal disc or blastoderm, and a series of layers of light and dark yolk, which are enclosed by the vitelline membrane (Fig. 1.2). The albumen is made of four layers, from the inside to the outside of the egg, that includes the chalaziferous that extends as a rope-like structure and keeps the yolk in the center of the egg, the adjacent inner thin layer, the dense albuminous sac, and the surrounding outer thin layer (Fig. 1.2). The outer covering consists of two keratin-like inner and outer membranes, with 0.01-1.02 mm total thickness, encircled by the shell. The eggshell is composed of 94% calcium carbonate, 1% magnesium carbonate, 1% calcium phosphate, and 4% protein (Fig. 1.2). The shell is a porous structure (~ 10,000 pores/shell), has an average thickness of 0.31 mm, and is covered by the cuticle, which is a protein-rich coating that constitutes the most external layer of the egg (Romanoff and Romanoff, 1949; Stadelman, 1995b; Okubo *et al.*, 1997).

Contamination of shell eggs with *Salmonella* Enteritidis

Microbial contamination of poultry production facilities with human pathogens, including *Salmonella* spp., plays a key role in the contamination of shell eggs. Jones *et al.* (1996) isolated more than eight different serovars of the microorganism in 72% of samples collected from an egg processing facility and its laying house. *Salmonella* Heidelberg and *Salmonella* Montevideo were the most prevalent serovars isolated from eggshells before egg processing.

Contamination of shell eggs with *Salmonella* Enteritidis occurs by trans-shell and transovarian routes (Humphrey *et al.*, 1991; Braun and Fehlhaber, 1995; Fajardo *et al.*, 1995). Trans-shell *Salmonella* transmission, also known as horizontal contamination, occurs after exposure of shell eggs to a contaminated environment, and results from *Salmonella* penetration through the eggshell pores, with the subsequent proliferation of the microorganism in the egg contents (Hammack *et al.*, 1993; Fajardo *et al.*, 1995). Previous reports indicated that egg exposure to fecal matter was one of the most common forms of eggshell contamination with salmonellae (Gast and Beard, 1990; Humphrey, 1994). Presence of *Salmonella* spp. on the eggshell, its potential egg penetration, and the subsequent migration in egg contents, may be facilitated by a series of factors that include eggshell moisture (Bruce and Drysdale, 1994), storage at ambient temperature (Rizk *et al.* 1966), shell damage (Humphrey *et al*., 1989; Todd, 1996), and eggshell-cuticle condition (Padron, 1990; Bruce and Drysdale, 1994).

Transovarian transmission, also known as vertical contamination, occurs when the ovaries of the hen are infected with the microorganism, which results in contamination of egg contents with *Salmonella* Enteritidis during egg formation (Fajardo *et al.*, 1995;

Thiagarajan, 1995; Humphrey, 1999). Previous studies, based on artificial and natural infection of laying hens, indicated that both yolk and albumen could be contaminated with *Salmonella* Enteritidis by the transovarian route (Shivaprasad *et al*., 1990; Humphrey, 1994). Timoney *et al.* (1989) reported that hens infected with *Salmonella* Enteritidis phage type 4 produced contaminated eggs, and the microorganism was present in both albumen and yolk. Similarly, Shivaprasad *et al*. (1990) observed that *Salmonella* Enteritidis, infecting the ovaries of hens, could be transmitted to the albumen and the yolk of shell eggs. However, Gast and Beard (1990) reported that hens, artificially infected with *Salmonella* Enteritidis phage type 13a produced contaminated eggs, in which the microorganism was present in the albumen, the whole yolk, but not in the yolk interior, therefore suggesting that the site of contamination could be the vitelline membrane around the yolk.

In a similar study, Humphrey *et al.* (1991) analyzed 5700 shell eggs, from hens that were naturally infected with *Salmonella* Enteritidis, and suggested that the site of contamination was the albumen immediately surrounding the yolk. In addition, these researchers reported that most of the contaminated eggs (72%) contained < 20 cells of *Salmonella* Enteritidis.

Egg safety in the United States

The U.S. is the second largest producer of shell eggs worldwide with a yearly production of approximately 64 billion eggs and product consumption per capita of approximately 254 eggs per year. Iowa is the largest egg producing U.S. state closely followed by the states of Ohio and Pennsylvania. (American Egg Board, 2003).

Although it is estimated that only 1 in 20,000 raw eggs contain *Salmonella* Enteritidis in the U.S., egg-transmitted salmonellosis is a prevalent public health problem (Ebel and Schlosser, 2000). In 1999, the President's Council on Food Safety developed the egg safety action plan to control *Salmonella* Enteritidis on eggs. The objectives of the plan were to reduce egg-transmitted human salmonellosis in the U.S. by 50% in the year 2005, and to eradicate this disease by the year 2010. The Food and Drug Administration (FDA) published in 2000 a final rule called "Food Labeling, Safe Handling Statements, Labeling of Shell Eggs; Refrigeration of Shell Eggs Held for Retail Distribution" that requires egg producers to use a safe handling statement on the label of shell eggs not processed for the elimination of salmonellae (CFR, 2000a).

Control of *Salmonella* spp. on the surface of shell eggs

Methods to inactivate external contamination by *Salmonella* on shell eggs can be divided into chemical and physical procedures (Table 1.3).

Chemical methods

Washing

The main objectives of egg washing are to remove contaminants on the shell surface, to improve the appearance of shell eggs to the consumer, and to guarantee the safety and quality of the product (Zeidler, 2001a). Egg washing is a regular practice in the U.S. that is required in egg producing plants operating under Federal Grading Service, and modern egg washers utilize water containing sanitizers and detergents to reduce

microorganisms on eggshells (USDA, 1966; 2003). Although washing eggs is a common procedure in the U.S., Canada, Australia, and more recently Japan, this practice is prohibited in the European Union for grade A eggs (Bartlett *et al.*, 1993; Zeidler, 2001a; Hutchison, 2003; 2004).

Egg washing is usually a continuous process, and the stages of a typical commercial washing procedure are presented in Fig. 1.3. The washing process can be divided in four stages that include eggshell wetting, washing, rinsing, and drying (Hutchison *et al.*, 2003). During the wetting step, shell eggs positioned on a conveyor belt, are sprayed with warm water to allow the removal of adhered fecal matter on the shell (Fig. 1.3). The washing step includes the use of water at least 32.2ºC (90ºF) to ensure proper shell cleaning. In addition, water should be at least 11ºC (20ºF) warmer than the egg; shell eggs should not be dipped in the water throughout the washing process to prevent penetration of microorganisms across the shell (Zeidler, 2001a; USDA, 2003). During washing, shell eggs are brushed and sprayed with water usually at 37.8-46.1ºC (100-115ºF), which contains detergents to remove material adhered to the shells, and to ensure water alkalinity at pH ~ 11 (Zeidler, 2001a). Additionally, USDA (2003) indicates that eggs should be washed only with potable water containing ≤ 2-ppm iron to prevent microbial proliferation. Surface-active food-grade agents, composed of mineral oils, are usually added to the water to prevent foam formation; wash water must be replaced every 4 h to maintain hygienic conditions (Zeidler, 2001a). The next step of the washing procedure consists of rinsing, in which shell eggs are sprayed with clean water, at approximately 60°C, containing 50-200 ppm available chlorine or quaternary ammonium compounds (Hutchison *et al.*, 2003; USDA, 2003). The last step of egg washing is drying

that includes the mechanical removal of water from the egg surface using warm filtered air (USDA, 2003). Effectiveness of egg washing depends on a series of factors that include equipment adjustment, the quality, temperature, and pH of the water, and the characteristics of the sanitizer, detergent, and surface-active agents used during the process (Moats, 1978; Stadelman, 1995c). Zeidler (2001a) indicated that under optimal conditions, commercial egg washing could reduce the microbial load on the shell by approximately 2-3 $\log_{10}$. However, a recent study reported that egg washing using a standard procedure with wetting, washing, and rinsing water at 44°C, 44°C, and 48°C respectively, with water spraying pressure at 20-38 psi (138-262 kPa), and a commercial chlorine compound at 3 g/liter, reduced > 5-$\log_{10}$ *Salmonella* spp. on the surface of shell eggs (Hutchison *et al.*, 2004).

Earlier studies addressed egg-washing conditions, the microbial quality of the water used in the process, and the efficacy of the procedure to reduce *Salmonella* spp. on the surface of shell eggs (Moats, 1979; Bartlett *et al.*, 1993; Hutchison *et al.*, 2003). Previously, Anellis *et al.* (1954) and Cotterill (1968) demonstrated that *Salmonella* spp. were less resistant to heat at alkaline pH. Holley and Proloux (1986) indicated that *Salmonella* spp. proliferated in washwater at 38-42 ºC if the pH was ≤ 9.5. Similarly, Bartlett *et al.* (1993), indicated that wash water at 40°C and pH 10, obtained from egg grading facilities in Canada, contained high microbial load (≥ 10^5 CFU/ml); the authors recommended a minimum 0.45-ppm available chlorine concentration to ensure the microbial quality of the water. Kinner and Moats (1981) reported that bacterial counts decreased on shells washed with water at pH 11 regardless of its temperature. In addition, they observed reduction of microbial populations on shells with water at 50-55ºC

regardless of its pH. Catalano and Knabel (1994) indicated that *Salmonella* Enteritidis was effectively inactivated on shells processed with water at pH 11, followed by immediate egg chilling after washing.

Worley *et al.* (1992) tested the antimicrobial activity of N-halamine compounds against *Salmonella* Enteritidis on eggshells, and reported that the compounds 1-bromo-3-chloro-2,2,5,5-tetramethylimidazolidin-4-one (DBC) and the 1,3-dichloro-2,2,5,5-tetramethylimidazolidin-4-one (DC) were more effective than free chlorine against the microorganism, and suggested that these compounds could be used as chlorine replacements in the egg-processing industry. Kim and Slavik (1996) washed eggs with the quaternary ammonium compound, cetylpiridinium chloride, at 100 ppm and with trisodium phosphate at 5%, and reported *Salmonella* Enteritidis inactivation on shells by 1.2- and 0.5-$\log_{10}$, respectively. In addition these researchers indicated that both chemicals adversely affected the shell cuticle. Knape *et al.* (2001) tried an iodine-based disinfectant at approximately 75 ppm in a commercial egg washer, and reported 1.4-3.2 $\log_{10}$ *Salmonella* Enteritidis inactivation on shells. Nevertheless, efficacy of the sanitizer was affected by increased amount of solids suspended in the water.

Hydrogen peroxide

Egg treatment with hydrogen peroxide was tested for potential inactivation of *Salmonella* on shells of hatching eggs (Padron, 1995; Cox *et al.*, 2000). Padron (1995) reported that eggs, externally contaminated with *Salmonella* Typhimurium, treated by double dipping in hydrogen peroxide at 6% resulted in 95% reduction of the microorganism. In addition, this researcher indicated that application of 16-psi positive

pressure, during treatments, increased the penetrability of the sanitizer through the shell pores. Bailey *et al.* (1996) indicated that intermittent treatment of eggs with hydrogen peroxide at 2.5%, over a period of 3 days in a hatching cabinet, reduced *Salmonella* Typhimurium by 55% on the shell surface. Cox *et al.* (2000) reported that *Salmonella* Typhimurium-contaminated shell eggs, which were treated with hydrogen peroxide (1.4%) and a surfactant, at –6 psig and atmospheric pressures caused 65% and 33% microbial reduction, respectively, therefore suggesting that vacuum improved the penetration of the sanitizer into the shells, and enhanced its effectiveness.

Ozone

Ozone in its gaseous and aqueous phase has been tested against *Salmonella* spp. and natural contaminants on the surface of shell eggs (Whistler and Sheldon, 1988; Ito *et al.*, 1999; Koidis *et al.*, 2000). Whistler and Sheldon (1988) reported that treatment of shell eggs for 2 h using ozone at 3% by weight with water mist effectively inactivated natural microbial flora on shells by 2.5 $\log_{10}$. Bailey *et al.* (1996) tested the effectiveness of gaseous ozone, at 0.2-0.4 ppm for 3 days, on the decontamination of shell eggs in a hatching cabinet, and reported that after this treatment, *Salmonella* Typhimurium was recovered in 91% of the ozonated eggshells. Conversely, Ito *et al.* (1999) indicated that treatment of hatching quail eggs with ozone, in gaseous phase at 10 ppm for 6 h, resulted in ≥ 3-$\log_{10}$ *Salmonella* reduction on shells. Koidis *et al.* (2000) studied aqueous ozone activity (1.4-3.0 ppm) against *Salmonella* Enteritidis on shell eggs, using two treatment temperatures, and reported that treatments at 22°C with 1.4-ppm ozone inactivated the microorganism by 1 $\log_{10}$ in 90 sec, while treatments at 4°C using 3.0-ppm ozone

reduced 2 $\log_{10}$ in the same time period. On the other hand, Davies and Breslin (2003) treated eggs with gaseous ozone (concentration not indicated) for up to 30 min, and reported limited reduction of *Salmonella* Enteritidis PT4 by 42% on the eggshells.

Electrolyzed oxidative water

Water containing radicals with antimicrobial activity can be generated by passing mineralized tap water across an electrochemical cell, which results in the production of positively charged water that contains hypochlorite, ozone, and hydroperoxyl ($HO_2^{\cdot}$) and hydroxyl ($^{\cdot}OH$) radicals (Davies and Breslin, 2003). Rusell (2003) electrolyzed saline solution (20%), which contained 8-ppm free chlorine (pH 2.1 and 1,150 mV redox potential), and treated *Salmonella* Typhimurium-contaminated shell eggs using electrostatic spraying over a period of 24 h. The author reported inactivation of the microorganism on the surface of shell eggs by 4-6 $\log_{10}$. In a different study, Davies and Breslin (2003) immersed shell eggs, previously contaminated with *Salmonella* Enteritidis, in electrolyzed water that contained radicals and chlorine for 5 min. These researchers reported ≥ 4-$\log_{10}$ reduction on eggshells.

Physical methods

Boiling water

Efficacy of boiling water to inactivate *Salmonella* Enteritidis on the surface of shell eggs has been previously explored. Gast (1993) reported that shell eggs, which were previously dipped in a *Salmonella* Enteritidis PT13a cell suspension (10^9 CFU/ml), and

subsequently immersed in boiling water for 5 sec, had non-detectable levels of the microorganism after plating on brilliant green agar. In a similar study, Himathongkham *et al.* (1999) indicated that when eggs were dipped in a cell suspension of *Salmonella* Enteritidis (10^8 CFU/ml), and immersed in boiling water for 3 sec, the microorganism was not detected on eggshells and membranes. However, these researchers indicated that these treatments sometimes resulted in shell break.

Ultraviolet radiation

Ultraviolet (UV) radiation at 254 nm is effective against microorganisms on surfaces, in air, and in liquids (Wong *et al.*, 1998; Bintsis *et al*, 2000; Shama, 2000). Utilization of UV radiation in processing is relatively economical, easy to use, and has lethal effect to most types of microorganisms (Bintsis *et al.*, 2000).

In addition, earlier studies suggested that UV radiation was effective against *Salmonella* spp. and natural contaminants on the surface of shell eggs (Latala and Dobrzanski, 1989; Berrang *et al.*, 1995; Goerzen and Scott, 1995).

According to Kuo *et al.* (1997a), UV radiation, at 620 $\mu W/cm^2$, effectively inactivated *Salmonella* Typhimurium on the surface of eggs by 4.6 $\log_{10}$ in 5 min. Similar UV treatments for 30 min inactivated natural microflora on eggshells by 2.4 $\log_{10}$. In a different study, Kuo *et al.* (1997b) reported that egg rotation at 1 rpm during UV exposure significantly improved the level of microbial inactivation on shell eggs, and indicated that treatments at 4,350 $\mu W/cm^2$ for 20 min reduced natural eggshell microflora by 2.5 $\log_{10}$.

Conversely, Bailey *et al.* (1996) reported that treatment of shell eggs, with UV at 146 μW/sec, for as long as 3 days, was ineffective against *Salmonella* Typhimurium on eggshells.

Pulsed Light

Pulsed energy processing is a new technology that includes the use of short, potent flashes of light emitted at wavelengths in the range of the UV (25%), visible (45%), and infrared (30%) radiation. Light flashes are short (200-300 μs), and their intensity is ~ 20,000 times that of the sunlight. Pulse light is produced through the conversion of alternating current voltage to direct current high-voltage pulses in a tubular lamp; emitted light is efficient against all types of microorganisms (Dunn, 1996; 2001). Dunn (1996) reported that treating shell eggs, which were externally contaminated with *Salmonella* Enteritidis, with pulsed light at 4 J/cm^2, resulted in ≥ 8-log$_{10}$ microbial reduction on shells in ≤ 1 second. Brackett (2000) cautioned that pulse light technology was only effective on surfaces directly exposed to the light and that its application was relatively costly.

Gamma radiation and X-rays

Gamma and X-rays are forms of radiation that have different origins. Gamma radiation is produced during the transition of an atomic nucleus from an excited to a stable state in radioactive materials.

On the other hand, X-rays are produced as a result of electron transitions from a low to a high energy level in an atom, and they can be generated in an accelerator by bombarding a heavy metal with fast electrons (Hansen and Shaffer, 2001).

Radiation treatments have been previously studied to inactivate *Salmonella* spp. on the shell surface and within eggs (Tellez *et al.*, 1995; Lith *et al.*, 1995). Tellez *et al.*, (1995) reported inactivation of *Salmonella* Enteritidis on eggshells by ≥ 8-$\log_{10}$ using cobalt-60 (^{60}Co) gamma radiation at 2 kGy. In a different study, Serrano *et al.* (1997) treated shell eggs, externally contaminated with ~ 10^6 *Salmonella* Enteritidis, utilizing doses of X-rays at 0.5-1.5 kGy, and reported that 0.5 kGy effectively inactivated the microorganism to non-detectable levels.

Plasma

Plasma is considered the fourth state of the matter because energetically it is different from solids, liquids and gases. Ionized gases are considered plasma if they possess approximately the same amount of positive and negative charges. Plasma can be produced by very high temperatures or by electric or magnetic fields, and can be used to inactivate a wide variety of microorganisms (Jacobs and Lin, 2001).

Davies and Breslin (2003) tested the effectiveness of ionized air against *Salmonella* Enteritidis on the surface of shell eggs, and reported that treatments for up to 20 min produced limited inactivation (15%) of the microorganism on eggshells.

Control of *Salmonella* spp. within shell eggs

Inactivation of *Salmonella* spp. within shell eggs is mainly based on the use of gamma and X-ray radiation, and pasteurization (Table 1.4).

Gamma and X-ray radiation

Radiation processing effectively inactivates microorganisms in foods, without drastically increasing the temperature of the treated product during its application. However, consumer acceptance of irradiated foods in the U.S. is still limited (Cottee *et al.*, 1995; Hunter, 2000). The Food and Drug Administration (FDA) recently approved the use of ionizing radiation at ≤ 3-kGy doses for the reduction of *Salmonella* in shell eggs (CFR, 2000b). However, irradiated eggs are not commercially available.

Tellez *et al.* (1995) reported that gamma radiation, from a ^{60}Co source, effectively inactivated *Salmonella* Enteritidis located inside the eggshell membranes, and reported that doses at 2-3 kGy reduced 10^8 salmonellae to non-detectable levels. However, these researchers indicated that egg quality was drastically reduced after the treatments. Lith *et al.* (1995) irradiated shell eggs, which were contaminated with *Salmonella* Enteritidis PT4 inside the yolk, using ^{60}Co doses of 1.0-6.0 kGy, and reported that 2.5 kGy-treatment inactivated the microorganism by 6-$\log_{10}$, however, the treatment adversely affected egg quality. Serrano *et al.* (1997) reported that irradiation of shell eggs internally contaminated with *Salmonella* Enteritidis, with 1.5 kGy X-ray, was effective against the microorganism. These doses resulted in 0.32-0.41 kGy *Salmonella* D-values. The investigators recommended an irradiation dose of 1.5 kGy to inactivate the microorganism by 4 $\log_{10}$ within eggs, and indicated that this dose did not impact egg

quality. Although gamma and X-ray irradiation are rapid and highly effective against *Salmonella* within eggs, the marked decrease in egg quality after treatments is a major drawback of this technology (Tellez *et al.*, 1995; Ma, 1996, Moon and Song, 2000). In addition, FDA indicated that egg irradiation at the approved dose (≤ 3 kGy) did not ensure microbial inactivation by 5 $\log_{10}$ within shell eggs (CFR, 2000b).

Ma (1996) indicated that treatment of shell eggs with 1-3 kGy gamma radiation (^{60}Co) markedly reduced albumen viscosity with aggregation and denaturation of egg proteins, and caused loss of yolk color. In addition, Moon and Song (2000) reported that shell eggs treated with high doses of ^{60}Co-gamma radiation (1-30 kGy), and subsequently stored for 30 days, showed a decrease in albumen viscosity, an increase in protein denaturation, and a rise in albumen pH.

Pasteurization

Pasteurization of shell eggs is a technology that has been approved by the U.S. Department of Agriculture (USDA) for commercial application (USDA, 1997). In earlier studies, Lith *et al.* (1995) heated shell eggs, containing 10^1-10^3 *Salmonella* Enteritidis PT4 inside the yolk, in water at 57°C for 20-30 min and reported that these treatments were not sufficient to inactivate the microorganism. In addition, these investigators suggested that treatments at ≥ 57°C for > 20 min could result in albumen coagulation and subsequently reduced egg quality. Hou *et al.* (1996) reported that *Salmonella* Enteritidis was effectively inactivated by 5 $\log_{10}$, in the yolk of shell eggs, after treatment in a hot-air oven at 55°C for 3 h.

In addition, these researchers reported that heating internally contaminated shell eggs in water at 57°C for 25 min, followed by their treatment with hot air at 55°C for 60 min resulted in microbial reductions by 7-$\log_{10}$.

Stadelman *et al.* (1996) heated shell eggs, containing ~10^7 *Salmonella* Enteritidis in the yolk, in a microwave oven to achieve ~55ºC, and then held contaminated eggs in a convection oven or in water at 56ºC for different periods of time. These researchers reported 7-$\log_{10}$ reduction for the microwave-convection oven and the microwave-water treatments in 120 and 30 min, respectively. In addition they indicated that these treatments did not adversely affect egg quality. Although microwave heating is effective and fast, the thermal distribution in microwave-heated eggs is not uniform enough for commercial applications (Schuman, 2000). Schuman *et al.* (1997) heated shell eggs, inoculated in the yolk with ~10^7 *Salmonella* Enteritidis, in water at 57 and 58 °C and reported microbial reductions by ≥ 5.6 $\log_{10}$ after 55 min and ≥ 5.8 $\log_{10}$ after 43 min, respectively. Additionally, these researchers calculated decimal reduction times (D-values) for the microorganism of 4.5 and 6 min at 57 and 58°C, respectively. According to this study, albumen clarity and its functionality was affected by the treatments. Brackett *et al.* (2001) heated yolk-contaminated shell eggs, by convection currents of humid air, and reported D-values of 5.4-6.1 min for a salmonellae cocktail treated at 57.2°C. Egg heating using this procedure for ≥ 70 min resulted in non-detectable levels of the microorganism.

Michael Foods Egg Products Co. in Minneapolis, Minnesota, introduced commercial egg pasteurization in the market in 1996, and Pasteurized Eggs Corp. from New Hampshire started to market shell eggs under the Davidson's Pasteurized Eggs

brand name since 1998 (Mermelstein, 2001). The pasteurization process consists of prolonged heating of shell eggs by immersion treatments in water baths at 56-62°C or by hot air in convection ovens (Schuman, 2000; Zeidler, 2001a; Brackett *et al.*, 2001). The steps of a shell egg pasteurization process are presented in Fig 1.4. The procedure includes the washing and grading of shell eggs for uniformity. Sets of 30 egg flats are loaded in the pasteurizer, pre-heated in warm water, and subsequently maintained at 56-62°C over time. After heating, shell eggs are cooled, off loaded, and inspected. The last steps of the process consist of spray rinsing of shell eggs, eggshell coating, and packaging (Schuman, 2000).

Heat and ozone

Cox *et al.* (1995) reported in a patent application the inactivation of *Salmonella* spp. in the content of shell eggs using heat, vacuum, and a mix of gases including ozone. The procedure consisted of heating shell eggs at 59.4ºC for extended time, followed by generation of vacuum in a treatment chamber, and application of ozone. The authors claimed that combined treatments reduced the level of microorganisms in shell eggs and extended the shell-life of the product.

Prevention of *Salmonella* spp. proliferation in eggs by rapid cooling

Low temperatures could preserve the quality and safety of shell eggs along the production, storage, transportation and retail stages (Mermelstein, 2000). According to regulations enforced by the Food Safety and Inspection Service (FSIS-USDA), shell eggs must be kept at 7.2°C (45°F) after processing, during transportation, and throughout

storage (CFR, 1998). Anderson *et al.* (1995) reported that cryogenic systems based on liquid carbon dioxide were able to effectively reduce internal egg temperature from 43.3°C (110°F) to 7.2°C (45°F) within 80 to 90 sec. Mermelstein (2000) indicated that a cryogenic system, based on liquid carbon dioxide cooling, increased the strength of the eggshell membranes, and in addition, reduced *Salmonella* Enteritidis contamination by 2 $\log_{10}$. However, Gast and Beard (1992) indicated that there was no significant reduction of *Salmonella* Enteritidis in artificially contaminated whole eggs during the process of freezing and thawing.

REFERENCES

Adler. M.G., and G.R. Hill. 1950. The kinetics and mechanism of hydroxide iron catalyzed ozone decomposition in aqueous solution.

American Egg Board. 2003. U.S. egg industry fact sheet. Available at http://www.aeb.org/eii/facts/industry-facts-10-2003.htm. Accessed 20 February, 2003.

American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. Standard methods for the examination of water and wastewater, 19th edition, Washington, DC.

Anderson, K.E., P.A. Curtis, and F.T. Jones. December 1995. Rapid chilling of shell eggs using cryogenic gases. U.S. patent 5,474,794.

Andrews, C.C., and O.J. Murphy. 2002. Generation and delivery device for ozone gas. U.S. patent 6,461,487.

Anellis, A., J. Lubas, and M.M. Rayman. 1954. Heat resistance in liquid eggs of some strains of the genus *Salmonella*. Food Res. 19: 377-395.

Angulo, F.J., and D.L. Swerdlow. 1999. Epidemiology of human *Salmonella enterica* serovar Enteritidis infections in the United States. pp. 33-41. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Bader, H. and J. Hoigné. 1981. Determination of ozone in water by the indigo method. Water Res. 15: 449-456.

Bailey, J.S., R.J. Buhr, N.A. Cox, and M.E. Berrang. 1996. Effect of hatching cabinet sanitation treatments on *Salmonella* cross-contamination and hatchability of broiler eggs. Poult. Sci. 75: 191-196.

Bartlett, F.M., J.M. Laird, C.L. Addison, and R.C. Mckellar. 1993. The analysis of egg wash water for the rapid assessment of microbial quality. Poult. Sci. 72:1584-1591.

Bell, C., and A. Kyrikiades. 2002. *Salmonella*. Blackwell Science Ltd., London.

Berrang, M.E., N.A. Cox, J.S. Bailey, and R.J. Buhr. 1995. Efficacy of ultra violet light treatment for elimination of *Salmonella* on hatching eggs. Poult. Sci. 74:50 (abstr.).

Bintsis, T., E. Litopoulou-Tzanetaki, and R.K. Robinson. 2000. Existing and potential applications of ultraviolet light in the food industry – a critical review. J. Sci. Food Agric. 80: 637-645.

Brackett, R. 2000. Commercial processing technologies. Presented on the 6th annual symposia series on food safety in the 21st century: Shell eggs and egg products – safety issues. Inst. Food Technologists, Chicago, IL, May 18.

Brackett, R.E., J.D. Schuman, H.R. Ball, and A.J. Scouten. 2001. Thermal inactivation kinetics of *Salmonella* spp. within intact eggs heated using humidity-controlled air. J. Food Prot. 64: 934-938.

Brady, J.E., and G.E. Humiston. 1978. General Chemistry Principles and Structure. John Wiley & Sons. New York.

Braun, P., and K. Felhaber. 1995. Migration of *Salmonella* Enteritidis from the albumen into the egg yolk. Int. J. Food Microbiol. 25: 95-99.

Bruce, J., and E.M. Drysdale. 1994. Trans-shell transmission, p. 63-91. *In* R.G. Board and R. Fuller (ed.), Microbiology of the avian egg. Chapman and Hall, New York.

Catalano, C.R. and S.J. Knabel. 1994. Destruction of *Salmonella* Enteritidis by high pH and rapid chilling during simulated commercial egg processing. J. Food Prot. 57:592-595, 603.

CDC. 1988. Update: *Salmonella* Enteritidis infections and *Salmonella* Enteritidis grade A shell eggs-United States. Morbid. Mortal. Weekly Rep. 37: August 19, 1988.

CDC. 1990. Epidemiologic notes and reports update: *Salmonella* Enteritidis infections and grade A shell eggs-United States, 1989. Morbid. Mortal. Weekly Rep. 38: 877-880.

CDC. 1993. Outbreaks of *Salmonella* Enteritidis gastroenteritis-California, 1993. Morbid. Mortal. Weekly Rep. 42: October 22, 1993.

CDC. 1996. Outbreaks of *Salmonella* serotype Enteritidis infection associated with consumption of raw eggs-United States, 1994-1995. Morbid. Mortal. Weekly Rep. 45: August 30, 1996.

CDC. 2003. Outbreaks of *Salmonella* serotype Enteritidis infection associated with eating shell eggs-United States, 1999-2001. Morbid. Mortal. Weekly Rep. 51: 1149-1152.

CFR. 1998. Refrigeration and labeling requirements for shell-eggs. Vol. 63. No. 166. Office Federal Register, Washington, DC.

CFR. 2000a. Food labeling, safe handling statements, labeling of shell eggs; refrigeration of shell eggs held for retail distribution. Vol. 65. No. 234. Office Federal Register, Washington, DC.

CFR. 2000b. Irradiation in the production, processing and handling of food. Vol. 65. No. 141. Office Federal Register, Washington, DC.

CFR. 2001. Secondary direct food additives permitted in food for human consumption. Vol. 66. No.123. Office Federal Register, Washington, DC.

Cohen, M.L., and R.V. Tauxe. 1986. Drug-resistant *Salmonella* in the United States: an epidemiologic perspective. Science. 234: 964-969.

Cottee, J., P. Kunstadt, and F. Fraser. 1995. Consumer acceptance of irradiated chicken and produce in the U.S.A. Radiat. Phys. Chem. 46: 673-676.

Cotterill, O.J. 1968. Equivalent pasteurization temperatures to kill *Salmonella* in liquid egg white at various pH levels. Poult. Sci. 72: 1584-1591.

Cox, J.P., J.M. Cox, and R.W.D. Cox. 1995. Hyperpasteurization of food. U.S. patent 5,431,939.

Cox, N.A., M.E. Berrang, R.J. Buhr, and J.S. Bailey. 2000. Bactericidal treatment of hatching eggs IV. Hydrogen peroxide applied with vacuum and surfactant to eliminate *Salmonella* from hatching eggs. J. Appl. Poult. Res. 9: 530-534.

D'Aoust, J.-Y. 1989. *Salmonella*, p.327-445. *In* M.P. Doyle (ed.), Foodborne bacterial pathogens, Marcel Dekker, New York.

D'Aoust, J.-Y. 2001. *Salmonella*, p.163-191. *In* R.G. Labbé, and S. Garcia (ed.), Guide to foodborne pathogens, Wiley-Interscience, New York.

Davies, R.H., and M. Breslin. 2003. Investigations into possible alternative decontamination methods for *Salmonella* Enteritidis on the surface of table eggs. J. Vet. Med. 50: 38-41.

Dillon, D., R. Combes, M. McConville, and E. Zeiger. 1992. Ozone is mutagenic in *Salmonella*. Environ. Mol. Mutagen. 19: 331-337.

Dunn, J. 1996. Pulsed light and pulsed electric field for foods and eggs. Poult. Sci. 75: 1133-1136.

Dunn, J. 2001. Pulsed-light disinfection of water and sterilization of blow/fill/seal manufactured aseptic pharmaceutical products, p. 765-793. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Ebel E. and W. Schlosser. 2000. Estimating the annual fraction of eggs contaminated with *Salmonella enteritidis* in the United States. Int. J. Food Microbiol. 61: 51-62.

Ewell, A.W. 1946. Recent ozone investigation. J. Appl. Physics. 17: 908-911.

Fajardo, T.A., R.C. Ananthesawaran, V.M. Puri, and S.J. Knabel. 1995. Penetration of *Salmonella* Enteritidis into eggs subjected to rapid cooling. J. Food Prot. 58: 473-477.

Finke, M., P. Shillam, T. McGivern, X. Wang, D. Vugia, S. Segler, D. Gerber, S. Stenzel, S. Zanski, M. Kennedy, R. Marcus, and the EIP FoodNet working group. Hospitalizations among cases with the most common serotypes of *Salmonella*: FoodNet, 1996-2000. International Conference on Emerging Infectious Diseases. Atlanta, GA, March 2002.

Frenzen, P.D., J.C. Buzby, and T. Roberts. 1999. An updated estimate of the economic costs of human illness due to foodborne *Salmonella* in the Unites States. Proceedings of the 3rd. international symposium on the epidemiology and control of *Salmonella* in pork, August, Washington, D.C.

Gast, R.K. 1993. Immersion in boiling water to disinfect egg shells before culturing egg contents for *Salmonella* Enteritidis. J. Food Prot. 56: 533-535.

Gast, R.K. and C.W. Beard. 1990. Production of *Salmonella* Enteritidis contaminated eggs by experimentally infected hens. Avian Dis. 34: 438-446.

Gast, R.K. and C.W. Beard. 1992. Detection and enumeration of *Salmonella* Enteritidis in fresh and stored eggs laid by experimentally infected hens. J. Food Prot. 55: 152-156.

Goerzen, P.R., and T.A. Scott. 1995. Ultraviolet light sanitation for broiler hatching eggs. Poult. Sci. 74: 83 (abstr.).

Gomez, T.M., Y. Molatjemi, S. Miyagawa, F.K. Kaferstein, and K. Stohr. 1997. Foodborne salmonellosis. World Health Stat. Q. 50: 81-89.

Gordon, G., W.J. Cooper, R.G. Rice, and G.E. Pacey. 1988. Methods for measuring disinfectant residuals. J. Am. Water Works Assoc. 80: 94-108.

Graham, D.M. 1997. Use of ozone for food processing. Food Technol. 51: 72-75.

Grimes, H.D., K.K. Perkins, and W.F. Boss. 1983. Ozone degrades into hydroxyl radical under physiological conditions. Plant Physiol. 72: 1016-1020.

Grimont, P.A.D., P. Bouvet, F. Grimont, and J.-C. Desenclos. 1999. *Salmonella enterica* serovar Enteritidis in France: epidemiology, prevention, and control. pp. 43-49. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Guard-Petter, J. 2001. The chicken, the egg and *Salmonella* Enteritidis. Environ. Microbiol. 3: 421-430.

Guthrie, R.K. 1992. *Salmonella*, CRC, Boca Raton, FL.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchel, and W.H. Andrews. 1993. Growth of *Salmonella* Enterirtids in grade A eggs during prolonged storage. Poult. Sci. 72: 373-377.

Hansen, J.M., and H.L. Shaffer. 2001. Sterilization and preservation by radiation sterilization, p. 729-746. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Hennessy, T.W., C.W. Hedberg, L. Slutsker, K.E. White, J.M. Besser-Wiek, M.E. Moen, J. Feldman, W.W. Coleman, L.M. Edmonson, K.L. MacDonald, and M.T. Osterholm.1996. A national outbreak of *Salmonella* Enteritidis infections from ice cream. New Eng. J. Med. 334: 1281-1286.

Himathongkham, S., H. Riemann, and R. Ernst. 1999. Efficacy of disinfection of shell eggs externally contaminated with *Salmonella* Enteritidis. Implications for egg testing. Int. J. Food Microbiol. 49: 161-167.

Hoigné, J., and H. Bader. 1975. Ozonation of water; role of hydroxyl radicals as oxidizing intermediates. Science. 190: 782-784.

Holley, R.A. and M. Proloux. 1986. Use of egg washwater pH to prevent survival of *Salmonella* at moderate temperatures. Poult. Sci. 65: 922-928.

Horváth, M., L. Bilitzky, and J. Huttner. 1985. Ozone. Elsevier, New York.

Hou, H., R.K. Singh, P.M. Muriana, and W.J. Stadelman. 1996. Pasteurization of intact shell eggs. Food Microbiol. 13: 93-101.

Humphrey, T.J., A. Baskerville, S.L. Mawer, B. Rowe, and S. Hopper. 1989. *Salmonella* Enteritidis PT4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. 103: 415-423.

Humphrey, T.J., A. Whitehead, A.H.L. Gawler, A. Henley, and B. Rowe. 1991. Numbers of *Salmonella* Enteritidis in the contents of naturally contaminated hen's eggs. Epidemiol. Infect. 106: 489-496.

Humphrey, T.J. 1994. Contamination of eggs with potential human pathogens, p. 93-116. *In* R.G. Board and R. Fuller (ed.), Microbiology of the avian egg. Chapman and Hall, New York.

Humphrey, T.J. 1999. Contamination of eggs and poultry meat with *Salmonella enterica* serovar Enteritidis, p. 183-192. *In* A.M. Saeed, R.K.Gast, M.E. Potter, and P.G. Wall (ed.), *Salmonella enterica* serovar Enteritidis in humans and animals: epidemiology, pathogenesis, and control, Iowa State University Press, Ames, IA

Hunter, C. 2000. Changing attitudes to irradiation throughout the food chain. Radiat. Phys. Chem. 57: 239-243.

Hutchison, M.L., J. Gittins, A. Walker, A. Moore, C. Burton, and N. Sparks. 2003. Washing table eggs: a review of the scientific and engineering issues. World's Poult. Sci. J. 59: 233-248.

Hutchison, M.L., J. Gittins, A. Walker, N. Sparks, T.J. Humphrey, C. Burton, and A. Moore. 2004. An assessment of the microbiological risks involved with egg washing under commercial conditions. J. Food Prot. 67: 4-11.

Ito, H., H. Nakatani, H. Bamba, and T. Hayashi. 1999. Influence of the hatchability of Japanese quail eggs (hatching eggs) and sterilization effect for *Salmonella* by ozone gas sterilization. Res. Bull. Aichi Agric. Res. 31: 305-310.

Jacobs, P.T., and S-M. Lin 2001. Sterilization process utilizing low-temperature plasma, p. 747-763. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Jay, J.M. 1996. Modern food microbiology. Chapman & Hall, New York.

Jones, F.T., D.V. Rives, and J.B. Carey. 1995. *Salmonella* contamination in commercial eggs and an egg production facility. Poult. Sci. 74: 753-757.

Khadre, M.A., A. E. Yousef, and J.-G. Kim. 2001. Microbiological aspects of ozone applications in food: A review. J. Food Sci. 66: 1242-1252.

Kim, J-G. 1998. Ozone as an antimicrobial agent in minimally processed foods. Ph.D. Thesis. The Ohio State University, Columbus, OH.

Kim, J.-W and M.F. Slavik. 1996. Changes in eggshell surface microstructure after washing with cetylpyridinium chloride or trisodium phosphate. J. Food Prot. 59: 859-863.

Kim, J-G., A.E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Prot. 62: 1071-1087.

Kim, J.-G., A.E. Yousef, and M.A. Khadre. 2003. Ozone and its current and future application in the food industry. Adv. Food Nutr. Res. 45: 167-218.

Kinner, J.A. and W.A. Moats. 1981. Effect of temperature, pH, and detergent on the survival of bacteria associated with shell eggs. Poult. Sci. 60: 761-767.

Koidis, P., M. Bori, and K. Vareltzis. 2000. Efficacy of ozone treatment to eliminate *Salmonella* Enteritidis from eggshell surface. Arch. Lebensmittelhyg. 51: 4-6.

Koike, K., G. Inoue, M. Suzuki, J. Saida, and I. Komatsu. 1998. Decomposition characteristics of concentrated ozone. J. Chem. Eng. Jap. 31: 195-200.

Komanapalli, I.R., and B.H.S. Lau. 1996. Ozone-induced damage of *Escherichia coli* K-12. Appl. Microbiol. Biotechnol. 46: 610-614.

Knape, K.D., J.B. Carey, and S.C. Ricke. 2001. Response of foodborne *Salmonella* spp. marker strains inoculated on egg shell surfaces to disinfectants in a commercial egg washer. J. Environ. Sci. Health. 36: 219-227.

Kuo, F.L., J.B. Carey, and S.C. Ricke. 1997a. UV irradiation of shell eggs: effect on populations of anaerobes, molds, and inoculated *Salmonella* Typhimurium. J. Food Prot. 60: 639-643.

Kuo, F.L., S.C. Ricke, and J.B.Carey. 1997b. Shell egg sanitation: UV radiation and egg rotation to effectively reduce populations of aerobes, yeasts, and molds. J. Food Prot. 60: 694-697.

Latala, A. and Z. Dobrzanski. 1989. Effect of ultraviolet radiation on the microbiological contamination of the shells of eggs for human consumption. Med. Wet. 45: 420-422.

Li-Chan, E.C.Y., W.D. Powrie, and S. Nakai. 1995. The chemistry of eggs and egg products. p. 105-175. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Lith, L.A.J.T. van, F.F. Putirulan, and R.W.A.W Mulder. 1995. Pasteurization of table eggs to eliminate *Salmonellae*. Arch. Geflügelk. 59: 157-160.

Lynntech, 1998. LT 1 Electrochemical ozone generator instruction's manual. College Station , TX.

Ma, C.Y. 1996. Effects of gamma irradiation on physicochemical and functional properties of eggs and egg products. Radiat. Phys. Chem. 48: 375.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5: 607-625.

Merck Index, 1989. 11th Edition. Budavari, Merck & Co., New Jersey.

Mermelstein, N.H. 2000. Cryogenic system rapidly cools eggs. Food Technol. 54: 100-103.

Mermelstein, N.H. 2001. Pasteurization of shell eggs. Food Technol. 55: 72-73, 79.

Moats, W.A. 1978. Egg washing-a review. J. Food Prot. 41: 919-925.

Moon, S., and K.B. Song. 2000. Effect of high dose γ-irradiation on the physicochemical properties of shell eggs during storage. Han'guk Nonghwa Hakhoechi. 43: 260-265.

Murray, R.G., S. Pamela, and H.E. Elson. 1965. Location of mucopeptide of selection of the cell wall of *E. coli* and other gram-negative bacteria. Can. J. Microbiol. 11: 547-560.

Okubo, T., S. Akachi, and H. Hatta. 1997. Structure of hen eggs and physiology of egg laying, p. 1-12. *In* T. Yamamoto, L.R. Juneja, H. Hatta, and M. Kim (ed.), Hen eggs. CRC Press, Boca Raton.

Padron, M.N. 1990. *Salmonella* Typhimurium penetration through the eggshell of hatching eggs. Avian Dis. 34: 463-465.

Padron, M. 1995. Egg dipping in hydrogen peroxide solution to eliminate *Salmonella* Typhimurium from eggshell membranes. Avian Dis. 39: 627-639.

Poppe, C. 1999. Epidemiology of *Salmonella enterica* serovar Enteritidis. pp. 3-18. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Popoff, M.Y., J. Bockemül, and F.W. Brenner. 1998. Supplement 1997 (no. 41) to the Kauffmann-White scheme. Res. Microbiol. 149: 601-604.

President's Council on Food Safety, 1999. Egg safety from production to consumption: an action plan to eliminate *Salmonella* Enteritidis illnesses due to eggs. USDA, HHS, EPA, Dept. of Commerce.

Rice, R.G. 1986. Analytical aspects of ozone: treatment of water and wastewater. Lewis Publishers, Chelsea, MI.

Rizk, S.S., J.C. Ayres, and A.A. Craft. 1966. Effect of holding condition on the development of salmonellae in artificially inoculated hens' eggs. Poult. Sci. 45: 823-829.

Romanoff, A.L., and A.J. Romanoff. 1949. The avian egg. John Wiley & Sons, New York.

Rusell, S.M. 2003. The effect of electrolyzed oxidative water applied using electrostatic spraying on pathogenic and indicator bacteria on the surface of eggs. Poult. Sci. 82: 158-162.

Schuman, J.D., B.W. Sheldon, J.M. Vandepopuliere, and H.R. Ball. 1997. Immersion heat treatments for inactivation of *Salmonella* Enteritidis with intact eggs. J. Appl. Microbiol. 83: 438-444.

Schuman, J.D. 2000. Methods for thermal processing of shell eggs. Presented in the 6th annual symposia series on Food Safety in the 21st century: Shell eggs and egg products – safety issues. Inst. Food Technologists, Chicago, IL, May 18.

Scott, D.B.M., and E.C. Lesher. 1963. Effect of ozone on survival and permeability of *Escherichia coli*. J. Bacteriol. 85:567-576.

Serrano, L.E., E.A. Murano, K. Shenoy, and D.G. Olson. 1997. D values of *Salmonella* Enteritidis isolates and quality attributes of shell eggs and liquid whole eggs treated with irradiation. Poult. Sci. 76: 202-205.

Shama, G. 2000. Ultraviolet light. pp. 2208-2214. In R.K. Robinson, C. Batt, and P. Patel (ed.) Encyclopedia of Food Microbiology. Academic Press, London.

Shechter, H. 1973. Spectrophotometric method for determination of ozone in aqueous solutions. Water Res. 7: 729-739.

Shivaprasad, H.L., J.F. Timoney, S. Morales, B. Lucio, and R.C. Baker. 1990. Pathogenesis of *Salmonella* Enteritidis infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serological responses. Avian Dis. 34: 548-557.

Stadelman, W.J. 1995a. The egg industry, p. 1-37. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Stadelman, W.J. 1995b. Quality identification of shell eggs, p. 39-66. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Stadelman, W.J. 1995c. The preservation of quality in shell eggs, p. 67-79. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Stadelman, W.J., R.K. Singh, P.M. Muriana, and H. Hou. 1996. Pasteurization of eggs in the shell. Poult. Sci. 75: 1122-1125.

Tellez, I.G., R.M. Trejo, R.E. Sanchez, R.M. Ceniceros, Q.P. Luna, P. Zazua, and B.M. Hargis. 1995. Effect of gamma irradiation on commercial eggs experimentally inoculated with *Salmonella* Enteritidis. Radiat. Phys. Chem. 46: 789-792.

Thiagarajan, D. 1995. Studies on the mechanism of transovarian transmission of *Salmonella* Enteritidis in laying hens. Ph.D. thesis. Purdue University, West Lafayette.

Timoney, J.F., H.L. Shivaprasad, R.C. Baker, and B. Row. 1989. Egg transmission after infection of hens with *Salmonella* Enteritidis phage type 4. Vet. Rec.125: 600-601.

Todd, E.C.D. 1996. Risk assessment of use of cracked eggs in Canada. Int. J. Food Microbiol. 30: 125-143.

Tschäpe, H., A. Liesegang, B. Gericke, R. Prager, W. Rabasch, and R. Helmuth 1999. Ups and downs of *Salmonella enterica* serovar Enteritidis in Germany. pp. 51-61. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

USDA. 1966. Improved methods, techniques and equipment for cleaning eggs. Marketing Research Report No. 757.

USDA, Agricultural Marketing Service. 1997. Pasteurized shell eggs (pasteurized in-shell eggs). Vol. 162. No. 185: 49955-57. Fed. Regist. Washington, DC.

USDA. 2003. Egg-grading manual. Handbook no. 75. Available at http://www.ams.usda.gov/poultry/pdfs/EggGrading manual.pdf. Accessed 7 February, 2003.

Wall, P.G., and L.R. Ward. 1999. Epidemiology of *Salmonella enterica* serovar Enteritidis phage type 4 in England and Wales. pp. 19-25. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Watkins, B.A. 1995. The nutritive value of the egg, p. 177-194. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Weavers, L.K., and G.B. Wickramanayake. 2001. Disinfection and sterilization using ozone, p. 205-214. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Whistler, P.E., and B.W. Sheldon. 1989. Bactericidal activity, eggshell conductance, and hatchability effects of ozone versus formaldehyde disinfection. Poult. Sci. 68:1074-1077.

Wickramanayake, G.B. 1984. Kinetics and mechanism of ozone inactivation of protozoan cysts. Ph.D. thesis. The Ohio State University, Columbus.

Wojctowicz, J.A. 1996. Ozone, p. 953-994. *In* R.E. Kirk, and D.E. Othmer (ed.), Encyclopedia of Chemical Technology, vol. 17. Wiley-Interscience, New York.

Wong, E., R.H. Linton, and D.E. Gerrad. 1998. Reduction of *Escherichia coli* and *Salmonella* Senftenberg on pork skin and pork muscle using ultraviolet light. Food Microbiol. 15: 415-423.

Worley, B.S., W.B. Wheatley, S.D. Lauten, D.E. Williams, E.C. Mora, and S.D. Worley. 1992. Inactivation of *Salmonella* Enteritidis on shell eggs by novel N-halamine biocidal compounds. J. Ind. Microbiol. 11: 37-42.

Wynn, C.S., B.S. Kirk, and R. McNabey. 1973. Pilot plant for tertiary treatment of wastewater with ozone. EPA report R2-73-146, US EPA, Municipal Environ. Res. Lab., Cincinnati, OH.

Zeidler, G. 2001a. Processing and packaging shell eggs, p. 1129-1161. *In* D.D. Bell, and D. Weaver Jr. (ed.), Commercial chicken meat and egg production, Kluwer Academic Publishers, Norwell, MA.

Species	Subspecies	Number of serovars
Salmonella enterica	*enterica*	1443
	salamae	448
	arizonae	94
	diarizonae	323
	houtenae	70
	indica	11
Salmonella bongori		20
Total		2449

Table 1.1: Taxonomy of *Salmonella* (Modified from Popoff *et al.*, 1998).

Year	Location	Food implicated	Cases	Reference
1988	New Jersey	Homemade ice cream prepared with raw eggs	88	CDC, 1988
1988	New Jersey	Scrambled eggs	47	CDC, 1988
1988	New York	Omelets	12	CDC, 1988
1989	New York	Pasta dish with raw eggs	21	CDC, 1990
1989	Pennsylvania	Egg based custard pies	12	CDC, 1990
1989	Tennessee	Hollandaise/Béarnaise sauce (undercooked eggs)	27	CDC, 1990
1993	California	Omelets, scrambled eggs, egg salad	4	CDC, 1993
1993	California	Hollandaise/Béarnaise sauce (raw eggs)	23	CDC, 1993
1993	California	Mayonnaise	22	CDC, 1993
1994	Nationwide	Commercially produced ice cream (raw egg contamination)	224,000 (estimated)	Hennessy *et al.*, 1996
1994	Washington, D.C.	Hollandaise sauce (raw eggs)	56	CDC, 1996
1995	Indiana	Baked eggs	70	CDC, 1996
1995	New York	Caesar salad dressing	76	CDC, 1996
1995	New York	Homemade beverage with raw eggs	3	CDC, 1996
2001	South Carolina	Tuna salad with undercooked eggs	688	CDC, 2003
2001	North Carolina	Shell eggs	51	CDC, 2003

Table 1.2: Selected salmonellosis outbreaks in the United States implicating eggs and egg products for the transmission of *Salmonella enterica* serovar Enteritidis.

Method		References
Chemical	Commercial washing	Moats, 1978; Hutchison *et al.*, 2003; USDA, 2003
	Washing with cetylpyridinium chloride and trisodium phosphate	Kim and Slavik, 1996
	Washing with iodine-based disinfectant	Knape *et al.*, 2001
	Washing with N-halamine compounds	Worley *et al.*, 1992
	Hydrogen peroxide	Padron, 1995; Bailey *et al.*, 1996; Cox *et al.*, 2000
	Ozone	Whistler and Sheldon, 1988; Bailey *et al.*, 1996; Ito *et al.*, 1999; Koidis *et al.*, 2000; Davies and Breslin, 2003
	Electrolyzed oxidative water	Davies and Breslin, 2003; Rusell, 2003
Physical	Boiling water	Gast, 1993; Himathongkham *et al.*, 1999.
	Ultraviolet radiation	Berrang *et al.*, 1995; Bailey *et al.*, 1996; Kuo *et al.*, 1997a;
	Pulsed light	Dunn, 1996
	X-rays	Serrano *et al.*, 1997
	Gamma radiation (^{60}Co)	Tellez *et al.*, 1995
	Plasma	Davies and Breslin, 2003

Table 1.3: Selected chemical and physical methods to control *Salmonella* spp. on the surface of shell eggs.

Method	References
Gamma radiation (^{60}Co)[a]	Tellez *et al.*, 1995; Lith *et al.*, 1995
X-rays[a]	Serrano *et al.*, 1997
Pasteurization in water bath[b]	Lith *et al.*, 1995; Schuman *et al.*, 1997
Pasteurization in convection oven[b]	Hou *et al.*, 1996
Pasteurization in water bath and convection oven[b]	Hou *et al.*, 1996
Pasteurization in microwave, water bath, and convection oven	Stadelman *et al.*, 1996
Combination of heat and ozone	Cox *et al.*, 1995

[a] FDA-approved at ≤ 3kGy (CFR, 2000); not commercially available
[b] USDA-approved (USDA, 1997); commercially available

Table 1.4: Selected methods to control *Salmonella* spp. within shell eggs.

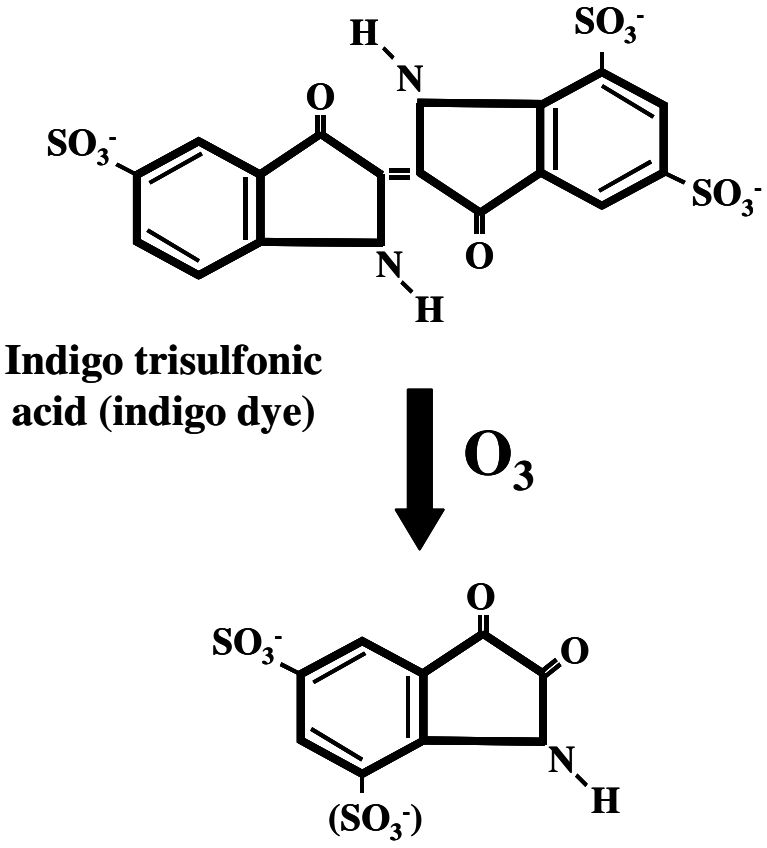

Figure 1.1: Reaction between ozone and indigo dye (Modified from Bader and Hoigné, 1981).

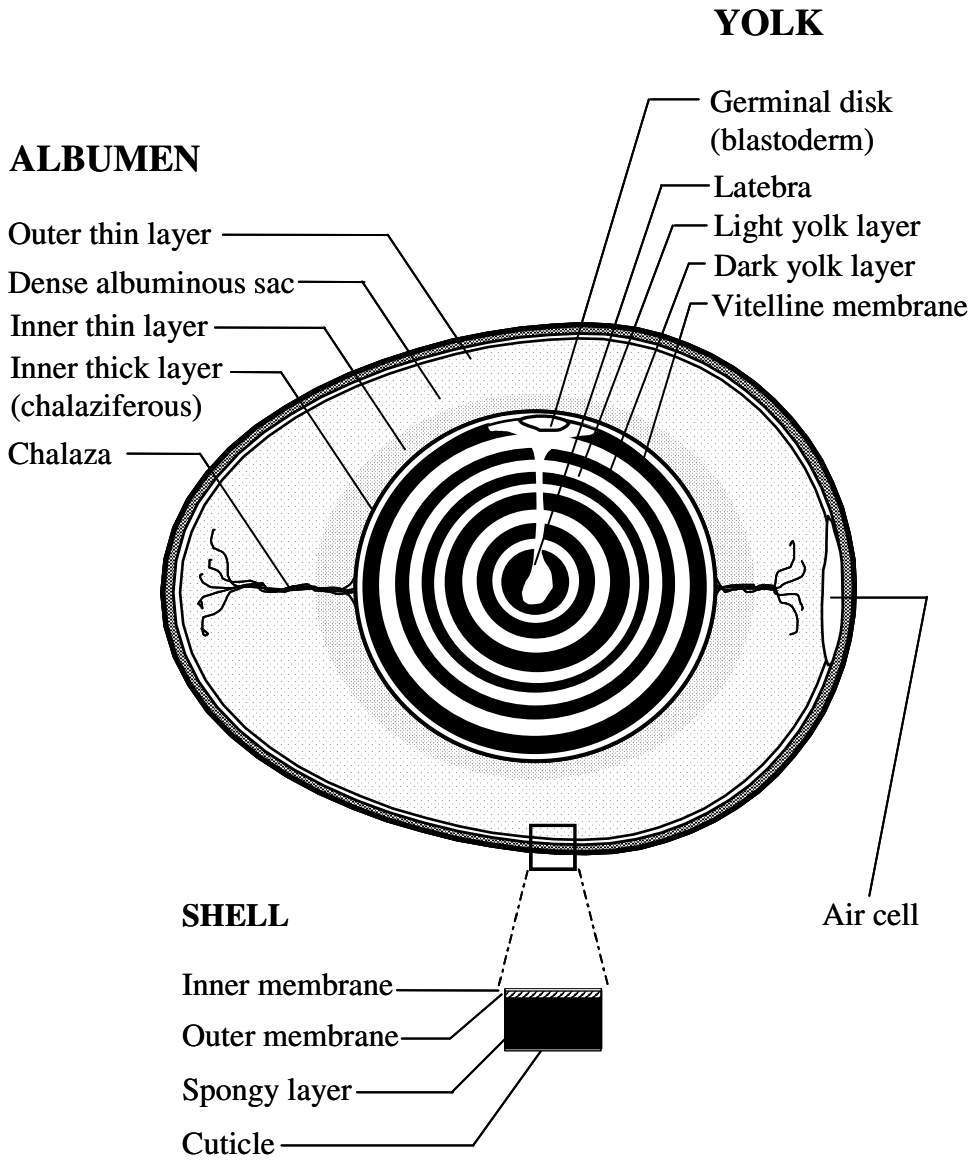

Figure 1.2: Schematic representation of the parts of the egg (Modified from Romanoff and Romanoff, 1949)

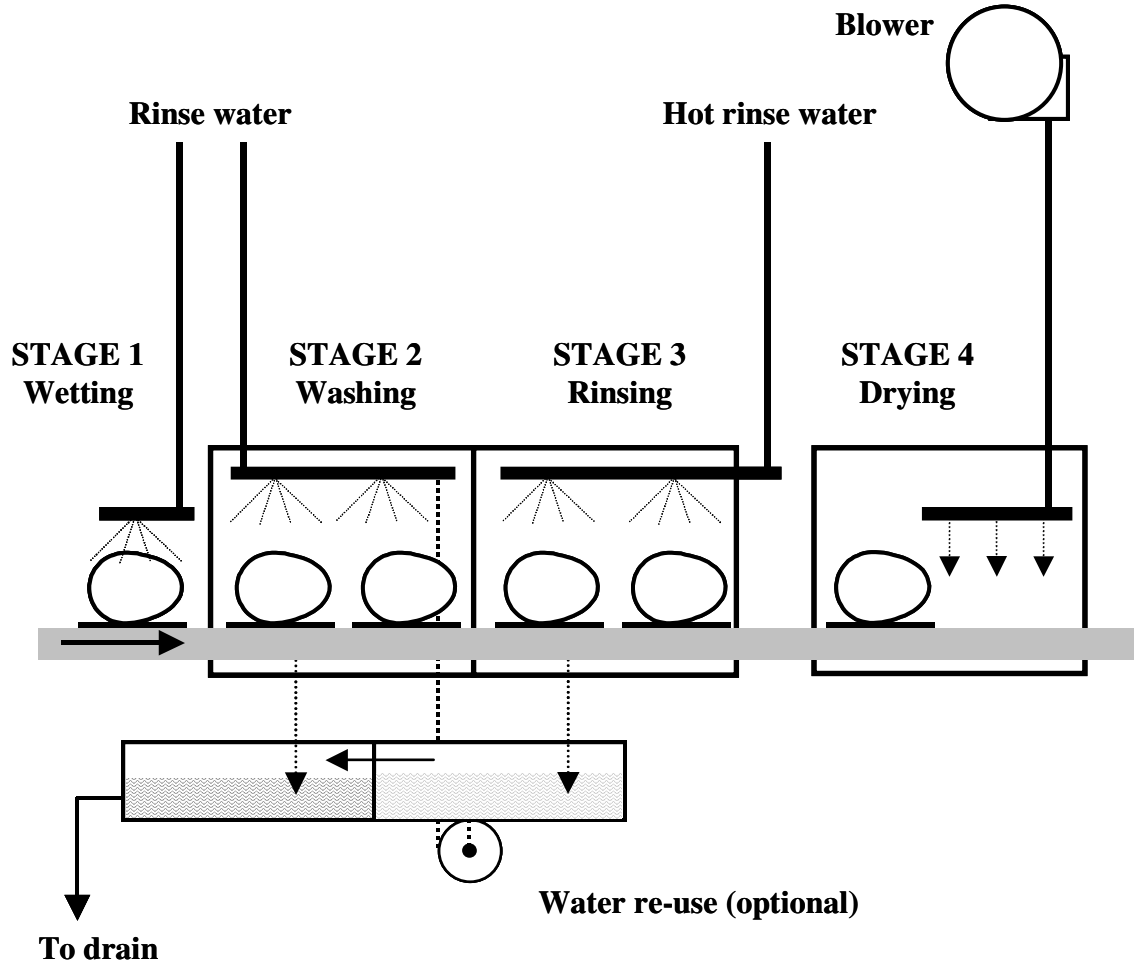

Figure 1.3: Stages of commercial egg washing (modified from Hutchison *et al.*, 2003).

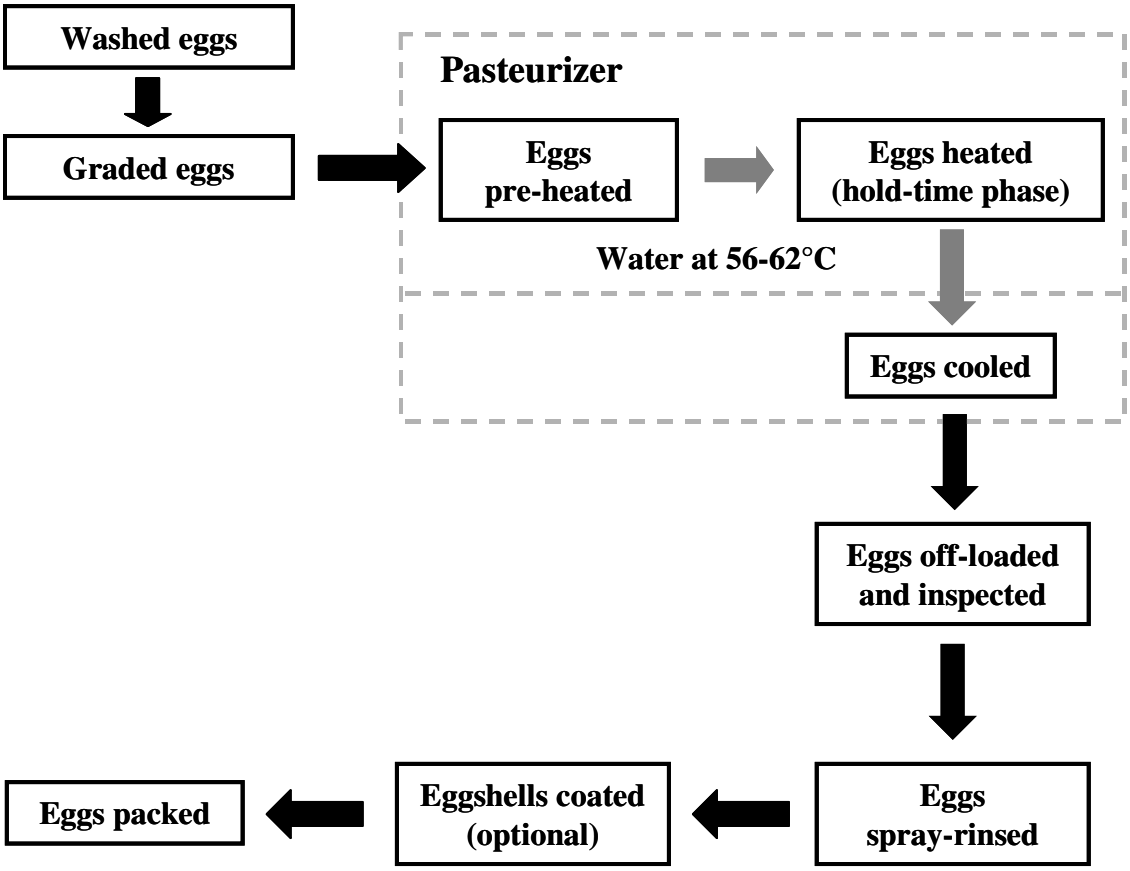

Figure 1.4: Commercial egg pasteurization (Modified from Schuman, 2000).

CHAPTER 2

INACTIVATION OF EXTERNAL *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS ON SHELL EGGS BY OZONE AND ULTRAVIOLET RADIATION

ABSTRACT

Presence of *Salmonella* Enteritidis in shell eggs has serious public health implications. Several treatments have been developed to control *Salmonella* on eggs with mixed success. Currently, there is a need for time-saving, economical, and effective egg sanitization treatments. In this study, shell eggs, externally contaminated with *Salmonella* ($8.0 \times 10^5 - 4.0 \times 10^6$ CFU/g), were treated with gaseous ozone (O_3) at 0-15 psig for 0-20 min. In other experiments, contaminated shell eggs were exposed to ultraviolet radiation (UV) at 100-2500 $\mu W/cm^2$ for 0-5 min. Treatment combination included exposing contaminated eggs to UV (1500-2500 $\mu W/cm^2$) for 1 min, followed by O_3 at 5 psig for 1 min. Eggs that were non-contaminated and untreated, contaminated and untreated, and contaminated and treated with air, were used as controls.

Results indicated that treating shell eggs with O_3 and UV separately, or in combination, significantly ($p < 0.05$) reduced *Salmonella* on shell eggs. For example, contaminated eggs treated with O_3 at 4-8°C and 15 psig for 10 min, or with UV (1500-

2500 μW/cm^2) at 22-25°C for 5 min produced ≥ 5.9 or 4.3 $\log_{10}$ microbial reductions, respectively, when compared to contaminated untreated controls. Combination in sequence using UV, followed by O_3 treatment resulted in synergistic inactivation of *Salmonella* by ≥ 4.6 $\log_{10}$ in ~ 2 min total treatment time. In conclusion, *Salmonella* was effectively inactivated on shell eggs in a short time, and at low temperatures utilizing gaseous ozone, ultraviolet radiation, and their combination.

INTRODUCTION

Chemical and physical sanitation procedures have been tested against *Salmonella* spp. on shell eggs with variable success. Some of these decontamination procedures include treatments with boiling water (Himathongkham *et al.*, 1999), chlorine and iodine (Knape *et al.*, 1999), hydrogen peroxide (Padron, 1995), pulsed light (Dunn, 1996), gas plasma and water with radicals (Davies and Breslin, 2003), and electrolyzed oxidative water (Russell, 2003). Regardless of efficacy of some of these procedures, currently there are no studies on low-temperature treatments able to inactivate large populations of *Salmonella* Enteritidis on shell eggs within an acceptable treatment time or without affecting the quality of the product.

Technologies with potential to decontaminate shell eggs at low temperatures include ultraviolet (UV) radiation, ozone, and their combinations (Kuo *et al.*, 1997a; Koidis *et al.*, 2000). Ultraviolet radiation inactivated microorganisms on surfaces, in air, and in liquids (Bintsis *et al.,* 2000; Shama, 2000). Furthermore, UV radiation effectively

reduced aerobic bacteria, molds, and *Salmonella* spp. on the surface of shell eggs (Latala and Dobrzanski, 1989; Kuo *et al.*, 1997a). Equipment for UV radiation is relatively inexpensive and easy to use, treatment with UV results in small amounts of heat if low-pressure UV lamps are used for a short time, and the process induces lethal effects to most types of microorganisms (Bintsis *et al.,* 2000; Blatchley and Peel, 2001).

Ozone (O_3) is a strong antimicrobial agent that has been extensively studied for its potential food industry applications (Kim *et al.*, 1999; 2003). The US Food and Drug Administration (FDA) has recently approved the use of ozone as an antimicrobial agent in food (CFR, 2001). Ozone decomposes spontaneously to a non-toxic product (*i.e.*, oxygen), and it can be used effectively at low temperatures (Koidis *et al.*, 2000; Achen and Yousef, 2001). Ozone has been tested for disinfection of poultry facilities and products such as hatcheries, hatching eggs, poultry-processing chiller water, and poultry carcass (Bailey *et al.*, 1996; Ito *et al.*, 1999, Kim *et al.*, 2003). In addition, ozone inactivates poultry associated pathogens that routinely contaminate the surface of shell eggs, setters, and hatchers (Whistler and Sheldon, 1989; Ito *et al.*, 1999; Koidis *et al.*, 2000). Combination of ozone and UV radiation is effective against pathogenic microorganisms, including *Salmonella* spp. when present in poultry-processing chiller water (Diaz *et al.*, 2001). Therefore, considering the advantages of ozone and UV radiation, the objective of this study is to develop a sanitation procedure at low temperature to eliminate *Salmonella* Enteritidis on the surface of shell eggs, using gaseous ozone under mild pressure, UV radiation, and their combination.

MATERIALS AND METHODS

Bacterial cultures and growth conditions

Salmonella Enteritidis was obtained from the culture collection of the Department of Microbiology at The Ohio State University (Columbus, OH). Stock culture was transferred to brain heart infusion (BHI) broth (Difco™; Becton, Dickinson and Co. Sparks, MD), and incubated at 37°C for 24 h. Aliquots of grown cultures were subsequently transferred in duplicate to 150 ml MacConkey broth (Difco)(0.1% inoculum), and incubated at 37°C for 24 h in orbital shaker (New Brunswick Scientific Co. Inc., Edison, NJ) under mild agitation. Aliquots (50 ml) of *Salmonella* Enteritidis cultures were centrifuged in duplicate (Sorval RC-5B, Dupont Instruments, Bannorbuck, IL) at 3020 × g for 10 min. Cell pellets were resuspended in 20 ml sterile phosphate buffer (0.1 M, pH 7.0) at 22-25°C, and mixed in vortex mixer (Fisher Scientific Industries, Inc., Bohemia, NY) for approximately 10 sec. Resulting cell suspension was centrifuged again and resuspended as previously described. This concentrated suspension was used to prepare 200 ml working cell suspension in phosphate buffer. This working suspension, in 400-ml beaker, had an OD_{600} ~ 0.4 and the cell density was 2.7×10^7- 1.0×10^8 CFU/ml.

Inoculation of shell eggs with *Salmonella* Enteritidis

Fresh, unfertilized shell eggs (53 ± 3 g/egg) were obtained from a poultry farm at The Ohio State University (Columbus, OH). Shell eggs were refrigerated at 4°C and used within 3 weeks of laying. Selected refrigerated eggs were warmed to ~ 22°C internal

temperature, washed with tap water (22-25°C), and gently scrubbed with plastic brush. Washed eggs were rinsed with distilled deionized water (22-25°C), and subsequently submerged in ethanol (70% vol/vol) for 30 min as described by Hammack *et al.* (1993). Sanitized shell eggs were transferred to sterile carton trays and aseptically dried at ambient temperature for ~ 40 min before inoculation. Dried, sanitized shell eggs were dipped for approximately 10 sec into stirred *Salmonella* Enteritidis cell suspension prepared as described previously. Contaminated shell eggs were transferred to sterile carton trays and permitted to dry for approximately 30 min before treatments. *Salmonella* Enteritidis count on externally contaminated shell eggs was $8.0 \times 10^5 - 4.0 \times 10^6$ CFU/g eggshell. Sanitized, non-contaminated shell eggs dipped into sterile deionized water at 22-25°C were used as negative controls.

Ozone generation

Gaseous ozone (O_3) was produced in electrochemical ozone generator (Model LT 1, Lynntech, Inc., College Station, TX). The generator produced 12-14% wt/wt ozone in oxygen at 1.45-liters/min gas mix. Experimental set up was kept inside chemical fume hood and all safety precautions were followed during experiments. Excess ozone was destroyed in heated catalyst (Lynntech).

Ozone treatment

Ozone treatment setup is shown schematically in Fig. 2.1. Eggs were treated with ozone in gasket-sealed stainless-steel vessel (4000 ml, 21.6 cm diam × 15.5 cm height; Alloy Products Corp., Waukesha, WI), adapted with a 30-psig pressure gauge (Ashcroft®,

Dresser Inc., Stratford, CT). Prior to every experiment, the treatment vessel was kept in refrigeration at 4°C overnight, and subsequently during experiments it was immersed in ice to maintain its temperature at 4-8°C. Temperature inside vessel was measured before ozone treatments with a glass thermometer (Ertco™, Ever Ready Thermometer Co., West Paterson, NJ). Sets of two eggs, inoculated as described previously, were placed within treatment vessel. Gaseous ozone, generated as previously described, was delivered to the cold vessel (1.45 liters/min O_3 in O_2 mix) without pressure for ≤ 8 min in a continuous treatment system. In a different experiment, shell eggs were treated in a batch mode with gaseous ozone at ≤ 15 psig (103 kPa) for ≤ 20 min. Come up time to achieve target pressure was approximately 2 min. After treatments, pressure was slowly released from vessel in 2-3 min. Contaminated non-treated shell eggs were used as controls. Additional controls included shell eggs treated with compressed air (Medipure™, Praxair, Inc. Danbury, CT) under conditions previously described. Sets of treated separated eggshells were tested for enumeration of *Salmonella* as described later.

Ultraviolet radiation treatment

Shell eggs, contaminated as previously described, were placed under a short-wave UV lamp (254 nm; 15 Watt, G15T8 General Electric, Co., Cleveland, OH) mounted on two cast-iron supports that allowed adjustment of light intensity by increasing or decreasing vertical distance between lamp and treated eggs. Protective UV-absorbing face shields were used during experiments and all safety precautions were observed when operating UV lamp. Contaminated shell eggs, prepared as previously described, were aseptically transferred to sterile glass base petri plates, placed under UV lamp and

irradiated at previously calibrated vertical distance to achieve 100 μW/cm^2 or 1500-2500 μW/cm^2 light intensity for ≤ 5 min. Prior to experiments, UV lamp was turned on for ~ 15 min to achieve stable irradiation intensity. During UV treatments, shell eggs were constantly, manually rotated with sterile metallic tongs to allow uniform surface exposure. Intensity of UV radiation was monitored with 254-nm radiometer probe (Model UVX-25; Ultraviolet Products, Inc., San Gabriel, California), and measured by a digital radiometer (UVX-Digital Radiometer; Ultraviolet Products, Inc., San Gabriel, California). Treated shell eggs were aseptically transferred to sterile carton trays and placed in the dark until analyzed. Contaminated, untreated shell eggs were used as controls. *Salmonella* spp. on shells of treated eggs were counted 5 min after UV treatments as described later.

Combination of treatments

Externally contaminated shell eggs were treated with UV radiation (254 nm; 1500-2500 μW/cm^2 intensity) at 22-25°C for 1 min. Immediately after UV radiation treatment, shell eggs were aseptically transferred to the ozone treatment vessel and gaseous ozone was applied at 5 psig (34 kPa) and 4-8°C for 1 min. Come up time required to achieve target treatment pressure was 30-40 sec. After treatments, pressure inside vessel was released in approximately 30 sec. Treated and control shell eggs were analyzed for *Salmonella* count as described later.

Enumeration of *Salmonella*

Ten untreated eggs were cracked, their shells were separated and weighed, and average shell weight was determined; average was 7 ± 1 g. Individual treated and control shell eggs were aseptically placed with tongs inside the upper part of a sterile 18 × 30 cm polyethylene stomacher bag (Fisherbrand®, Labplas, Inc., Quebec, Canada). Each egg was manually held from outside the bag, and cracked by carefully knocking on the bag with the blunt end of a knife blade. Egg contents (yolk and albumen) were recovered into stomacher bag. Eggshells, separated from egg contents, were aseptically recovered from upper part of stomacher bags, placed in blender jars, and used for enumeration of *Salmonella*. Sample preparation of eggshells was performed as previously described (Board *et al.*, 1964) with modifications. Briefly, shells of two eggs, which were treated under the same conditions, were aseptically placed into sterile 500-ml glass blender jar (Fisher Scientific). Chilled, sterile peptone water (0.1%, 126 ml)(Difco) was mixed with shells in a blender (Waring™, Model WPB05; Dynamics Corporation of America, New Hartford, CT) at high speed for 1 min. Homogenized shells were permitted to sediment for approximately 1 min. Serial dilutions in peptone water were made, and 1-ml aliquots were inoculated on plate count agar (PCA; Difco) using pour-plating technique. Plates were incubated at 37°C for 48 h and colonies were counted. Detection limit of the procedure was 10 CFU/g eggshell. Selected colonies were confirmed for *Salmonella* by streaking samples onto Xylose Lysine Desoxycholate agar (XLD; Difco). Plates were incubated at 37°C for 24 h and characteristic colonial morphology of *Salmonella* spp. was observed.

Statistical analyses

Experiments were performed in duplicate with four shell eggs per experimental condition. Statistical estimations included analyses of variance (ANOVA), and comparisons of means by Tukey with $\alpha = 0.05$. Data were analyzed in JMP IN® version 4.0.4 software (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Inactivation of *Salmonella* Enteritidis by ozone

Ozone treatment at atmospheric pressure (0 psig) for 3 min significantly ($p < 0.05$) reduced *Salmonella* Enteritidis on the surface of shell eggs by 3.1 $\log_{10}$ when compared to untreated control (Table 2.1). However, further ozone treatment for 5 and 8 min, resulted in significantly ($p < 0.05$) higher microbial counts when compared to the 3 min treatment. In spite of this trend, ozone treatments for 5 and 8 min significantly ($p < 0.05$) reduced the microorganism by 2.3 and 2.6 $\log_{10}$ respectively when compared to untreated control (Table 2.1). Significant ($p < 0.05$) apparent microbial reduction by 0.9-1.1 $\log_{10}$ was observed in eggs treated with air for 3-8 min with respect to untreated control (Table 2.1). Ozone treatment, in general, caused a rapid decrease in count in 3 min, but longer treatment time did not cause additional inactivation. This two-stage inactivation trend was previously observed when *Escherichia coli* O157:H7 was treated with aqueous ozone in a batch mode (Achen and Yousef, 2001). Koidis *et al.* (2000) reported a similar *Salmonella* Enteritidis inactivation pattern on surface-contaminated

shell eggs during treatment with aqueous ozone. An explanation for this inactivation pattern was provided by Hunt and Marinas (1999), who suggested that cellular debris from killed organisms could account for a greater ozone demand, therefore leaving less ozone available to inactivate the rest of the microbial population. Studies indicate that calcium carbonate, the main mineral component of the eggshell, is almost inert to ozone (Alebić-Juretić *et al.*, 2000). However, organic matter and proteins embedded in the eggshell matrix could consume ozone and reduce its availability for microbial inactivation (Koidis *et al.*, 2000).

Apparent microbial reduction was observed when eggshells were subjected to air (Table 2.1). Airflow may have helped bacteria to penetrate into pores and crevices, which made it difficulty to recover eggshell-embedded microorganisms by the sample preparation and counting technique used in this study (Board *et al.*, 1964; Berrang *et al.*, 1991). Contaminated untreated shell eggs kept at 22ºC for 8 min were used as additional controls; no significant ($p > 0.05$) difference in microbial counts was observed on these shell eggs when compared to initial counts at 0 min (data not shown).

A previous study indicated that use of pressure increased penetration and effectiveness of sanitizers through eggshells (Padron, 1995). In the present study, pressurized ozone was applied at low temperature to *Salmonella*-contaminated shell eggs. Treatments were performed in batch system with gaseous ozone at 15-psig (103 kPa). Application of pressurized ozone for 10 and 20 min resulted in inactivation of microorganism in a two-stage trend, similar to the one observed in previous experiment where ozone was applied at atmospheric pressure (Table 2.1 and Fig. 2.2). *Salmonella* Enteritidis was significantly ($p < 0.05$) reduced after 10 min treatment of shell eggs by ≥

4.5 and ≥ 5.9 $\log_{10}$ when compared to air-treated and untreated control, respectively (Fig. 2.2). However, further ozone treatment for 20 min did not significantly ($p > 0.05$) reduce the microorganism when compared to the 10-min treatment (Fig. 2.2). Nevertheless, treatment with pressurized ozone for 20 min significantly ($p < 0.05$) reduced microbial contamination on shell eggs by ≥ 3.7 and ≥ 5.7 $\log_{10}$ when compared to air-treated and untreated control, respectively (Fig. 2.2). Application of pressurized air resulted in an apparent microbial reduction by 1.4 and 2.0 $\log_{10}$ after 10 and 20 min, respectively, when compared to untreated controls (Fig. 2.2). This apparent microbial reduction may have resulted from increased penetration of cells into pores and crevices of eggshells, and the difficulty in recovering these embedded bacteria by the counting procedure (Berrang *et al.*, 1991). Nonetheless, ozone treatment under pressure at low temperature proved to be effective in reducing *Salmonella* Enteritidis on shell eggs by ≥ 4.5 $\log_{10}$ in 10 min regardless of air effect (Fig. 2.2). Padron (1995) observed that application of hydrogen peroxide under pressure increased effectiveness of this sanitizer against *Salmonella* Typhimurium on the surface of shell eggs. Conversely, pressure was unable to increase aqueous ozone effectiveness to decontaminate other food products (Sharma *et al.*, 2003). Previous studies suggest that microorganisms are inactivated by ozone because of its reaction with unsaturated lipids and lipopolysaccharides in the bacterial envelope of Gram-negative bacteria, leading to changes in cell permeability and subsequent lysis and death of microorganisms (Kim *et al.*, 2003). In addition, ozone may affect intracellular proteins and damage microbial genetic material (Komanapalli and Lau, 1996).

Limited effectiveness of ozone against *Salmonella* on shell eggs in previous studies (Koidis *et al.*, 2000) could be the result of its use in aqueous phase, and at low

concentrations. Moreover, it has been demonstrated that ozone stability is higher in gas than in aqueous phase (Kim *et al.*, 2003). In addition, ozone in gas phase is stable at low temperatures and high concentrations (Koike *et al.*, 1998). Sanitizers under pressure have enhanced antimicrobial efficacy on shell eggs (Padron, 1995). Therefore, use of gaseous ozone at high concentrations, low temperature, and mild pressure could result in higher number of ozone molecules available for microbial inactivation, and increased effectiveness when applied as a shell egg sanitizer as indicated by experiments in this study.

Inactivation of *Salmonella* Enteritidis by UV radiation

Treatment of *Salmonella* Enteritidis contaminated shell eggs with UV radiation (100 $\mu W/cm^2$) resulted in a rapid decline of bacterial population with short treatment time, followed by a stage of no additional inactivation (Table 2.2). This trend also was observed with gaseous ozone treatments (Table 2.1 and Fig. 2.2). Lack of efficacy with extended treatment may be caused by the limited penetrability of UV radiation in shells, and to the shielding effect of shell's porous surface that could limit direct exposure of bacteria inside eggshell pores to UV light (Kuo *et al.*, 1997a; Blatchley and Peel, 2001). Ultraviolet radiation treatments for 2 and 4 min significantly ($p < 0.05$) decreased *Salmonella* Enteritidis by 2.6 and 2.0 $\log_{10}$, respectively, when compared to untreated controls (Table 2.2). In another experiment, *Salmonella*-contaminated shell eggs were treated with higher UV radiation intensity (1500-2500 $\mu W\ cm^2$) for up to 5 min (Fig. 2.3). Microbial reduction followed a two-stage trend already observed in previous experiment (Table 2.2). Radiation treatments with UV for 1, 3, and 5 min resulted in

significant ($p < 0.05$) microbial reductions by 3.4, 3.0, and 4.3 $\log_{10}$ respectively when compared to untreated controls (Fig. 2.3). No significant ($p > 0.05$) difference was observed when *Salmonella* reductions after 1, 3, and 5 min were compared (Fig. 2.3). Despite possible shielding effects, *Salmonella* Enteritidis decreased ≤ 4.3 $\log_{10}$ on the shell surface in a short period of time (Fig. 2.3). A previous study also reported a 4.6-$\log_{10}$ inactivation of *Salmonella* on shell eggs after a treatment with UV having lower intensity than that used in the current study (Kuo *et al*., 1997a).

Ultraviolet radiation inactivate microorganisms by inducing formation of cross-linking between DNA pyrimidine nucleotide bases, that subsequently results in inhibition of DNA transcription and replication mechanisms, and leads eventually to death of cells (Blatchley and Peel, 2001). In addition, UV radiation affects cell membrane integrity, induces protein modifications, and inhibits oxidative phosphorilation (Kuo *et al.,* 1997a; Bintsis *et al.*, 2000). Ability of some microbial cells to recover after UV radiation by enzymatic repair mechanisms could be a possible limitation to the use of UV treatments in food products (Shama, 2000). However, Kuo *et al.* (1997a) observed that *Salmonella* Typhimurium treated by UV radiation on shell eggs did not recover after subsequent incubation under either dark or light conditions. Furthermore, Shama (2000) suggested that UV-resistant mutants have only been generated under laboratory conditions, and concerns about possible occurrence of such mutants when UV treatments are applied in commercial facilities may be unsubstantiated.

Salmonella Enteritidis inactivation by combination of treatments

Results in this study indicate that increased penetrability of gaseous ozone applied under mild pressure could effectively eliminate *Salmonella* Enteritidis on shell eggs (Fig. 2.2). In addition, treatment with UV radiation could inactivate shell egg external microbial contamination in a short period of time (Fig. 2.3). Despite these results, antimicrobial effectiveness of ozone applied alone may be tampered when used in foods with high organic content (Novak and Yuan, 2003). In addition, UV radiation, though effective against microorganisms, can not achieve product sterilization and its use alone is not recommended to sanitize foods (Juneja and Novak, 2003). Previous studies suggest that ozone applied in combination with other treatments has enhanced antimicrobial activity when compared to treatments where ozone is used alone (Diaz *et al.*, 2001; Unal *et al.*, 2001; Novak and Yuan, 2003). Synergism is defined as the capability of combined treatments to produce a greater antimicrobial effect than the sum of treatments applied individually (Shama, 2000). In the food industry, combination of treatments is becoming a popular preservation technique that is based on the ability of combined selected hurdles in food to produce an additive or synergistic effect against pathogens (Unal *et al.*, 2001). *Salmonella* Enteritidis-contaminated shell eggs were treated with UV radiation (254 nm; 1500-2500 $\mu W/cm^2$) for 1 min, followed by gaseous ozone at 5 psig (34 kPa) and low temperature for 1 min. Results indicated that population of *Salmonella* Enteritidis significantly ($p < 0.05$) decreased on shell eggs after treatment with UV radiation alone by 2.5 $\log_{10}$, compared to untreated control (Fig. 2.4). Decrease in *Salmonella* population on contaminated eggs with gaseous ozone under pressure was not significant ($p > 0.05$)(Fig. 2.4). However, treatment of shell eggs with UV radiation followed by gaseous

ozone under pressure decreased *Salmonella* count significantly ($p < 0.05$). *Salmonella* survivors decreased ≥ 4.6, ≥ 4.1, and ≥ 2.1 $\log_{10}$ by the combined treatment when compared to untreated control, ozone, and UV radiation treatment, respectively (Fig. 2.4). Moreover, microbial reduction after combination treatment was calculated from estimated plate counts with detection limit of 10 CFU/g eggshells. A previous study reported enhanced effectiveness when ozone, hydrogen peroxide, and UV radiation were applied simultaneously (Diaz *et al.*, 2001). Simultaneous application of these sanitizing factors may cause photolysis of ozone and hydrogen peroxide by UV radiation to generate hydroxyl radicals with the purpose of inactivating microorganisms in water, and not in solid foods (Diaz *et al.*, 2001; Kim *et al.*, 2003). In the present study, and according to the definition of synergism given previously, use of UV radiation, followed by treatment with gaseous ozone produced synergistic 1.6-$\log_{10}$ reduction of *Salmonella* Enteritidis on shell eggs (Fig. 2.4). Furthermore, inactivation of the microorganism by ≥ 4.6 $\log_{10}$ was achieved in approximately 2 min total treatment time, compared to the same degree of inactivation obtained in 5 and 10 min by UV radiation and gaseous ozone, respectively, when these treatments were applied separately (Fig. 2.2 and 2.3).

Using UV and ozone in sequence should have minimized the possibility of microbial reactivation that may happen when UV radiation is used alone (Shama, 2000). In addition, cells surviving sublethal UV radiation doses may synthesize stress proteins and become more resistant to subsequent lethal treatments (Bintsis *et al.*, 2000; Blatchley and Peel, 2001, Juneja and Novak, 2003). In order to prevent recovery and possible stress adaptation of microorganisms after UV radiation treatments, shell eggs were immediately treated with gaseous ozone. Increased penetrability of gaseous ozone under pressure

could inactivate microorganisms previously injured by UV radiation, and those shielded by porous surface of eggshells. Use of treatment combinations in a reverse order was not attempted in this study. However, Novak and Yuan (2003) observed that vegetative cells of *Clostridium perfringens* previously exposed to aqueous ozone on meat were highly susceptible to the effect of subsequent heat treatments.

Results in this study suggest that shell eggs externally contaminated with *Salmonella* Enteritidis could be effectively sanitized with gaseous ozone under mild pressure, UV radiation, and their combination. Furthermore, elimination of microorganism could be achieved at low temperature, in a short period of time, and under relatively dry conditions. Therefore, use of combined treatments including UV radiation and ozone to eliminate *Salmonella* Enteritidis on shell eggs should be considered for possible applications in the egg industry.

REFERENCES

Achen, M., and A.E. Yousef. 2001. Efficacy of ozone against *Escherichia coli* O157:H7 on apples. J. Food Sci. 66: 1380-1384.

Alebić-Juretić, A., T. Cvitaš, and L. Klasnic. 2000. Kinetics of heterogeneous ozone reactions. Chemosphere. 41: 667-670.

Bailey, J.S., R.J. Buhr, N.A. Cox, and M.E. Berrang. 1996. Effect of hatching cabinet sanitation treatments on *Salmonella* cross-contamination and hatchability of broiler eggs. Poult. Sci. 75: 191-196.

Berrang, M.E., N.A. Cox, J.S. Bailey, and L.C. Blankenship. 1991. Methods for inoculation and recovery of *Salmonella* from chicken eggs. Poult. Sci. 70: 2267-2270.

Bintsis, T., E. Litopoulou-Tzanetaki, and R.K. Robinson. 2000. Existing and potential applications of ultraviolet light in the food industry - a critical review. J. Sci. Food Agric. 80: 637-645.

Blatchley III, E.R., and M.M. Peel. 2001. Disinfection by ultraviolet irradiation, p. 823-851. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Board, R.G., J.C. Ayres, A.A. Kraft, and R.H. Forsythe. 1964. The microbial contamination of eggshells and egg packing materials. Poult. Sci. 43: 584-595.

Code of Federal Regulations (CFR). 2001. Secondary direct food additives permitted in food for human consumption. Vol. 66. No. 123. Office Federal Register, Washington, D.C.

Davies, R.H., and M. Breslin. 2003. Investigations into possible alternative decontamination methods for *Salmonella* Enteritidis on the surface of table eggs. J. Vet. Med. 50: 38-41.

Diaz, M.E., S.E. Law, and J.F. Frank 2001. Control of pathogenic microorganisms and turbidity in poultry-processing chiller water using UV-enhanced ozonation. Oz. Sci. Eng. 23: 53-64.

Dunn, J. 1996. Pulsed light and pulsed electric field for foods and eggs. Poult. Sci. 75: 1133-1136.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchell, and W.H. Andrews.1993. Growth of *Salmonella* Enteritidis in grade A eggs during prolonged storage. Poult. Sci. 72: 373-377.

Himathongkham, S., H. Riemann, and R. Ernst. 1999. Efficacy of disinfection of shell eggs externally contaminated with *Salmonella* Enteritidis. Implications for egg testing. Int. J. Food Microbiol. 49: 161-167.

Hunt, N.K., and B.J. Marinas. 1999. Inactivation of *Escherichia coli* with ozone: chemical and inactivation kinetics. Wat. Res. 33: 2633-2641.

Ito, H., H. Nakatani, H. Bamba, and T. Hayashi. 1999. Influence of the hatchability of Japanese quail eggs (hatching eggs) and sterilization effect for *Salmonella* by ozone gas sterilization. Res. Bull. Aichi Agric. Res. 31: 305-310.

JMP IN. 2001. JMP IN statistical software for Windows, 4.0.4. version. SAS Institute Inc., Cary, NC.

Juneja, V.K., and J.S. Novak. 2003. Adaptation of foodborne pathogens to stress from exposure to physical intervention strategies, p. 31-53. *In* A. E. Yousef and V. K. Juneja (ed.), Microbial stress adaptation and food safety, CRC Press, Boca Raton, FL.

Kim, J.-G., A.E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Prot. 62: 1071-1087.

Kim, J.-G., A.E. Yousef, and M.A. Khadre. 2003. Ozone and its current and future application in the food industry. Adv. Food Nutr. Res. 45: 167-218.

Knape, K.D., J.B. Carey, R.P. Burgess, Y.M. Kwon, and S.C. Ricke. 1999. Comparison of chlorine with an iodine-based compound on eggshell surface microbial populations in a commercial egg washer. J. Food Safety. 19: 185-194.

Koidis, P., M. Bori, and K. Vareltzis. 2000. Efficacy of ozone treatment to eliminate *Salmonella* Enteritidis from eggshell surface. Arch. Lebensmittelhyg. 51: 4-6.

Koike, K., G. Inoue, M. Suzuki, J. Saida, and I. Komatsu. 1998. Decomposition characteristics of concentrated ozone. J. Chem. Eng. Jap.31: 195-200.

Komanapalli, I.R., and B.H.S. Lau. 1996. Ozone-induced damage of *Escherichia coli* K-12. Appl. Microbiol. Biotechnol. 46: 610-614.

Kuo, F.L., J.B. Carey, and S.C. Ricke. 1997a. UV irradiation of shell eggs: effect on populations of aerobes, molds, and inoculated *Salmonella* Typhimurium. J. Food Prot. 60: 639-643.

Latala, A. and Z. Dobrzanski. 1989. Effect of ultraviolet radiation on the microbiological contamination of the shells of eggs for human consumption. Med. Wet. 45: 420-422.

Novak, J. S., and J. T. C. Yuan. 2003. Viability of *Clostridium perfringens*, *Escherichia coli*, and *Listeria monocytogenes* surviving mild heat or aqueous ozone treatment on beef followed by heat, alkali, or salt stress. J. Food Prot. 66: 382-389.

Padron, M. 1995. Egg dipping in hydrogen peroxide solution to eliminate *Salmonella* Typhimurium from eggshell membranes. Avian Dis. 39: 627-630.

Russell, S. M. 2003. The effect of electrolyzed oxidative water applied using electrostatic spraying on pathogenic and indicator bacteria on the surface of eggs. Poult. Sci. 82: 158-162.

Shama, G. 2000. Ultraviolet light. p. 2208-2214. *In* R.K. Robinson, C. Batt, and P. Patel (ed.) Encyclopedia of Food Microbiology. Academic Press, London.

Sharma, R. R., A. Demirici, L. R. Beuchat, and W. F. Fett. 2003. Application of ozone for inactivation of *Escherichia coli* O157:H7 on inoculated alfalfa sprouts. J. Food Process. Preserv. 27: 51-64.

Unal, R., J-G. Kim, and A.E. Yousef. 2001. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulse electric field. J. Food Prot. 64: 777-782.

Whistler, P.E., and B.W. Sheldon. 1989. Bactericidal activity, eggshell conductance, and hatchability effects of ozone versus formaldehyde disinfection. Poult. Sci**.** 68:1074-1077.

Treatments[a]	Treatment time (min)	*Salmonella* Enteritidis (log_{10} CFU/g eggshell)[b]	Log_{10} reduction
Control	0	6.3 ± 0.2A[c]	-
Air	3	5.2 ± 0.1B	1.1
O_3		3.2 ± 0.3C	3.1
Air	5	5.3 ± 0.1B	1.0
O_3		4.0 ± 0.1D	2.3
Air	8	5.4 ± 0.1B	0.9
O_3		3.7 ± 0.1D	2.6

[a] Control, contaminated untreated shell eggs; Air, air treatment at atmospheric pressure (0 psig); O_3, ozone treatment with 12-14 % wt/wt O_3 in O_2 mix.

[b] Mean ± S.D. obtained in experiments performed in duplicate with four eggs per experimental condition.

[c] Means within columns not followed by the same letter are significantly different ($p < 0.05$).

Table 2.1: Inactivation of *Salmonella* Enteritidis on shell eggs by gaseous ozone at atmospheric pressure (0 psig) and ambient 4-8ºC.

Treatments[a]	Treatment time (min)	*Salmonella* Enteritidis (log_{10} CFU/g egg shell)[b]	Log_{10} reduction
Control	0	5.8 ± 0.1A[c]	-
UV	2	3.2 ± 0.1B	2.6
	4	3.8 ± 0.1B	2.0

[a] Control, contaminated untreated shell eggs; UV, ultraviolet radiation treatment (254 nm) at 100 μW/cm^2.

[b] Mean ± S.D. obtained in experiments performed in duplicate with four shell eggs per experimental condition.

[c] Means within columns not followed by the same letter are significantly different ($p < 0.05$).

Table 2.2: Inactivation of *Salmonella* Enteritidis on shell eggs by ultraviolet radiation at 100 μW/cm^2.

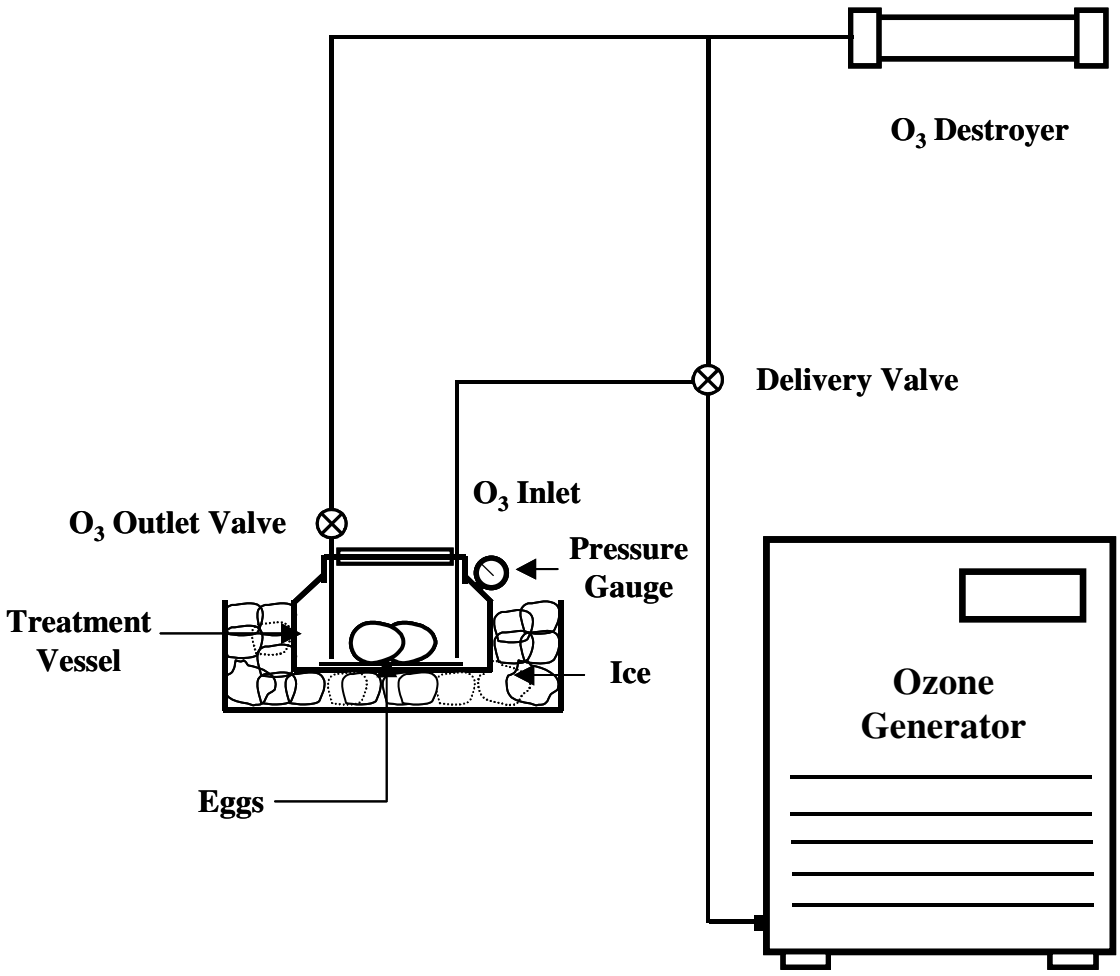

Figure 2.1: Experimental setup to treat shell eggs with gaseous ozone under pressure at low ambient temperature.

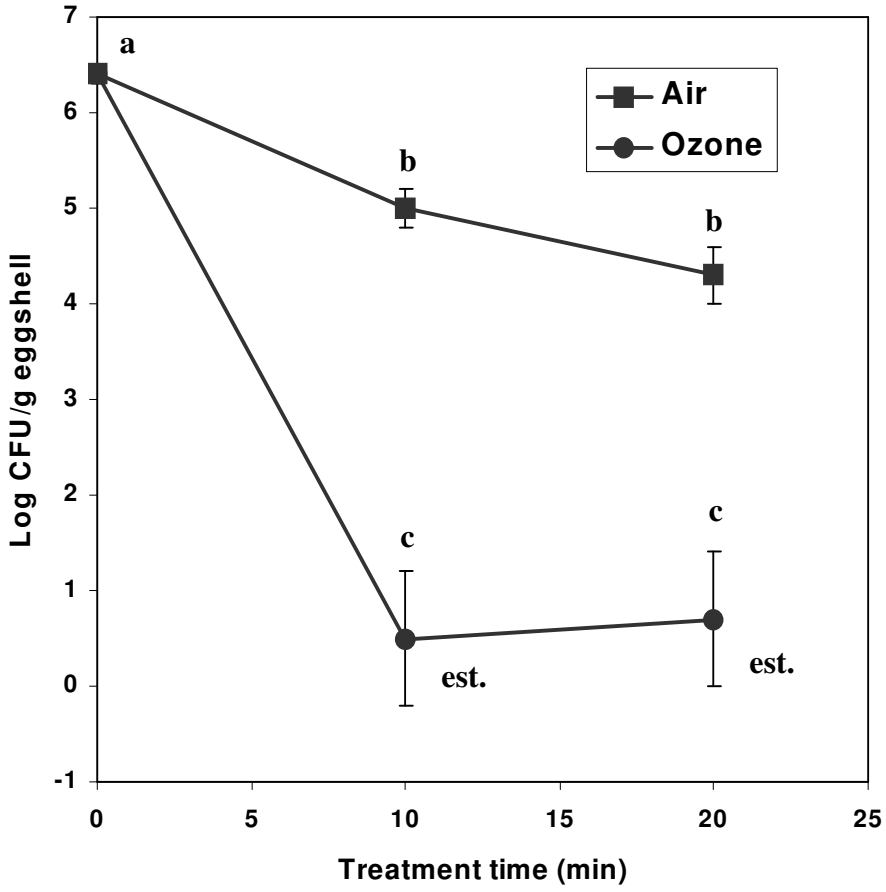

Figure 2.2: Inactivation of *Salmonella* Enteritidis on shell eggs by gaseous ozone (12-14% wt/wt) under 15 psig (103 kPa). Come up time to achieve target pressure in treatment vessel at 4-8°C was 2 min. Points represent the mean of microbial counts in experiments performed in duplicate with four shell eggs per experimental condition. Error bars indicate standard deviation. Different letters indicate significant ($p < 0.05$) difference among treatments. Estimated counts (est.) were obtained in plate count with a detection limit of 10 CFU/g eggshell

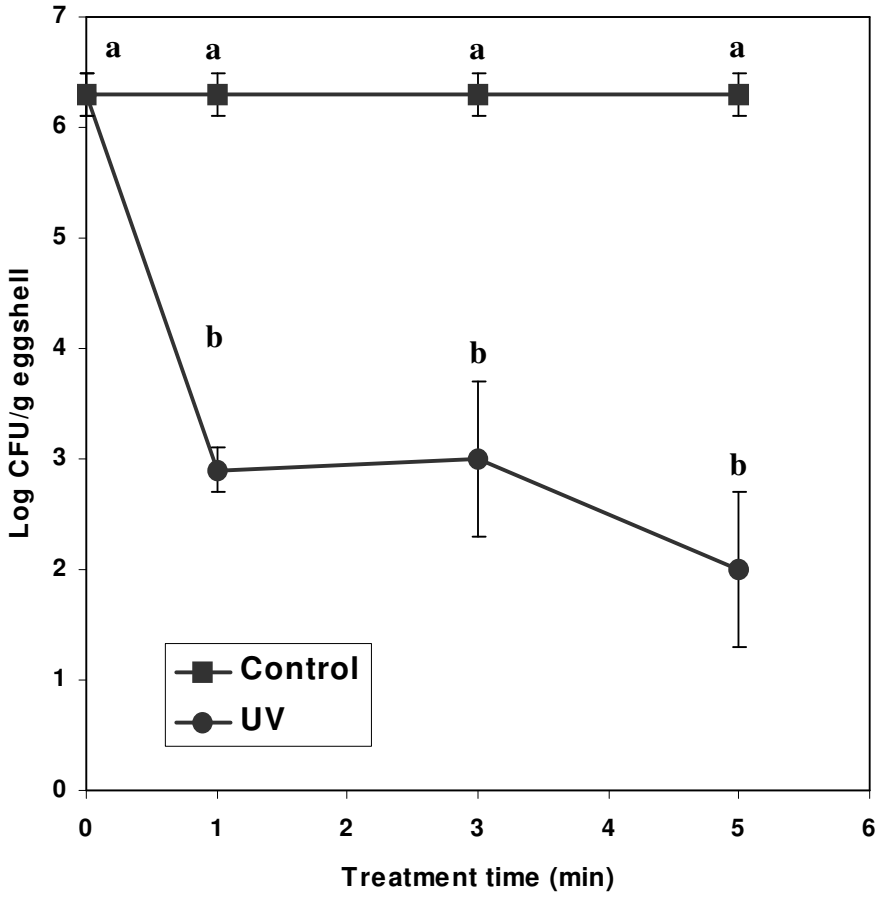

Figure 2.3: Inactivation of *Salmonella* Enteritidis on shell eggs by ultraviolet radiation (254 nm; 1500-2500 μW/cm^2) at 22-25°C. Points represent the mean of microbial counts in experiments performed in duplicate with four shell eggs per experimental condition. Error bars indicate standard deviation. Different letters indicate significant ($p < 0.05$) difference among treatments.

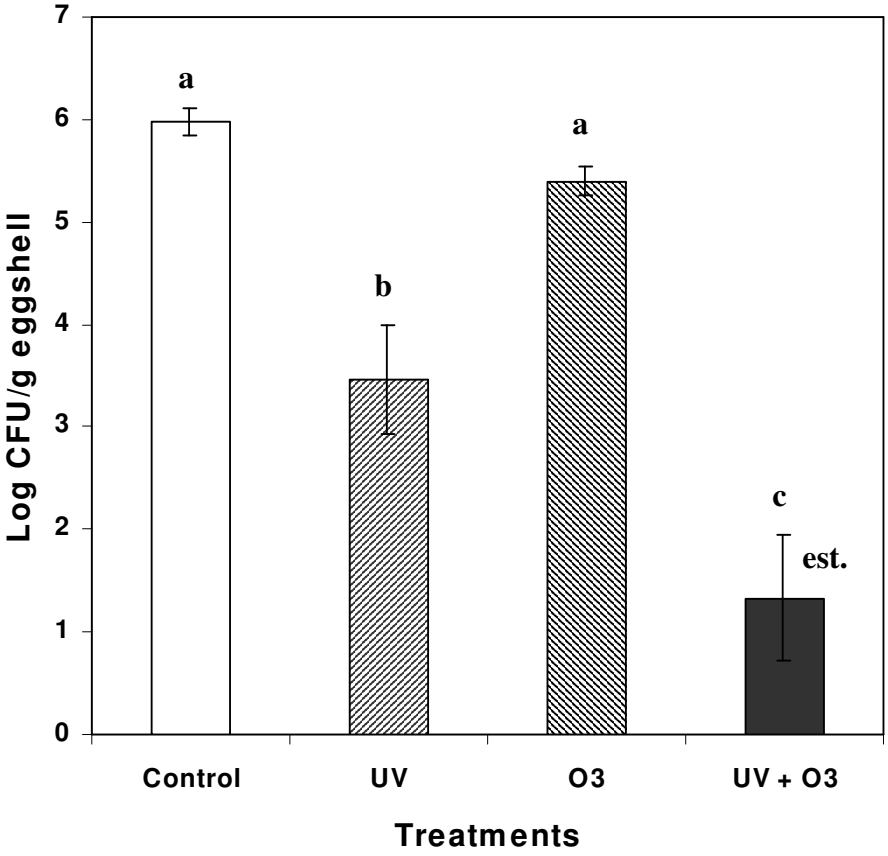

Figure 2.4: Inactivation of *Salmonella* Enteritidis on shell eggs by combination of UV radiation (254 nm; 1500-2500 μW/cm^2) at 22-25°C for 1 min, followed by gaseous ozone treatment in vessel at 4-8°C and 5 psig (34 kPa) for 1 min (plus 30-40 sec come up time). Bars represent mean of microbial counts in experiments performed in duplicate with four shell eggs per experimental condition. Error bars indicate standard deviation. Different letters indicate significant ($p < 0.05$) difference among treatments. Estimated counts (est.) were obtained in plate count with detection limit of 10 CFU/g eggshell.

CHAPTER 3

EVALUATION OF A PASTEURIZATION PROCEDURE TO INACTIVATE *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS IN SHELL EGGS, AND IMPACT OF THERMAL TREATMENTS ON EGG QUALITY

ABTRACT

Thermal treatments effectively inactivate *Salmonella* Enteritidis inside shell eggs. However, heat sufficient to eliminate the microorganism affects egg appearance and quality. In the present study, an inoculation protocol was developed to simulate natural microbial contamination onto or around the vitelline membrane of shell eggs, and thermal inactivation of *Salmonella* Enteritidis in contaminated shell eggs was studied. A total of 105 contaminated shell eggs (1.6-4.0 $\times$ 10^6 CFU/g egg) were heated by immersion in water at 57, 58, and 59°C for up to 40 min. Resulting microbial inactivation curves had sigmoid patterns with shoulders and tails. *Salmonella* population decreased 4.8, 5.1, and 5.4 $\log_{10}$ during 30-40 min of heating at 57, 58, and 59°C, respectively. Albumen quality, measured as Haugh units, increased significantly ($p < 0.05$). Albumen turbidity significantly ($p < 0.05$) increased in eggs treated with 58 and 59°C, and was less affected at 57°C. Albumen analysis by differential scanning calorimetry (DSC) indicated a

change in the pattern of protein denaturation after treatment at 59°C for 40 min. Treatment necessary to inactivate ≥ 5 $\log_{10}$ *Salmonella* increased albumen turbidity or protein denaturation.

INTRODUCTION

Salmonella Enteritidis is a foodborne pathogen responsible for salmonellosis, which has a considerable economic impact on the poultry and egg industry. It is estimated that 1.4 million cases of foodborne salmonellosis occur annually in the United States, and a high proportion is associated to consumption of *Salmonella*-contaminated egg or egg products (Buzby, 2002). Public health concerns regarding contamination of fresh eggs with *Salmonella* Enteritidis prompted USDA approval of a thermal process to eliminate this microorganism in shell eggs (USDA, 1997). Pasteurization of eggs (in-shell pasteurization) is a commercially available process, which consists of extended heating of shell eggs by immersion in water baths at 55-60ºC or by hot air in convection ovens (Schuman *et al.*, 1997; Zeidler, 2001a; Brackett *et al.*, 2001). Thermal inactivation processes decrease ≥ 5 $\log_{10}$ *Salmonella* Enteritidis, inoculated inside the yolk (Stadelman *et al.*, 1996; Schuman *et al.*, 1997). Although effective, extensive heating during in-shell pasteurization processes causes albumen turbidity due to protein denaturation (Hou *et al.*, 1996; Schuman *et al.*, 1997).

In-shell pasteurization procedures have been developed on the assumption that *Salmonella* Enteritidis naturally resides inside the yolk, and extensive heat

treatments must target the whole egg in order to transfer appropriate thermal energy to the center of the product to effectively inactivate the microorganism (Stadelman *et al.*, 1996; Schuman *et al.*, 1997; Brackett *et al.*, 2001). Previous reports indicated that *Salmonella* Enteritidis could be isolated from inside the yolk of naturally contaminated shell eggs, *i.e.*, by transovarian route (Timoney *et al.*, 1989, Shivaprasad *et al.*, 1990). However, more recent studies provided evidence that naturally contaminated shell eggs contain the microorganism on the vitelline membrane or in the albumen surrounding the yolk, and not inside yolk contents (Gast and Beard, 1990; Humphrey *et al.*, 1991). The assumption that *Salmonella* Enteritidis resides inside the yolk may result in overprocessing of outer egg parts during treatments, with consequential increase in protein denaturation and negative impact on albumen clarity and functionality (Fleischman *et al.*, 2003).

Targeting *Salmonella* on the vitelline membrane should produce safe eggs without quality deterioration. Therefore, this study investigates the thermal inactivation of *Salmonella* Enteritidis inside shell eggs by heat, after an inoculation procedure that simulates natural microbial contamination in shell eggs. Thermal treatments were performed at 57-59°C; a range of temperature previously reported for inactivation of *Salmonella* in eggs (Lith *et al.*, 1995; Schuman *et al.*, 1997). In addition, effect of immersion heat treatments on albumen quality was evaluated. Minimum heat treatment sufficient to reduce the risk of salmonellosis without sacrificing egg quality will be sought.

MATERIALS AND METHODS

Shell eggs

Fresh, unfertilized hen shell eggs (63 ± 2 g/egg) were provided by Ohio Poultry Association (Columbus, OH). Shell eggs were kept at 4ºC and used within 2 weeks of laying. Prior to every experiment, the breadth of selected refrigerated shell eggs was measured with a Vernier caliper (Manostat Co., Switzerland). Selected shell eggs with 4.4-4.5 cm breadth were transferred to clean carton trays and then held at 22-25ºC for ~ 2 h. Individual shell eggs were washed with tap water and gently scrubbed with a plastic brush. Clean eggs were submerged in ethanol (70% vol/vol) for 30 min as described previously (Hammack, *et al.*, 1993). Sanitized shell eggs were immediately transferred to sterile carton trays and permitted to dry at 22-25°C for 40 min before inoculation with *Salmonella*.

Bacterial culture and growth conditions

Salmonella Enteritidis, isolated from eggs, was kindly provided by Ohio Department of Agriculture (Reynoldsburg, OH). Stock cultures were transferred to brain heart infusion (BHI) broth (Difco; Becton, Dickinson and Co. Sparks, MD), and incubated at 37°C for 24 h. Grown cultures were subsequently transferred in duplicate to 250-ml flasks (Pyrex, Corning Inc., Acton, MA) containing 150 ml of fresh MacConkey broth (Difco); inoculation level was 0.1%. Transferred cultures were incubated at 37°C for 24 h in orbital shaker (G-24, New Brunswick Scientific Co. Inc., Edison, NJ)

under mild agitation. Aliquots of *Salmonella* Enteritidis grown cultures were centrifuged (Sorval RC-5B, Dupont Instruments, Bannorbuck, IL) at 3050 × g for 10 min. Cell pellets were resuspended in 3.2 ml sterile chilled phosphate buffer (0.1 M, pH 7). Concentrated cell suspension of *Salmonella* Enteritidis contained $1.7 \times 10^9 – 2.3 \times 10^{10}$ CFU/ml.

Growth of *Salmonella* in albumen and yolk

Preliminary experiments were performed in order to establish the most suitable *Salmonella* inoculum placement site, and to determine effect of egg components on microbial growth. Breadth and length of ten shell eggs were measured with a Vernier caliper (Manostat); average breadth and length were 4.5 and 6.0 cm respectively. Two inoculation sites were independently tested in preliminary inoculation trials. In a first inoculation site trial, eggshells of individual sanitized eggs were carefully drilled in the approximate center of pointed end of shell eggs (opposite to the air cell) with 2.54-cm/18-gauge sterile needle coupled to 1-ml disposable syringe (Becton Dickinson & Co., Franklin Lakes, NJ). Inoculum placement was standardized beforehand using tracer dye as previously described (Chantarapanont *et al.*, 2000). *Salmonella* Enteritidis cell suspension (100 µl) prepared as described earlier was inoculated into yolk using a sterile high-precision chromatography syringe with fixed needle (100 µl; Model 710, Hamilton, Co., Reno, NV). Depth of inoculation was controlled with sterile rubber stopper previously coupled to the needle and located 2.5 cm from needle tip. In a second inoculation site trial, eggshells of individual shell eggs were perforated as described earlier. *Salmonella* Enteritidis cell suspension (100 µl) was inoculated into

albumen of individual shell eggs by adjusting the rubber stopper distance to 0.8 cm from needle tip. After inoculation, drilled perforations on eggshells of individual eggs were covered with droplet of commercial glue (Instant Krazy Glue®, Elmer's Products Inc., Columbus, OH); glue was permitted to dry for approximately 5 min. Experiments were performed with three shell eggs per inoculation site trial. Sanitized shell eggs without microbial inoculation were used as controls. Final concentration of *Samonella* Enteritidis inside shell eggs was ~ 10^7 CFU/g egg for albumen and yolk inoculation procedures. Internally contaminated shell eggs were immediately placed on sterile carton trays and incubated at 37°C for 72 h. After incubation, contaminated and control eggs were analyzed for *Salmonella* survivors as described later.

Inoculation protocol

Inoculation procedure included placing the inoculum onto the vitelline membrane or in albumen surrounding the yolk to simulate natural transovarian (*i.e.*, vertical) contamination. Eggshells of individual clean eggs were carefully perforated with sterile needle in the approximate center of egg's blunt end. In preliminary experiments, 10 µl crystal violet solution 0.4%; (J.T. Baker Chemical Co., Phillipsburg, NJ), were injected inside shell eggs using 25-µl high-precision chromatography syringe with fixed needle (Model 702, Hamilton Co.). Needle was previously coupled to rubber stopper located at predetermined distance from needle tip. Injection of dye in shell eggs was monitored in egg candler (Model 8-200, Virglas, Virtis Research Equipment, Gardiner, NY). After injection of dye, shell eggs were carefully placed on clean carton trays. Shell eggs were

carefully cracked on the rim of clean glass beaker and whole egg contents were placed on disposable polystyrene weighing dishes (Fisher Scientific, Pittsburgh, PA). Eggs were visually examined and consistency of dye placement was observed in experiments performed at least ten times. Additional experiments to confirm dye placement inside shell eggs consisted on hard-boiling of eggs and visual inspection of tracer dye in cooked eggs as described previously (Chantarapanont *et al.*, 2000). These trials showed that inserting the needle 5-6 mm into the egg allowed placement of dye in close proximity to the yolk. Optimized inoculation protocol was tried with cell suspension of *Salmonella* Enteritidis prepared as described before. Aliquots of cell suspension (10 µl) were aseptically inoculated in shell eggs using the technique previously performed for injection of dye. Immediately after inoculation, drilled perforations on eggshells were covered with approximately 1 cm^2 of polytetrafluoroethylene (PTFE) films with silicone adhesive backing (Cole-Parmer Instrument Co., Vernon Hills, IL). Contaminated shell eggs were held at 22-25°C for approximately 10 min before heat inactivation trials.

Inactivation of *Salmonella* Enteritidis in shell eggs by heat

Heat treatments of *Salmonella*-contaminated eggs were performed in water bath with a circulating pump (Model 260; Precision Scientific, Inc., Chicago, IL). Water bath was previously calibrated to attain 57, 58, or 59ºC with ± 0.2ºC variations. In this study, we refer to ambient temperature as the temperature of the water bath during treatments, so that it will not be confused with egg's internal temperature. Sets of five internally contaminated shell eggs per experimental condition were placed in aluminum baskets (15

× 15 × 15 cm; Fisher Scientific), and submerged in distilled deionized water at ambient 57, 58, and 59°C for up to 40 min. During heat treatments, level of water above shell eggs was ~ 4 cm. Contaminated and non-contaminated untreated shell eggs were used as controls in all experiments. Eggs were taken from water bath at predetermined intervals (5-10 min). Sampled shell eggs were cooled in 2-liter glass beakers, containing 1.5 liters of sterile deionized distilled water at 22-25°C, for 10 min. Individual cooled eggs were gently dry wiped with clean paper tissue (Kimwipes, Kimberly-Clark, Roswell, GA). *Salmonella* in treated eggs was enumerated as described later. Internal temperature of non-contaminated shell eggs was measured in separate experiments during heating as follows. Eggshells of individual eggs were carefully perforated in the approximate center of egg's blunt end as described previously. Flexible wire thermocouples (K-type; Cole-Parmer Instrument Co.), previously attached to rubber stopper, were inserted in shell eggs at same depth of optimized inoculation site. Thermocouples positioned inside shell eggs were fixed on eggshell surface of individual eggs with a commercial glue (Instant Krazy Glue) applied between rubber stopper and eggshell. Glue was permitted to dry for approximately 5 min. Shell eggs were treated at ambient 57-59°C for up to 40 min, and internal egg temperature during heat treatments was monitored using a digital thermometer (± 0.1% + 0.7°C accuracy)(Fluke Model 51II, Fluke Co., Everett, WA). Internal egg temperature measurements were performed in triplicate trials per experimental condition.

Enumeration of *Salmonella*

Ten shell eggs were broken and their contents were weighed to determine their average weight; average was 52 ± 2 g. Treated or control eggs were aseptically placed with metallic tongs in a sterile 18 × 30 cm polyethylene stomacher bag (Fisherbrand®, Labplas, Inc., Quebec, Canada). Shell eggs were carefully broken from outside of stomacher bag with the blunt end of a clean knife's blade. Egg contents (albumen and yolk) were recovered into stomacher bags and eggshells were discarded as a biohazard waste. Chilled peptone water (0.1%, 477 ml) was aseptically poured into stomacher bags and the mixture was homogenized in a stomacher (Stomacher lab-blender 400, Cooke Laboratory Products, VA.) for 1 min. Serial dilutions of homogenate were immediately prepared in peptone water, 1 ml of diluted samples was plated onto plate count agar (PCA; Difco). Aliquots of least dilute sample, consisting of 0.4, 0.3 and 0.3 ml (1 ml total) were inoculated on three different plates. Inoculated plates were incubated at 37°C for 48 h, colonies were counted, and selected colonies were confirmed as *Salmonella* by streaking onto xylose lysine desoxycholate agar (XLD; Difco). Inoculated XLD plates were incubated at 37°C for 24 h and characteristic colonial morphology of *Salmonella* spp. was observed. Detection limit of microorganism in plate counts was 10 CFU/g egg contents.

Albumen quality evaluation

Sanitized uninoculated shell eggs were heat treated in water bath at ambient 57, 58, and 59° for up to 40 min as previously described. Selected eggs were taken after

different heating intervals, and immediately cooled in sterile deionized distilled water at 22ºC for 10 min. Cooled shell eggs were gently dry wiped with clean paper tissue. Treated and control shell eggs were transferred to clean carton trays and additionally permitted to cool at 22-25°C for 2 h before egg quality measurements. All experiments were performed in triplicate with appropriate controls.

Haugh units. Eggs treated as described before were carefully broken, and contents were placed on clean plastic surface where Haugh units of individual eggs were measured with calibrated micrometer (Haugh Meter™, Mattox & Moore, Inc., Indianapolis, IN) as previously described (Haugh, 1937). Measurements were performed in triplicate per experimental condition with appropriate controls.

Turbidity and thermal protein denaturation. Shell eggs were carefully broken, contents were divided with plastic egg separator, and eggshells and yolks were discarded. Albumen of individual eggs was recovered and placed on disposable polystyrene weighing dishes (Fisher Scientific). Albumen samples were taken for turbidity measurement and protein denaturation trials. Aliquots (1 ml), containing thick and thin albumen, were transferred to plastic disposable cuvets (Fisher Scientific). Turbidity was measured at 600 nm in spectrophotometer (Spectronic 1202, Milton Roy Co., Rochester, NY). Measurements were performed in triplicate using distilled water as a reference (Shimada and Matsushita, 1980). Selected thin albumen samples were analyzed for thermal protein denaturation using differential scanning calorimetry (DSC) in modulated calorimeter (Model 2920, MDSC® modulated differential scanning calorimeter, TA Instruments, New Castle, DE) as described previously (Serrano *et al.*, 1997) with slight

modifications. Briefly, aliquots of albumen (10 mg) from treated and control shell eggs were weighed on DSC aluminum pans (PerkinElmer®, PerkinElmer Life and Analytical Sciences Inc., Boston, MA). Sample and empty reference pans were hermetically sealed in encapsulation press (TA Instruments). Sealed pans were placed inside chamber of DSC device. Calorimeter was connected to an IBM personal computer with capability for temperature control and data acquisition. Samples were heated from 30 to 100°C with 5°C temperature increase per minute. Thermograms of albumen samples were obtained and analyzed by a commercial software (Universal Analysis 2000, Thermal Advantage™, TA Instruments, New Castle, DE).

Statistical Analyses

Albumen quality measurements, in triplicate trials, were evaluated using analysis of variance (ANOVA), and Tukey's multiple comparison of means ($\alpha = 0.05$). Data analyses were performed in JMP IN®, version 4.0.4, software (SAS Institute, 2001, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Inoculation protocol

Preliminary experiments were performed in order to determine effect of *Salmonella* Enteritidis inoculum position within shell eggs on growth of the microorganism. Population of *Salmonella* was 3.4 $\log_{10}$ greater in yolk than in albumen

after incubation of contaminated eggs for 72 h at 37°C (Fig. 3.1). Results indicated that albumen environment decreased *Salmonella* population 1.8 $\log_{10}$ after 72 h at 37°C (Fig. 3.1). Conversely, *Salmonella* Enteritidis inoculated into yolk grew 1.6 $\log_{10}$ during egg incubation (Fig. 3.1). Microbial reduction could be attributable to albumen's high viscosity, alkaline pH, and antimicrobial proteins, e.g., ovotransferrin, lysozyme, and avidin (Li-Chan *et al.*, 1995). In addition, restricted iron availability in albumen could limit *Salmonella* Enteritidis growth (Humphrey, 1999). Inoculum size and temperature are key conditions for steady or delayed microbial growth in albumen (Humphrey, 1999; Cogan *et al.*, 2001). Limited or no growth of *Salmonella* Enteritidis was observed in artificially inoculated albumen (Humphrey *et al.*, 1991; Fleischman *et al.*, 2003). On the contrary, *Salmonella* Enteritidis grows rapidly in yolk contents regardless of microbial inoculum size, age of eggs, or absence of oxygen (Humphrey *et al.*, 1991; 1999; Fleischman, 2003).

In order to determine thermal inactivation of *Salmonella* Enteritidis in shell eggs, development of an optimized inoculation protocol (*in ovo* inoculation) was required for this study. Previous investigations described procedures to inoculate *Salmonella* into yolk prior to microbial thermal inactivation experiments (Lith *et al.*, 1995; Hou, *et al.*, 1996; Schuman *et al.*, 1997; Chantarapanont *et al.*, 2000; Brackett *et al.*, 2001). *Salmonella* could contaminate yolk in minor proportions by transovarian route (Timoney *et al.*, 1989, Shivaprasad *et al.*, 1990). Inoculation into yolk is inconsistent with more recent studies, which suggest that transovarian transmission of *Salmonella* to shell eggs occurs mainly onto the vitelline membrane or in the albumen around the yolk (Gast and Beard, 1990;

Humphrey *et al.*, 1991). Process validation based on microbial inactivation inside the yolk, could result in overprocessing of external part of shell eggs with resulting detrimental effect on albumen quality (Schuman *et al.*, 1997; Fleischman, *et al.*, 2003). Preliminary experiments, in this study, confirmed that inoculum placement into albumen and yolk resulted in significant ($p < 0.05$) microbial inhibition and growth, respectively (Fig. 3.1). Therefore, proper simulation of natural transovarian contamination necessitates development of optimized inoculation procedure for validation of thermal inactivation methods. Inoculum placement was tested using tracer dye and inoculum volume was reduced to 10 µl. Additionally, consistency of dye placement without drift was confirmed in dye-spiked eggs after hard-boiling as described by Chantarapanont *et al.* (2000). Furthermore, trials, performed at least 15 times, using the new inoculation procedure gave consistent recovery of inoculated *Salmonella* ($1.6\text{-}4.0 \times 10^6$ CFU/g egg).

Thermal inactivation of *Salmonella* Enteritidis in shell eggs

Treatment of inoculated shell eggs at ambient 57, 58, and 59°C resulted in gradual reduction of *Salmonella* Enteritidis as internal egg temperature increased (Fig. 3.2-3.4). Survivor curves showed sigmoid patterns with three distinct phases; shoulder (lag), rapid inactivation, and tailing. Shoulder observed in survivor curves occurred during the first 10 min of treatment at ambient 57 and 58°C, and during first 5 min of heating at ambient 59°C. During the shoulder phase, internal egg temperature increased from initial average of 23°C to 53.5, 55.2, and 49.3°C while heating at ambient 57, 58, and 59°C, respectively. As treatment time progressed, internal temperature of eggs treated at 59°C

was similar to that in eggs treated at 58°C. Previous studies have reported delayed inactivation of *Salmonella* Enteritidis inside shell eggs during immersion heat treatments (Hou *et al.*, 1996; Schuman *et al.*, 1997). Since treating *Salmonella* at > 50°C usually inactivates the pathogen, observed lag phase in microbial reduction is most likely caused by inefficient heat transfer during the early phase of immersion treatments (Stadelman *et al.*, 1996). Further heating of shell eggs produced gradual increase in internal egg temperature. Final internal temperatures for eggs treated at ambient 57, 58 and 59°C were 56.8, 58.2, and 59°C, respectively (Fig. 3.2-3.4).

Second phase of microbial inactivation coincided with internal temperature approaching its maximum value. Maximum *Salmonella* Enteritidis reduction by 4.8, 5.1, and 5.4 $\log_{10}$ was observed after 30 min heating at ambient 57, 58, and 59°C, respectively (Fig. 3.2-3.4).

Efficacy of thermal treatments is usually based on calculation of decimal reduction time (D-value). Calculation of D-value, however, implies that microbial inactivation follows a linear pattern under constant temperature conditions. Thermal inactivation of *Salmonella*, in this study, was not linear and internal egg temperature during the treatment was not constant. Therefore, calculation of D-value was unfeasible. Curvilinear inactivation pattern of *Salmonella* spp. was observed previously (Juneja *et al.*, 2001). Conversely, other studies using heat treatments to inactivate *Salmonella* Enteritidis in shell eggs have reported linear inactivation patterns (Schuman *et al.*, 1997; Brackett *et al.*, 2001). Schuman *et al.* (1997) reported apparent D-values of 6.0 and 4.5 min for *Salmonella* Enteritidis at 57 and 58°C, when microorganism was placed in

the yolk of eggs heated by immersion at ambient 57° and 58°C respectively. Brackett *et al.* (2001) performed two trials for inactivation of *Salmonella* Enteritidis inoculated in yolk of eggs treated with hot air, and reported D-values of 5.5-6.1 min in eggs heated at ambient 57.2°C. Nonetheless, D-values reported by these researchers were calculated with temperature transitions within shell eggs, or without reaching temperature of heating environment at site of inoculum placement. Other studies have addressed inactivation of *Salmonella* Enteritidis suspended in yolk medium, under constant temperature conditions using glass or capillary tubes (Humphrey *et al.*, 1990; Michalski *et al.*, 1999). Among these few studies, Humphrey *et al.* (1990) reported D-values for yolk suspended *Salmonella* Enteritidis of 21 and 1.1 min at 55 and 60°C, respectively. However, Michalski *et al.* (1999) calculated D-values for the microorganism in the same medium of 10.4, 1.5, and 0.27 min at 55, 58, and 61°C respectively. Therefore, considering the differences used in experimental conditions, it seems unfeasible to compare D-values for *Salmonella* Enteritidis, obtained under constant temperature to those obtained during variable internal temperature of shell eggs. Thompson *et al.* (1979) observed that *Salmonella* spp. inactivated by treatments at gradual rising temperatures from 25 to 60°C showed higher D-values than those treated at constant temperature (50°C). In the present study, extended heating of shell eggs at ambient 57, 58, and 59°C resulted in equilibrium between ambient and internal egg temperature after 30 min treatment (Fig. 3.3-3.5). During this time, *Salmonella* Enteritidis survivors were near the detection limit of the enumeration procedure (Fig. 3.3-3.5). Persistence of *Salmonella* in egg after prolonged heating (tailing effect) could be explained by clumping, protective effect of dead cells,

microbial debris produced after cell destruction, and localized sites with low water activity among many other conditions (Pflug *et al.*, 2001).

Albumen quality and thermal protein denaturation

Albumen quality measurements, including Haugh units and turbidity, were performed in sixty uninoculated shell eggs after heat treatments at ambient 57, 58, and 59°C (Table 3.1 and Fig. 3.5). The Haugh unit is an egg quality determination, which describes the relationship between egg weight and height of thick albumen (Stadelman, 1995). The present study showed that treatment of shell eggs at ambient 57, 58, and 59°C significantly ($p < 0.05$) increased Haugh units from 72, in untreated control, to 84.1 after 40 min treatment, regardless of temperature (Table 3.1). Significant ($p < 0.05$) increase in Haugh units was first observed after 10 min treatment in shell eggs heated at the three ambient temperatures when compared to untreated control (Table 3.1). This increase in Haugh units occurred when temperatures inside shell eggs reached ~ 55°C (Fig. 3.2-3.4). Treatment of shell eggs for > 10 min did not result in additional significant increase in Haugh units (Table 3.1). A previous study reported increase in Haugh units from 59.7 to 78.3 and from 66.8 to 80.7 in shell eggs heated by immersion for 35 min at ambient 57 and 58°C, respectively (Schuman *et al.*, 1997). Hou *et al.* (1996) indicated that Haugh units remained unaffected in eggs heated in water baths and with hot air at ambient 55-57°C for as long as 85 min. Haugh unit increase, in the present study, could be the result of rapid protein coagulation when albumen temperature is ≥ 56°C under alkaline pH (Cunningham, 1995).

In the present study, albumen turbidity was determined from changes in its absorbance (600 nm) after heating shell eggs at three ambient temperatures for up to 40 min (Fig. 3.5). Significant ($p < 0.05$) gradual increase in albumen absorbance by 0.24, 0.39, and 0.34 OD_{600} was observed after 40 min treatments at ambient 57, 58, and 59°C, respectively. Shell eggs heated for 20 min showed marked significant ($p < 0.05$) increase in albumen turbidity at ambient 58 and 59°C. However, turbidity in eggs heated at ambient 57°C was significantly ($p < 0.05$) lower than that of eggs heated at the other temperatures (Fig. 3.5). Extended egg heating (> 30 min) significantly ($p < 0.05$) increased turbidity regardless of temperature (Fig. 3.5). Similarly, Hou *et al.* (1996) reported increase in albumen absorbance by 0.3 in eggs heated in water bath at ambient 57°C for 40 min. Schuman *et al.* (1997), on the other hand, used similar conditions but observed higher albumen turbidity than that reported in the current study.

Differential scanning calorimetry (DSC) is a rapid method to detect changes in denaturation patterns of egg white proteins, which could be used as quality indicators after treatment of shell eggs (Serrano, *et al.*, 1997). In the present study, thermal denaturation of albumen proteins was studied after heating eggs at ambient 57, 58, and 59ºC for up to 40 min. Results (Fig. 3.6) indicated presence of three endotherms (peaks) at 67, 80, and 84ºC in untreated shell eggs, corresponding to denaturation of conalbumin (ovotransferrin), ovalbumin, and s-ovalbumin, respectively (Ferreira *et al.*, 1997). Ovalbumin and conalbumin are two of the main components of egg white and constitute 54 and 12%, respectively, of total albumen proteins (Li-Chan *et al.*, 1995). Endotherms of albumen proteins from untreated (control) and heated (57 and 58ºC for 40

min) were similar (data not shown). However, treatments at 59ºC for 40 min produced a difference in the denaturation pattern of ovalbumin and s-ovalbumin (Fig. 3.6). S-ovalbumin is a form of ovalbumin that when present in high proportions results in low quality egg-white gels (Yamasaki *et al.*, 2003). Therefore, change in the pattern of denaturation of albumen proteins, as a result of extensive treatments, could result in quality deterioration (Serrano *et al.*, 1997). These results are in agreement with observation of high albumen turbidity after longest time treatment at ambient 59ºC in the previous experiments (Fig. 3.5).

In conclusion, results in this study indicate that inoculum placement to simulate natural egg contamination during thermal-treatment validation, resulted in 5.1-$\log_{10}$ inactivation of *Salmonella* at 58°C for 30 min. Conversely, Schuman *et al.*, (1997), using the same temperature, reported ~ 5.6-$\log_{10}$ inactivation of yolk-inoculated *Salmonella* in 43 min. Extensive egg heating (> 30 min) at ambient 58 and 59°C inactivated ≥ 5-$\log_{10}$ *Salmonella*, but increased albumen turbidity and produced protein modification. Treatment at ambient 57°C for ≤ 30 min produced less albumen turbidity than those at 58 and 59°C, but it did not achieve 5-$\log_{10}$ inactivation. Prolonged egg heating at ambient 57°C necessary to effectively inactivate *Salmonella*, though not explored in this study, may produce pasteurized eggs with acceptable quality. Evidence from the current study confirms that thermal treatments at ≥ 57°C for > 10 min resulted in increased Haugh units, which is a quality attribute highly sought-after in the egg industry. Although extensive egg heating at ambient 57-59ºC affected albumen clarity, Schuman *et al.* (1997) reported much higher turbidity than that observed in this study while assuming

presence of *Salmonella* in yolk. Therefore, in the present study, treatment validation simulating natural contamination could prevent overprocessing of egg white. Thermal treatments (> 40 min) at ambient 57ºC should be considered to inactivate *Salmonella* in eggs without drastically affecting albumen quality.

REFERENCES

Brackett, R.E., J.D. Schuman, H.R. Ball, and J. Scouten. 2001. Thermal inactivation kinetics of *Salmonella* spp. within intact eggs heated using humidity-controlled air. J. Food Prot. 64: 934-938.

Buzby, J.C. 2002. Older adults at risk of complications from microbial foodborne illness. USDA Economic Research Service. FoodRev. 25: 30-35.

Chantarapanont, W., L. Slutsker, R.V. Tauxe, and L.R. Beauchat. 2000. Factors influencing inactivation of *Salmonella* Enteritidis in hard-cooked eggs. J. Food Prot. 63: 36-43.

Cogan, T.A., G. Domingue, H.M. Lappin-Scott, C.E. Benson, M.J. Woodward, and T.J. Humphrey. 2001. Growth of *Salmonella* Enteritidis in artificially contaminated eggs: the effects of inoculum size and suspending media. Int. J. Food Microbiol. 70: 131-141.

Cunningham, F.E. 1995. Egg product pasteurization, p. 289-321. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Ferreira, M., C. Hofer, and A. Raemy. 1997. A calorimetric study of egg white proteins. J. Thermal Anal. 48: 683-690.

Fleischman, G.J., C.L. Napier, D. Stewart, and S.A. Palumbo. 2003. Effect of temperature on the growth response of *Salmonella Enteritidis* inoculated onto the vitelline membrane of fresh eggs. J. Food Prot. 66: 1368-1373.

Gast, R.K., and C. W. Beard. 1990. Production of *Salmonella* Enteritidis-contaminated eggs by experimentally infected hens. Avian Dis. 34: 438-446.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchell, and W.H. Andrews. 1993. Growth of *Salmonella* Enteritidis in grade A eggs during prolonged storage. Poult. Sci. 72:373-377.

Haugh, R.R. 1937. The Haugh unit for measuring egg quality. U.S. Egg and Poultry Magazine. 43: 553-555, 572-573.

Hou, H., R.K. Singh, P.M. Muriana, and W. J. Stadelman. 1996. Pasteurization of intact shell eggs. Food Microbiol. 13: 93-101.

Humphrey, T.J., P.A. Chapman, B. Rowe, and R.J. Gilbert. 1990. A comparative study of the heat resistance of salmonellas in homogenized whole egg, egg yolk or albumen. Epidemiol. Infect. 104: 237-241.

Humphrey, T.J., A. Whitehead, A. H. L. Gawler, A. Henley, and B. Rowe. 1991. Numbers of *Salmonella* Enteritidis in the contents of naturally contaminated hen's eggs. Epidemiol. Infect. 106: 489-496.

Humphrey, T.J. 1999. Contamination of eggs and poultry meat with *Salmonella enterica* serovar Enteritidis, p. 183-192. *In* A.M. Saeed, R.K.Gast, M.E. Potter, and P.G. Wall (ed.), *Salmonella enterica* serovar Enteritidis in humans and animals: epidemiology, pathogenesis, and control, Iowa State University Press, Ames, IA

Juneja, V.K., B.S. Elben, and H.M. Marks. 2001. Modeling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. Int. J. Food Microbiol. 70: 37-51.

Li-Chan, E.C.Y., W.D. Powrie, and S. Nakai. 1995. The chemistry of eggs and egg products, p. 105-175. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Lith, L.A.J.T. van, F.F. Putirulan, and R.W.A.W Mulder. 1995. Pasteurization of table eggs to eliminate *Salmonellae*. Arch. Geflügelk. 59: 157-160.

Michalski, C.B., R.E. Brackett, Y.-C. Hung, and G.O.I. Ezeike. 1999. Use of capillary tubes and plate exchanger to validate U.S. department of agriculture pasteurization protocols for elimination of *Salmonella* Enteritidis from liquid egg products. J. Food Prot. 62: 112-117.

Pflug, I.J., R.G. Holcomb, and M.M. Gomez. 2001. Principles of thermal destruction of microorganisms, p. 79-129. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams & Wilkins, Philadelphia, PA.

SAS Institute. 2001. JMP IN® version 4.0.4. SAS Institute Inc., Cary, NC.

Schuman, J.D., B.W. Sheldon, J.M. Vandepopuliere, and H.R. Ball Jr. 1997. Immersion heat treatments for inactivation of *Salmonella* Enteritidis with intact eggs. J. Appl. Microbiol. 83: 438-444.

Serrano, L.E., E.A. Murano, K. Shenoy, and D.G. Olson. 1997. D values of *Salmonella* Enteritidis isolates and quality attributes of shell eggs and liquid whole eggs treated with irradiation. Poult. Sci. 76: 202-205.

Shimada, K., and S. Matsushita. 1980. Thermal coagulation of egg albumin. J. Agric. Food Chem. 28: 409-412.

Shivaprasad, H.L., J.F. Timoney, S. Morales, B. Lucio, and R.C. Baker. 1990. Pathogenesis of *Salmonella* Enteritidis infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serologic responses. Avian Dis. 34: 548-557.

Stadelman, W.J. 1995b. Quality identification of shell eggs, p. 39-66. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Stadelman, W.J., R.K. Singh, P.M. Muriana, and H. Hou. 1996. Pasteurization of eggs in the shell. Poult. Sci. 75: 1122-1125.

Thompson, W.S., F.F. Busta, D.R. Thomson, and C.E. Allen. 1979. Inactivation of *Salmonellae* in autoclaved ground beef exposed to constantly rising temperatures. 42: 410-415.

Timoney, J.F., H.L. Shivaprasad, R.C. Baker, and B. Rowe. 1989. Egg transmission after infection of hens with *Salmonella* Enteritidis phage type 4. Vet. Rec. 125: 600-601.

USDA, Agricultural Marketing Service. 1997. Pasteurized shell eggs (pasteurized in-shell eggs). Vol. 162. No. 185: 49955-57. Fed. Regist. Washington, DC.

Yamasaki, M., N. Takahasi, and M. Hirose. 2003. Crystal structure of s-ovalbumin as a non-loop-inserted thermostabilized serpin form. J. Biol. Chem. 278: 35524-35530.

Zeidler, G. 2001a. Processing and packaging shell eggs, p. 1129-1161. *In* D.D. Bell, and D. Weaver Jr. (ed.), Commercial chicken meat and egg production, Kluwer Academic Publishers, Norwell, MA.

Heating time (min)	Haugh units		
	Treatment temperature (ºC)		
	57	58	59
0	72 ± 3^{a}	72 ± 4^{a}	72 ± 3^{a}
5	72 ± 4^{a}	73 ± 3^{a}	74 ± 3^{a}
10	84 ± 1^{b}	82 ± 1^{b}	78 ± 5^{ab}
20	81 ± 1^{b}	84 ± 4^{b}	81 ± 2^{b}
30	83 ± 3^{b}	79 ± 4^{b}	87 ± 6^{b}
40	82 ± 2^{b}	86 ± 6^{b}	85 ± 1^{b}

Table 3.1: Haugh units in shell eggs during immersion treatments at ambient 57, 58, and 59°C. Results are averages of triplicate trials ± standard deviation. Within a column, average with similar superscripts are not significantly different ($p \geq 0.05$).

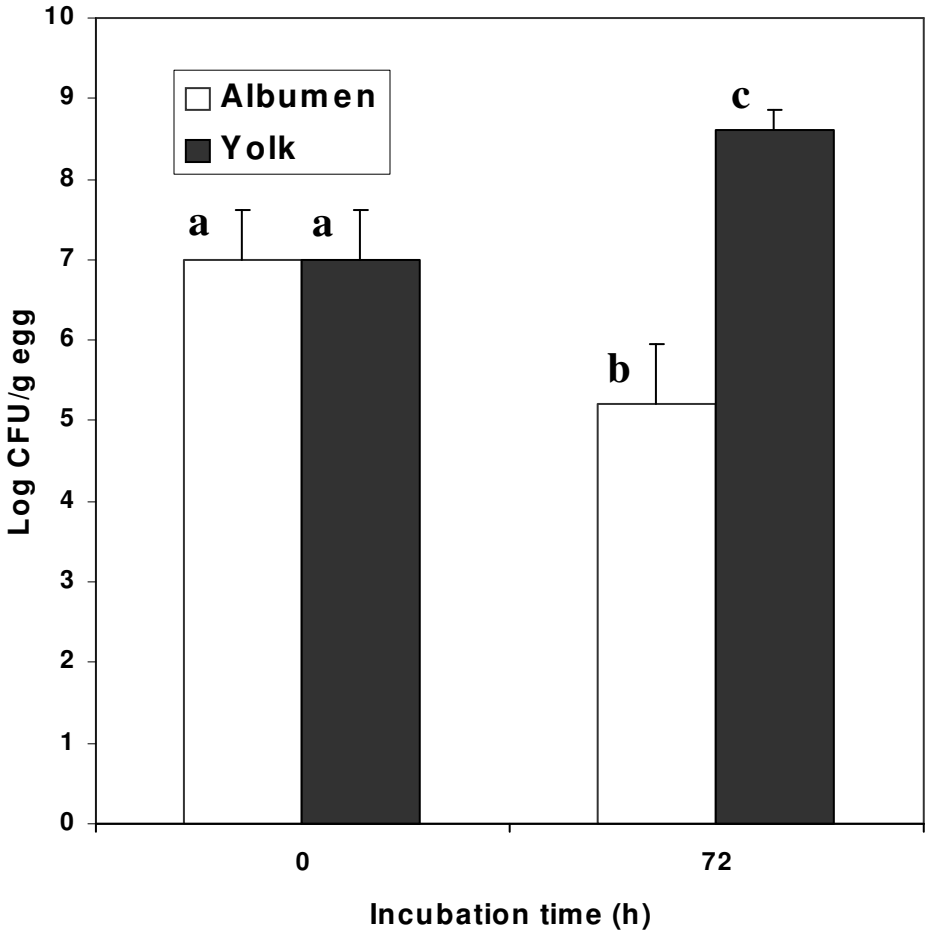

Figure 3.1: Effect of internal egg environment on *Salmonella* Enteritidis inoculated into albumen or yolk. Contaminated shell eggs were incubated at 37°C for 72 h. Experiments were performed in triplicate per experimental condition. Error bars indicate standard deviation. Different letters indicate significant ($p < 0.05$) difference among conditions.

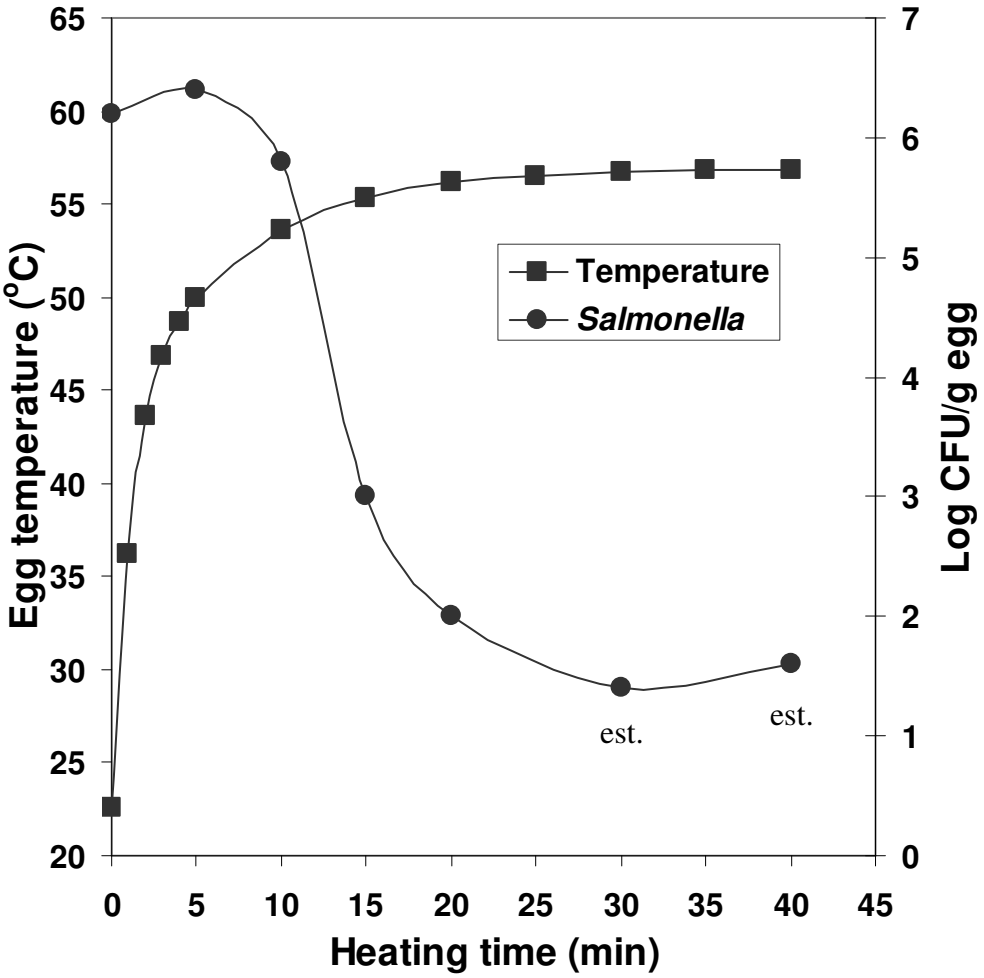

Figure 3.2: Inactivation of *Salmonella* Enteritidis in shell eggs by heat in a water bath at ambient 57°C, and increase of temperature inside shell eggs during treatment. *Salmonella* inactivation points represent the mean of microbial counts in experiments with five repeats. Points representing temperature were obtained in triplicate measurements. Estimated counts (est.) were obtained from enumeration on plates with detection limit of 10 CFU/g egg contents.

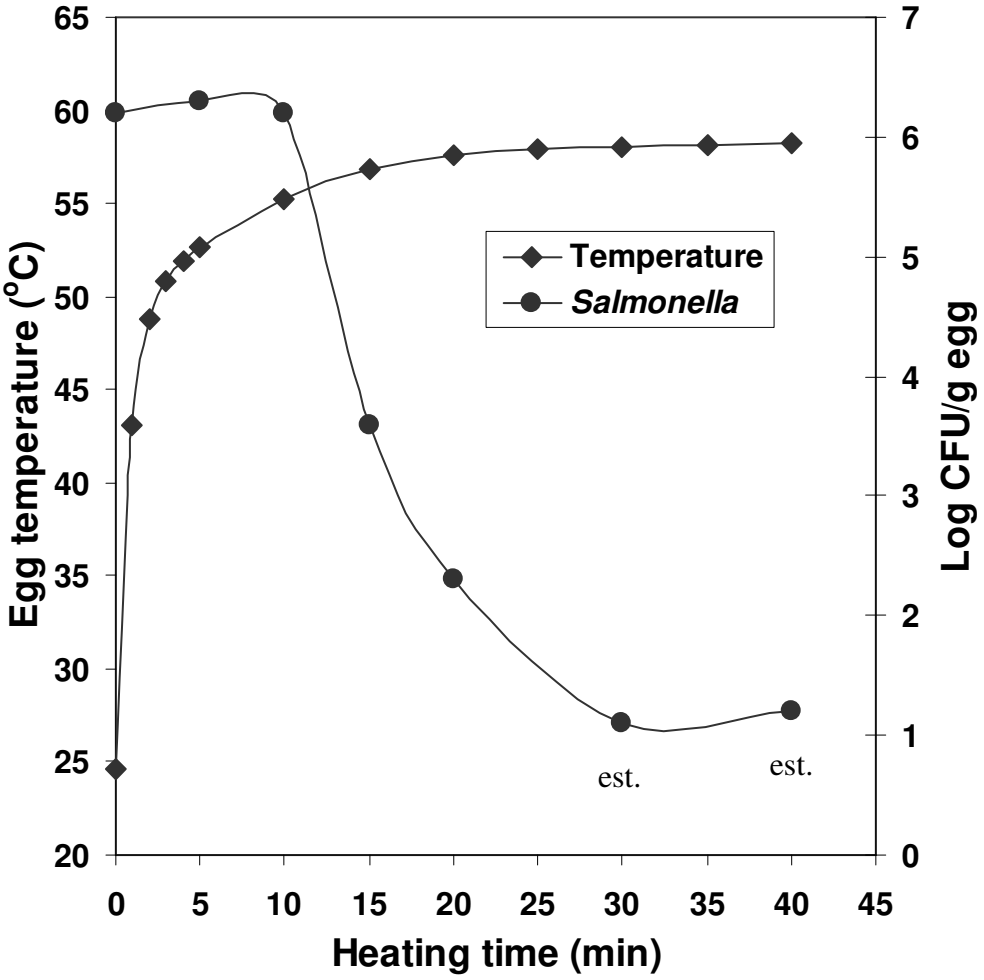

Figure 3.3: Inactivation of *Salmonella* Enteritidis in shell eggs by heat in a water bath at ambient 58°C, and increase of temperature inside shell eggs during treatment. Microbial inactivation points represent the mean of microbial counts in experiments performed five times. Points representing temperature were obtained in measurements performed three times. Estimated counts (est.) were obtained from enumeration on plates with detection limit of 10 CFU/g egg contents.

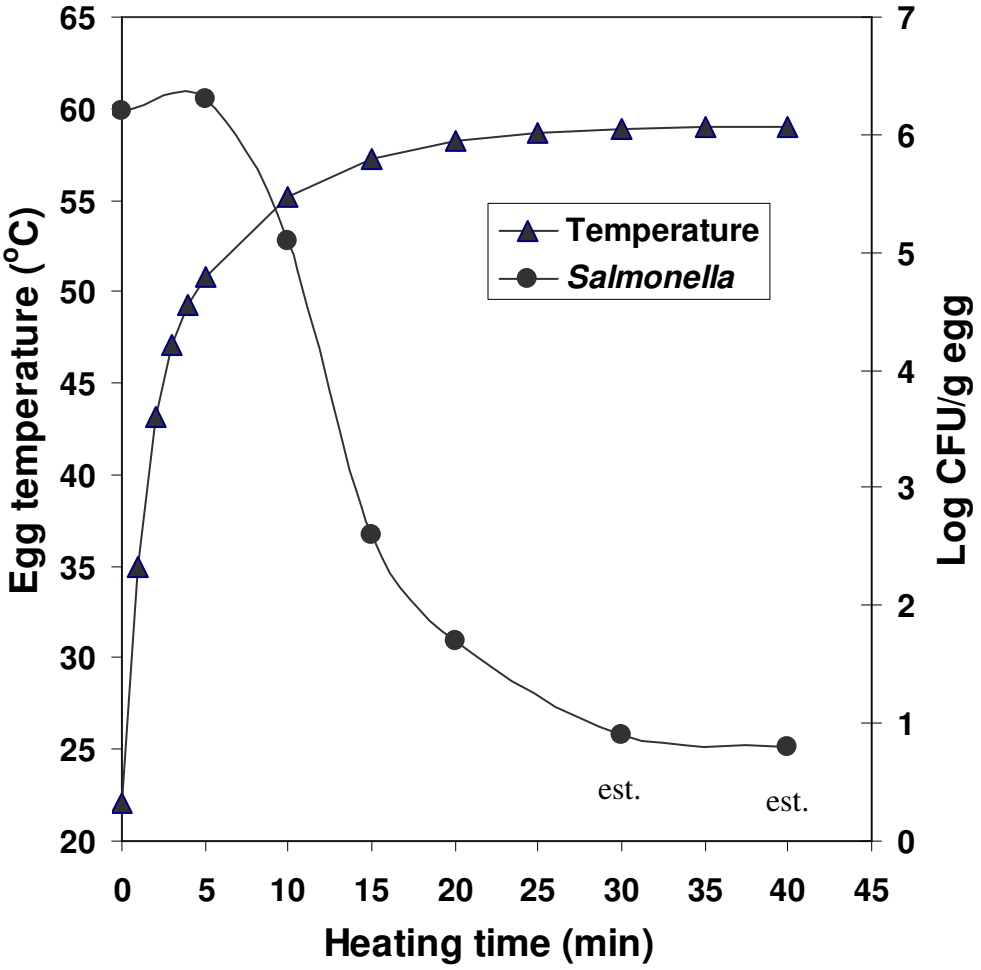

Figure 3.4: Inactivation of *Salmonella* Enteritidis in shell eggs by heat in a water bath at ambient 59°C, and increase of temperature inside shell eggs during treatment. Microbial inactivation points represent the mean of microbial counts in experiments with five repeats. Points representing temperature were obtained in measurements with three repeats. Estimated counts (est.) were obtained from enumeration on plates with detection limit of 10 CFU/g egg contents.

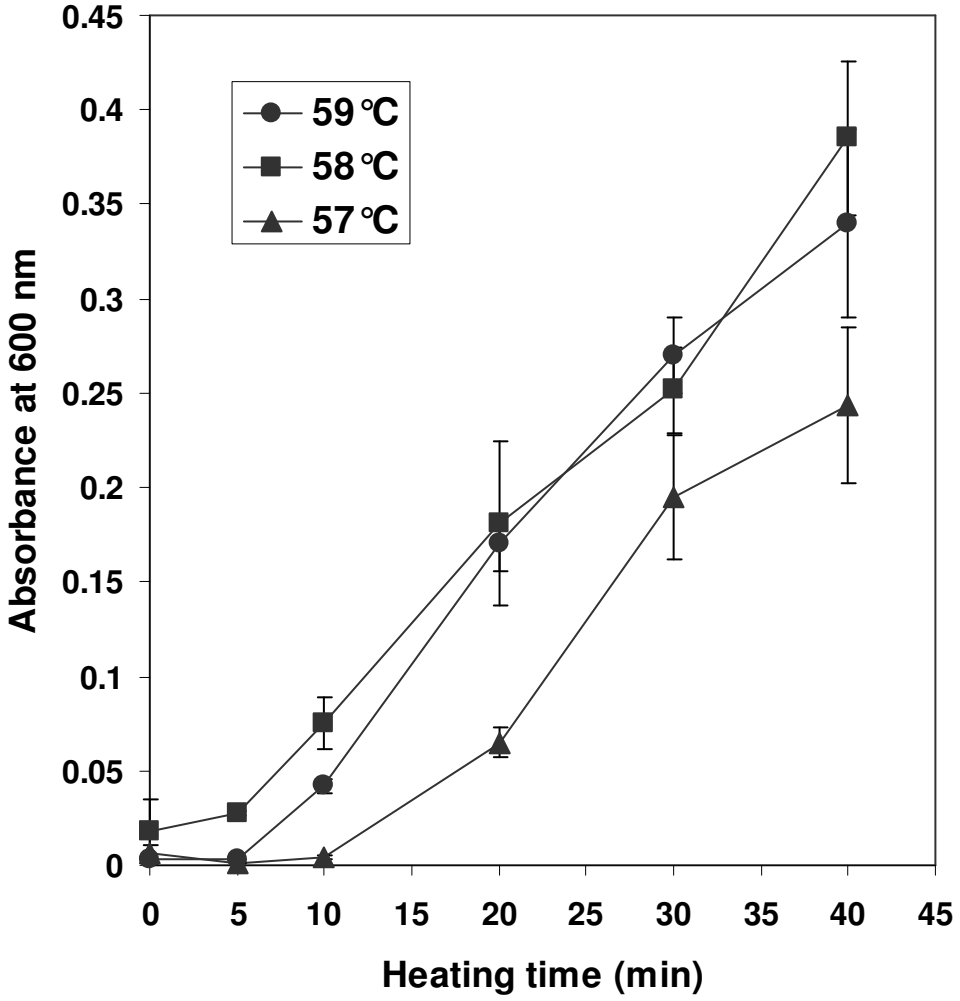

Figure 3.5: Albumen turbidity measured at 600 nm in shell eggs treated in water bath at ambient 57, 58, and 59°C for up to 40 min. Points represent the mean of experiments performed in triplicate per condition. Error bars indicate standard deviation

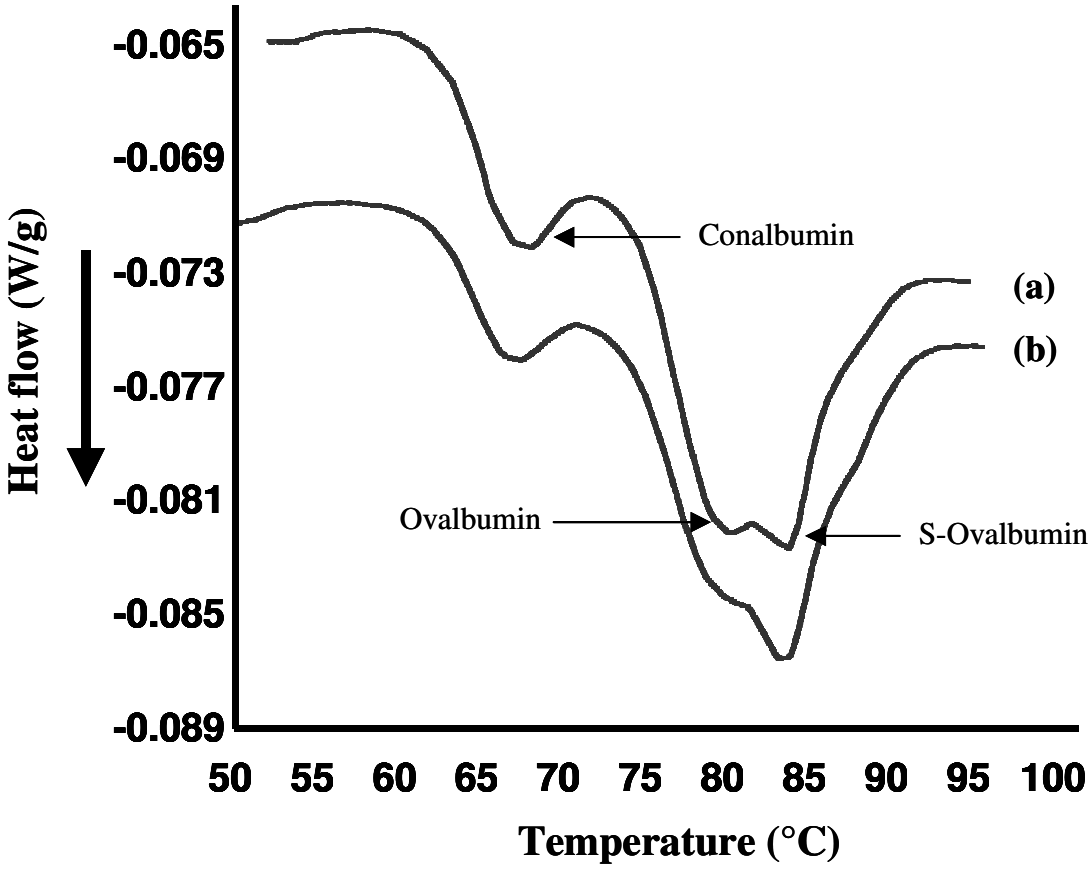

Figure 3.6: Differential scanning calorimetry thermograms of albumen obtained from (a) untreated shell eggs, and (b) shell eggs heated at ambient 59ºC for 40 min. Experiments were performed in triplicate and representative thermograms are presented.

CHAPTER 4

INACTIVATION OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS IN SHELL EGGS BY SYNERGISTIC COMBINATIONS OF HEAT AND OZONE

ABSTRACT

Contamination of shell eggs by *Salmonella* Enteritidis constitutes a hazard to consumers and an added liability to the food industry. Currently, methods to decontaminate shell eggs are limited. In this study, procedures to decontaminate shell eggs were developed using combinations of mild heat and gaseous ozone. Contaminated shell eggs (4.0-8.0 $\times$ 10^6 CFU/g egg) were heated at 57-59°C for up to 40 min, placed under vacuum (-7 to -10 psig), and immediately treated with gaseous ozone under pressure (12-14% wt/wt; $\leq$ 20 psig), or with mixtures of ozone-carbon dioxide for $\leq$ 40 min. Inactivation of *Salmonella* by heat (57-59°C for 0-40 min), or heat-ozone combination (57-59°C for 0-40 min heating, and O_3 at 12-14% wt/wt; $\leq$ 20 psig) was significant ($p < 0.05$), but pressurized ozone alone (12-14% wt/wt; $\leq$ 20 psig) was barely significant ($p = 0.05$). Synergy was observed between heating and ozonation times (0-40 min for both), and treatment temperature (57-59°C) and ozone pressure (up to 20 psig). However, carbon dioxide did not improve the efficacy of ozone against internal

Salmonella contamination. Response surface model, developed from data to optimize treatments, was significant ($p < 0.0001$; $n = 112$), and thus was used to predict treatment combinations to achieve microbial inactivation by 5-$\log_{10}$ inside eggs. For example, when eggs were heated at 57°C for 25 min followed by application of vacuum (-7 to -10 psig), and ozonation (10 psig) for 40 min, internal *Salmonella* population decreased ≥ 6.3-$\log_{10}$; the response surface model correctly predicted this microbial reduction. Egg quality was not drastically affected after treatments by selected effective combinations.

INTRODUCTION

Fresh egg is one of the vehicles for transmission of *Salmonella* Enteritidis to man, and consumption of contaminated shell eggs or egg products has been closely related to increase of salmonellosis in industrialized countries. In the United States, there are approximately 1.4 million cases, annually, of foodborne salmonellosis, and according to some studies 47% of them result from consumption of contaminated eggs (Frenzen *et al.*, 1999; Buzby, 2002). Furthermore, it has been calculated that economic cost of egg-associated salmonellosis in the United States is as high as $1.1 billion per year (Frenzen *et al.*, 1999).

In spite of the increase in cases of salmonellosis, and of the economic impact of the disease, control of *Salmonella* Enteritidis inside shell eggs has been attempted with a limited number of methods such as use of ionizing radiation and extensive heat treatments (Tellez *et al.*, 1995; Hou *et al.*, 1996). Recently, the Food and Drug

Administration (FDA) approved in the United States the use of radiation on shell eggs (CFR, 2000b). Although it has been demonstrated that gamma radiation effectively inactivates *Salmonella* Enteritidis inside shell eggs (Tellez *et al.*, 1995), other studies indicate that these treatments drastically affect egg quality (Ma *et al.*, 1994; Ma, 1996). Thermal treatment of shell eggs to inactivate *Salmonella* Enteritidis has been investigated, and approved by the US Department of Agriculture (USDA) for commercial use (Stadelman *et al.*, 1996; Schuman *et al.*, 1997; Vandepopuliere *et al.*, 1999; USDA, 1997). In spite of their effectiveness, heat treatment procedures are time consuming and extensive heating of shell eggs results in overprocessing of albumen with subsequent protein denaturation, and therefore affected egg functionality (Hou, *et al.*, 1996; Schuman *et al.*, 1997; Fleischman *et al.*, 2003).

Ozone (O_3) is a strong antimicrobial agent that has been extensively studied for its potential application in food products (Kim *et al.*, 1999; Khadre *et al.*, 2001). Recently, the FDA approved the use of ozone as antimicrobial in foods in the United States (CFR, 2001). In addition, ozone presents the advantage of its spontaneous decomposition to a non-toxic product, *i.e.*, oxygen (Kim *et al.*, 1999). Furthermore, ozone effectively reduced microorganisms in poultry facilities, processing chiller water, and carcasses (Bailey *et al.*, 1996; Dave, 1999; Diaz *et al.*, 2001). Moreover, ozone is potent against *Salmonella* Enteritidis and natural microbial flora on the surface of shell eggs (Whistler and Sheldon, 1989; Ito *et al.*, 1999; Koidis *et al.*, 2000). In spite of its effectiveness, several reports have indicated that ozone presents limited antimicrobial activity when applied in products with high organic matter content (Greer and Jones, 1989; Labbe and

Kinsley, 2001). However, combining ozone with other treatments could improve inactivation of microorganisms in food products (Scott, 1989; Unal *et al.*, 2001; Kim *et al.*, 2003). Furthermore, a recent study suggested that heat synergistically enhances the efficacy of ozone (Novak and Yuan, 2003). Although it is known that heat destroys ozone rapidly, sequential application of heat and ozone could be feasible (Kim *et al.*, 2003). In addition, there is evidence that ozone penetrates the eggshell, and a previous report suggested that use of heat and ozone could effectively inactivate microorganisms inside eggs (Krivopishin, 1970; Cox *et al.*, 1995). Therefore, considering the feasibility of using heat and ozone in sequence treatments, the objective of this study is to develop a procedure to inactivate *Salmonella* Enteritidis inside shell eggs, without drastically affecting interior egg quality, by using mild heat, vacuum, ozone, and mixtures of ozone-carbon dioxide under mild pressure, and their combinations.

MATERIALS AND METHODS

Shell eggs

Unfertilized, unwashed, fresh shell eggs (63 ± 2 g/egg) were obtained from Ohio Poultry Association (Columbus, OH). Shell eggs were kept under refrigeration at 4°C, and used in a period of 2 weeks after laying. Selected refrigerated eggs were measured with plastic Vernier caliper (Manostat Co., Switzerland). Shell eggs with 4.4-4.5 cm breadth were selected and stored at ambient 22-25°C for ~ 2 h. Shell eggs were washed

with tap water and scrubbed with plastic brush. Clean shell eggs were submerged in ethanol (70% vol/vol) for 30 min to eliminate external contaminants as previously described (Hammack, *et al*., 1993). Sanitized eggs were placed on sterile carton trays and permitted to dry at 22-25°C for approximately 40 min before inoculation.

Bacterial cultures

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis), an egg isolate, was provided by Ohio Department of Agriculture (Reynoldsburg, OH). Stock cultures were transferred to brain heart infusion (BHI) broth (Difco; Becton Dickinson, Sparks, MD), and incubated at 37°C for 24 h. Grown cultures were subsequently transferred in duplicate to 250-ml flasks (Pyrex®, Corning Inc., Acton, MA) containing 150 ml MacConkey broth (Difco); inoculum level was 0.1 %. Transferred cultures were incubated at 37°C for 24 h in orbital shaker (Model G-24, New Brunswick Scientific Co. Inc., Edison, NJ) under mild agitation. Aliquots of 80 ml *Salmonella* Enteritidis grown cultures were separated at 4°C in refrigerated centrifuge (Sorval RC-5B, Dupont Instruments, Bannorbuck, IL) at 3050 × g for 10 min. Harvested cells were resuspended in 2.5 ml sterile chilled phosphate buffer (0.1 M, pH 7), and cell suspension in tubes was agitated. Concentrated cell suspension contained $8.0 \times 10^9 - 4.0 \times 10^{10}$ CFU/ml *Salmonella* Enteritidis.

Inoculation of *Salmonella* Enteritidis

Shells of sanitized eggs were carefully perforated in the approximate center of egg blunt side with 2.54-cm/22-gauge sterile needle coupled to disposable 1-ml syringe. *Salmonella* Enteritidis cell suspension (10 µl) was inoculated into area surrounding the vitelline membrane outside the yolk of individual eggs. Inoculation was performed with sterile 50-µl high-precision chromatography syringe (Model 705; 22S needle gauge, Hamilton Co., Reno, NV) with fixed needle attached to rubber stopper located 5-6 mm from needle tip. Optimized inoculation protocol was previously developed by injection of tracer dye inside shell eggs and by visual examination of consistent dye placement as previously described (Chantarapanont *et al.*, 2000). Area surrounding eggshell perforations in contaminated eggs was wiped with 70% ethanol, and permitted to dry for approximately 2 min. Eggshell perforations were covered with droplet of commercial glue (Instant Krazy Glue®, Elmer's Products Inc., Columbus, OH); glue was permitted to dry for approximately 5 min. Perforations were additionally covered with approximately 1 cm^2 of polytetrafluoroethylene (PTFE) films with silicone adhesive backing (Cole-Parmer Instrument Co., Vernon, IL). Internal *Salmonella* Enteritidis concentration in shell eggs was 4.0-8.0 $\times$ 10^6 CFU/g egg. Contaminated eggs were held at 22-25°C for approximately 15 min before treatments.

Experimental design

Experimental design for inactivation of *Salmonella* Enteritidis inside shell eggs by heat and ozone was developed using commercial software (JMP IN® version

4.0.4; SAS Institute, Inc., Cary, NC). The design was based on response surface methodology (quadratic polynomial model) in central composite. Experimental design included heat treatments of shell eggs at 57, 58, and 59°C for 5-40 min. Gaseous ozone treatment conditions were at 0-20 psig (0-138 kPa) for 5-40 min. Carbon dioxide treatment conditions were at 0-10 psig (0-69 kPa). Vacuum was included in all gas treatments at -7 to -10 psig (-48 to -69 kPa) prior to use of ozone or ozone-carbon dioxide mixtures. Twenty-five different combinations of treatment factors, including appropriate controls, were obtained from the experimental design and are presented in Table 4.1. One hundred and twelve shell eggs treated by individual or combined factors were used to develop a response surface model. Experiments were performed in duplicate with a total of four shell eggs per experimental condition.

Ozone Generation

Gaseous ozone (O_3) was produced in electrochemical ozone generator (12-14% wt/wt; ozone in oxygen mixture, 1.45 liters/min flow rate)(LT 1 Model, Lynntech, Inc., College Station, TX) set at 90-95% capacity according to manufacturer's instructions. Experimental set up was kept inside chemical fume hood (Siemens Building Technologies, Inc., Buffalo Grove, IL), and all safety precautions were followed. Environmental ozone in treatment area was monitored with portable ozone detector (Model EZ-1X; Eco Sensors®, Eco Sensors, Inc., Santa Fe, NM) during experiments. Excessive gaseous ozone was destroyed in heated catalyst (Lynntech, Inc., College Station, TX).

Heat treatment

Shell eggs contaminated as previously described were placed inside aluminum basket (15 × 15 × 15 cm; Fisher Scientific, Pittsburgh, PA). Shell eggs inside basket were heat treated by immersion in water at 57, 58 or 59°C for 5-40 min. Treatment times were selected according to experimental design. Heat treatments were performed in previously calibrated water bath with circulating pump (Model 260, Precision Scientific, Inc., Chicago, IL) containing distilled deionized water at target temperature. Temperature variations during heat treatments in water bath were ± 0.2°C; water level above shell eggs was ~ 6 cm. Two eggs per experimental condition in duplicate series were taken from water bath, and immediately transferred to airtight vessel for ozone treatments as described later. In the case of eggs treated with heat only, sets of four eggs per experimental condition were removed from water bath at intervals, and placed in 2-liter glass beakers containing 1.5 liters of sterile distilled deionized water at 22°C. Shell eggs were held for 10 min in water for cooling. Cooled eggs were gently dry-wiped with clean paper tissue, and tested for *Salmonella* as described later.

Ozone treatment

Sets of two internally contaminated shell eggs were placed in gasket-sealed stainless-steel vessel (4000 ml, 21.6 cm diam × 15.5 cm height; Alloy Products Corp., Waukesha, WI), adapted with a 30-psig (207 kPa) pressure gauge (Ashcroft®, Dresser Inc., Stratford, CT). A schematic representation of set up, used for ozone/ozone-carbon dioxide treatments, is shown in Fig. 4.1. Shell eggs were mounted on adapters, made

of small silicone tubes (3.0-mm internal diam), and placed in the treatment vessel. The adapter allowed free gas contact with eggs, since only 10-15% of egg surface was in contact with it. The gas-treatment vessel was connected with approximately 40 cm of silicone tubing (6.4-mm internal diam; Masterflex®, Cole-Parmer Instrument Co., Vernon Hills, IL) to a vacuum pump (Fig. 4.1)(model 115V; Curtin Matheson Scientific, Houston, TX). The setup allowed, vacuum (-7 to -10 psig/-48 to -69 kPa) to be generated and held inside the vessel prior to ozone treatments. Immediately after vacuum generation, treatment vessel was disengaged from vacuum pump and gaseous ozone, produced as previously described, was injected into vessel at up to 20 psig (138 kPa)(approximately 3 min come up time). Ozone was delivered into the vessel, and passed through a 10-µm-pore size stainless-steel sparger (Solvent inlet filter, Upchurch®, Upchurch Scientific, Oak Harbor, WA) located at the bottom of the container to create gas turbulence (Fig. 4.1). Gaseous ozone was kept under pressure inside vessel by closing an inlet valve (0.6-cm diam tube fitting; Swagelok®, Swagelok Co., Solon OH) located between the ozone generator and the treatment vessel (Fig. 4.1). Shell eggs were treated with gaseous ozone under pressure for up to 40 min according to experimental design. Compressed oxygen (Praxair, Inc., Danbury, CT) was used instead of ozone as control in selected experiments. After treatments, gaseous ozone under pressure was slowly released from vessel by opening gas outlet valve (approximately 3-5 min total releasing time)(Fig. 4.1). In selected experiments, treatment vessel was placed on top of an orbital shaker (Model 361, Fisher Scientific, Pittsburgh, PA); vessel was agitated at 200 rpm throughout gas treatments (Fig. 4.1). Immediately after treatments, shell eggs

were taken from vessel and gently dry wiped with soft paper tissues. Sets of two treated shell eggs per experiment, in duplicate trials, were tested for enumeration of *Salmonella* as described later.

Treatment with mixture ozone-carbon dioxide (O_3-CO_2)

Gas mixtures of ozone and carbon dioxide were used in selected experiments. Contaminated eggs were placed inside the treatment vessel under -7 to -10 psig (-48 to -69 kPa) vacuum as previously described. Compressed carbon dioxide (Praxair, Inc., Danbury, CT) was injected into vessel at up to 10 psig (69 kPa). Vessel was filled subsequently with gaseous ozone to final maximum pressure of 20 psig (138 kPa). Different proportions of ozone-carbon dioxide (O_3-CO_2) were used according to experimental design. Selected gas mixtures included 1:1, 2:1, and 3:1 of O_3:CO_2 pressure proportions respectively. Experimental set up used for treatment of shell eggs with ozone/ozone-carbon dioxide mixtures is shown in Fig. 4.1. Sets of treated and control shell eggs in experiments performed in duplicate were taken from vessel. Treated shell eggs were dry wiped with clean paper tissues, and analyzed for enumeration of *Salmonella* as described later.

Combinations for process validation

Typical sequence of combinations included heating shell eggs, followed by gas treatment with ozone or ozone-carbon dioxide mixture under conditions described earlier. Analyzed microbial inactivation data, from 112 treated and control shell eggs, resulted in

development of a response surface model with capability to predict optimal combination of factors to reduce *Salmonella* Enteritidis inside shell eggs by ≥ 5 $\log_{10}$. Two sets of experimental conditions were selected for validation trials. In a first experiment (treatment I), contaminated shell eggs (4.0-8.0 × 10^6 CFU/g egg) prepared as described previously were immersed in water bath at 57 ± 0.2°C for 20 min. Immediately after heat treatment, eggs were placed inside the gas treatment vessel, and vacuum was generated inside the container (-7 to -10 psig/-48 to -69 kPa). Gaseous ozone was injected into treatment vessel to reach 10 ± 2 psig (69 ± 14 kPa), and pressure was maintained for 40 min; gas was vented intermittently and fresh O_3 gas was permitted in the vessel. Throughout ozonation, vessel was agitated at 200 rpm as previously described. In a variation of first experiment (treatment II), sets of contaminated shell eggs were heat-treated for 25 min at 57°C, and then were ozonated for 40 min (65 min total treatment time) under the conditions already described. Experiments were performed three times, and duplicate sets of eggs were used in each trial. *Salmonella* Enteritidis was enumerated in treated and control shell eggs as indicated later. Effect of single treatment factors on microbial inactivation was measured and compared to model predicted data.

Enumeration of *Salmonella*

Ten eggs were broken and their contents were weighed; average was 52.0 ± 2 g. Treated or control eggs were placed inside sterile 18 × 30 cm polyethylene stomacher bags (Fisherbrand®, Labplas, Inc., Quebec, Canada). Shell eggs were broken with the blunt end of a knife blade from outside of stomacher bags; whole egg contents

(albumen and yolk) were collected into bags and eggshells were discarded as biohazard waste. Egg contents were mixed with peptone water diluent in stomacher (Stomacher lab-blender 400, Cooke Laboratory Products, VA.) for 1 min. Serial decimal dilutions of homogenate were prepared and inoculated onto plate count agar (PCA; Difco). Aliquots of least dilute sample, consisting of 0.4, 0.3, and 0.3 ml (1 ml total) were inoculated on three different plates. Inoculated plates were incubated at 37°C for 48 h. Colonies on incubated plates were counted and confirmed as *Salmonella* by streaking onto xylose lysine desoxycholate agar (XLD; Difco). XLD plates were incubated at 37°C for 24 h and characteristic colonial morphology of the microorganism was observed. Minimum detection limit of this procedure was 10 salmonellae/g egg contents.

Interior egg quality measurements

Uninoculated shell eggs were treated with selected combinations of heat and ozone sufficient to inactivate ≥ 5 $\log_{10}$ internal *Salmonella* Enteritidis as described earlier. Immediately after treatments, shell eggs were placed on clean carton trays and permitted to cool at 25°C for ~ 2 h. Effect of treatments on interior egg quality was evaluated using eggs from triplicate trials with a total of six shell eggs per experimental condition. Appropriate controls were used in all experiments and subsequent measurements. Treated and control shell eggs were carefully broken with clean knife blade. Egg contents were carefully placed onto clean plastic surface; eggshells were discarded. Haugh units were measured in individual egg contents with calibrated micrometer (Haugh Meter™, Mattox & Moore, Inc., Indianapolis, IN) as described

previously (Haugh, 1937). Spherical yolk condition was determined by measurement of yolk index according to Funk (1948). Samples of thin albumen were analyzed for thermal protein denaturation by differential scanning calorimetry (DSC) as described later. A second set of treated and control shell eggs were carefully broken as described earlier, and egg contents were divided with a plastic egg separator. Separated albumen and yolk were individually recovered into clean 250-ml glass beakers (Kimax, Kimble Glass Inc., Vineland, NJ). Sampled albumen and yolk, from individual treated and control shell eggs, contained in beakers were homogenized on a magnetic stirrer (Corning, Model PC-320, Corning Inc., Corning, NY) at medium speed for approximately 5 min. Clarity of albumen was measured as absorbance at 600 nm in a spectrophotometer (Spectronic 1202, Milton Roy Co., Rochester, NY); water was used as standard during measurements according to Shimada and Matsushita (1980). Portions of stirred albumen and yolk samples were used to measure pH using a pH meter (Accumet®, Model 15, Fisher Scientific, Pittsburgh, PA).

Differential scanning calorimetry (DSC)

Thermal protein denaturation in albumen from shell eggs treated by two selected combinations, and from untreated controls was determined by differential scanning calorimetry (DSC) in modulated calorimeter (Model 2920 MDSC®, TA Instruments, New Castle, DE). Experiments were performed as described by Serrano *et al.* (1997) with some modifications, thin albumen aliquots (10 mg) from control and treated shell eggs, in triplicate trials, were weighed in aluminum pans (PerkinElmer®, PerkinElmer Life and

Analytical Sciences Inc., Boston, MA). Pans containing albumen samples were sealed in encapsulation press (TA Instruments, New Castle, DE). Individual sample pans were placed in calorimeter chamber, and an additional empty sealed pan was used as a reference. Samples were heated in calorimeter from 30 to 100°C with increases of 5°C per minute. Temperature and heating rate were set and controlled in an IBM personal computer connected to the calorimeter. Thermograms of samples were obtained and analyzed in commercial software (Universal Analysis 2000, Thermal Advantage™, TA Instruments, New Castle, DE). Thermograms of untreated control albumen samples were compared to those of samples obtained from eggs treated by selected combinations.

Measurement of lipid oxidation in yolk

Lipid oxidation in yolk of shell eggs treated by two selected treatments was measured in triplicate trials with six shell eggs per experimental condition (Table 5.7). Oxidation of yolk lipids was determined using 2-thiobarbituric acid (TBA) test, which includes acid extraction and detection of secondary lipid oxidation products (*i.e.*, malonaldehyde) as described previously (Pegg, 2001). Oxidation measurement in yolk samples of untreated shell eggs served as negative control. Positive control was the lipid oxidation measurement in yolks of shell eggs previously treated with gamma radiation at 6 kGy. Shell eggs, serving as positive control, were irradiated in triplicate in a Cobalt-60 (^{60}Co) source at The Ohio State University Nuclear Reactor Laboratory (Columbus, OH).

Statistical analyses

One hundred and twelve shell eggs (in experiments performed in duplicate with total of four eggs per experimental condition) were used in central composite design to develop response surface model for inactivation of *Salmonella* Enteritidis. Experimental design and data analyses were performed in JMP IN® version 4.0.4 software (SAS Institute, 2001, SAS Institute Inc., Cary, NC). Raw data of microbial counts (CFU/g egg) were converted into $\log_{10}$ before analyses. Interior egg quality measurements were performed in trials with 3-4 independent replicates per experimental condition. Statistical estimations included linear regression, paired t-tests ($\alpha = 0.05$), multiple and one-way analyses of variance (ANOVA) with 95% confidence limits, and multiple comparisons of means by Tukey ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Analyses of *Salmonella* Enteritidis inactivation factors

Shell eggs internally contaminated with *Salmonella* Enteritidis were treated with mild heat, gaseous ozone or mixes of ozone-carbon dioxide under pressure, and their combinations (Table 4.2). Microbial inactivation data resulted in development of highly significant ($p < 0.0001$) response surface model with a small standard error (0.5 $\log_{10}$ CFU/g egg)(Table 4.2). Statistical analyses of results, obtained from microbial inactivation in 112 treated shell eggs, indicated that ambient treatment temperature (57-59°C), and heating and ozonation time (up to 40 min for both) were highly significant

($p < 0.0001$) for inactivation of *Salmonella* Enteritidis inside eggs (Table 4.2). Previous investigators indicated that immersion heat treatments of eggs at ambient 57°C or above are required to effectively inactivate *Salmonella* Enteritidis inside shell eggs (Stadelman *et al.*, 1996; Hou *et al.*, 1996; Schuman, *et al.*, 1997). Furthermore, Cox *et al.* (1995) suggested that use of heat and ozone could reduce microbial load from the contents of shell eggs. In previous reports, Padron (1995) and Cox *et al.* (2000) indicated that application of sanitizers under pressure increased penetration of antimicrobial agents deep into the egg, and consequently improved effectiveness to inactivate *Salmonella* spp. However, in the current study, use of pressurized ozone alone (up to 20 psig/138 kPa) was barely significant ($p = 0.05$) for *Salmonella* Enteritidis inactivation in eggs (Table 4.2). Nonetheless, a significant ($p = 0.02$) interaction was observed between ambient treatment temperature (57-59°C) and pressurized ozone (up to 20 psig/138 kPa)(Table 4.2). Interaction between heating and ozonation time (up to 40 min for both) was significant ($p = 0.02$) for microbial inactivation (Table 4.2). Previous reports have suggested that combination of treatments using heat, and ozone in sequence could result in enhanced antimicrobial effect in food applications (Kim *et al.*, 2003; Novak and Yuan, 2003). On the other hand, a non-significant ($p = 0.07$) but potential interaction was observed between heating time and ozonation pressure (Table 4.2).

It has been previously reported that carbon dioxide inhibits decarboxylation enzymes, and consequently produces bacteriostatic effects on many microorganisms (King and Nagel, 1975; Mitsuda *et al.*, 1990). However, no significant ($p > 0.05$) effect of pressurized carbon dioxide alone was observed on *Salmonella* Enteritidis inside eggs

under experimental conditions used in this study (Table 4.2). Mitsuda *et al.* (1990) reported synergistic effect of ozone and carbon dioxide on microbial inactivation in foods, however, in this study there was no significant ($p = 0.07$) interaction between the two gases in the reduction of *Salmonella* Enteritidis contamination inside eggs (Table 4.2).

Response surface model

Models are mathematical descriptions of processes that could be useful to study and predict behavior of microorganisms in foods under multiple treatment conditions (Davey, 1992; McMeekin *et al.*, 1993). In the present study, response surface methodology was used to develop an empirical model to inactivate *Salmonella* Enteritidis inside eggs using combinations of heat, pressurized gaseous ozone, or mixes of pressurized ozone and carbon dioxide (Table 4.3). Response surface methodology is a cost-effective procedure that allows experimental design, fitting data to mathematical models, and selecting factors that produce a desired response with the purpose of process optimization (McMeekin *et al.*, 1993; Khuri and Cornell, 1996). In this study, a quadratic polynomial model was developed based on data obtained from microbial inactivation in shell eggs by single and combined treatment factors (Table 4.3). Model presented the simplified form $Y = \beta_0 + \beta_1 X_A + \beta_2 X_B + \beta_3 X_A X_B + \beta_4 X_A^2 + \beta_5 X_B^2 \pm \varepsilon$, where Y is the dependent variable (response or microbial inactivation), $\beta_{0\text{-}5}$ are coefficients obtained from experimental data, X_A and X_B are variables or treatment factors A and B respectively (for example: temperature and ozonation time), $X_A X_B$ represents interaction between

treatment factors A and B, X_A^2 and X_B^2 are quadratic terms of the equation, and ε is the standard error of model (Table 4.3). In the present study, model was developed based on multiple linear regression analysis, in which presence of quadratic terms accommodated a curvilinear trend of the response (Neter *et al.*, 1996). Results indicated that response surface model was highly significant ($p < 0.0001$), presented high correlation index ($r^2 = 0.97$) between actual and predicted data, and therefore it predicted optimal combination of treatment factors to inactivate *Salmonella* Enteritidis inside shell eggs within predetermined ranges of experimental conditions (Tables 4.2 and 4.3). Figure 4.2 is a three-dimensional representation of model-predicted data showing interaction between heating time at 57°C, and ozonation time (10 psig/69 kPa) on the elimination of *Salmonella* Enteritidis inside eggs.

Salmonella Enteritidis inactivation by selected treatments

Sets of selected combinations consisted on heating internally contaminated shell eggs (8.0×10^6 CFU/g egg) at ambient 57°C for 20 or 25 min in treatment I and II respectively, followed by ozonation under pressure for 40 min in both treatments (Tables 4.4 and 4.5). Results indicated that heat treatment alone for 20 and 25 min significantly ($p < 0.05$) reduced the microorganism by 4.4 and ≥ 5.2 $\log_{10}$ respectively when compared to untreated controls (Tables 4.4 and 4.5). Difference between observed and model-predicted microbial inactivation by heat alone was 0.3 and 0.5 $\log_{10}$ in selected treatments I and II, respectively (Tables 4.4 and 4.5). Internal egg temperature after heating shell eggs at ambient 57°C for 20 and 25 min was 56.1 and 56.5°C, respectively (data not

shown). These temperatures are close to the critical internal 55-56°C, in which maximum microbial inactivation occurs without substantial egg protein denaturation (Stadelman *et al.*, 1996). Although heat treatment alone at ambient 57°C for 25 min resulted in ≥ 5.2 log microbial reduction (Table 4.5), it seems unlikely that prolonged heating at this temperature could achieve higher inactivation without affecting albumen clarity, reducing lysozyme activity, or affecting egg functionality (Hou *et al.*, 1996; Schuman *et al.*, 1997). Moreover, it has been observed that prolonged heating usually results in tailing, a phase of microbial inactivation in which survivor cells present high resistance to further treatment (Pflug *et al.*, 2001). In contrast, ozone treatment alone for 40 min resulted in non-significant ($p > 0.05$) microbial reduction (0.3 $\log_{10}$) with respect to untreated controls in both combinations (Table 4.4 and 4.5). Difference between observed and model predicted reduction by ozone alone was 0.2 in both selected combinations (Table 4.4 and 4.5). Although it is known that ozone diffuses through eggshells and their membranes, it seems that antimicrobial activity of ozone alone is limited when applied to products with high organic matter content (Krivopishin, 1970; Greer and Jones, 1989; Labbe *et al.*, 2001). Nonetheless, sequence application of heat and ozone in selected treatment I produced significant ($p < 0.05$), synergistic microbial inactivation by ≥ 5.7 $\log_{10}$ with respect to untreated control (Table 4.4). Difference between observed and model predicted inactivation by selected combination I was 0.3 $\log_{10}$ (Table 4.4). Moreover, significant ($p < 0.05$) difference of 1.3 $\log_{10}$ was observed between inactivation produced by combination I and that resulted from heat alone (Table 4.4). Similarly, selected treatment combination II inactivated ≥ 6.3 $\log_{10}$ ($p < 0.05$) when

compared to untreated control (Table 4.5). A difference of 0.3 $\log_{10}$ in microbial reduction was measured between model-predicted inactivation and that observed in selected combination II. In addition, significant ($p < 0.05$) difference of 1.1 $\log_{10}$ was observed between inactivation resulting from combination and that obtained from heat treatment alone (Table 4.5). Differences between observed microbial inactivation after selected treatments, and those predicted by model ranged from 0.2 to 0.5 $\log_{10}$, which is within the standard error of the response surface model (0.5 $\log_{10}$)(Tables 4.2, and 4.4 - 4.5). Results from these experiments indicate that ozone could be effective in the inactivation of microorganisms in the tailing, since selected combinations resulted in non-detectable counts. It has been suggested that pressure and heat could modify properties of eggshell membranes, resulting in compression of membrane fibers and subsequent opening of internal eggshell pores (Froix *et al.*, 1977; Board and Tranter, 1995). Therefore use of ozone under pressure in heated shell eggs, previously held under vacuum, could result in increased ozone penetration and higher inactivation of microorganisms in egg contents (Cox *et al.*, 1995). Although heat easily destroys ozone, a previous study indicated that aqueous ozone effectively inactivated microorganisms in food products at temperature as high as 45°C (Achen and Yousef, 2001). Furthermore, Cox *et al.* (1995) patented a procedure to inactivate microorganisms inside shell eggs using heat treatments at ambient 59.4°C, and subsequent treatments with small concentrations of gaseous ozone.

Even though high temperature could increase ozone reactivity, previous reports suggest that sequential application of heat and ozone could be more beneficial than their concurrent use to inactivate microorganisms in foods (Kim *et al.*, 2003; Novak and Yuan, 2003).

Effect of selected treatments on interior egg quality

Egg quality was measured in non-contaminated shell eggs treated by the two previously described combinations of heat and ozone, which were effective to inactivate *Salmonella* Enteritidis by ≥ 5 $\log_{10}$. Selected treatment combinations I and II produced significant ($p < 0.05$) increase in Haugh units by 80.8 and 84.0, respectively, when compared to those in untreated control (72.5)(Table 4.6). The Haugh unit is a quality indicator calculated from the relationship between egg weight and albumen thickness (Stadelman, 1995b). Increased Haugh units are indication of high egg quality, and therefore it could be assumed that selected treatments I and II positively affected albumen quality by causing limited protein coagulation, and subsequently incremented albumen thickness (Cunningham, 1995; Zeidler, 2001b). Yolk index is a quality measurement that determines spherical yolk condition (Stadelman, 1995b). In the present study, yolk index was not significantly ($p > 0.05$) affected by treatments I and II when compared to untreated control (Table 4.6). Cox *et al.* (1995) reported changes in yolk dimensions in shell eggs treated with heat, vacuum, and ozone under pressure; they used of higher pressures than the ones tested in the present study.

Selected treatments I and II produced slight but significant ($p < 0.05$) increase in albumen turbidity by 0.07 and 0.09, respectively, with respect to that measured in untreated control (0.03)(Table 4.6). Nonetheless, this increase in albumen turbidity was minimal when compared to that previously reported for effective immersion heat treatments, in which heating shell eggs at 57°C for 75 min or at 58°C for 65 min increased albumen absorbance by 0.7 and 1.3, respectively (Schuman *et al.*, 1997). A minor, but significant ($p < 0.05$), decrease in albumen pH was observed in treatments I and II with respect to untreated control (Table 4.6). This slight pH reduction could be attributable to protein denaturation as a result of albumen exposure to ozone inside the egg (Kira and Ogata, 1988). Conversely, yolk pH was not significantly ($p > 0.05$) affected in treatments I and II when compared to untreated control (Table 4.6).

Effect of selected treatments on protein denaturation and lipid oxidation

Differential scanning calorimetry (DSC) is a reliable technique to determine effect of treatments on egg protein denaturation (Serrano *et al.*, 1997). Selected shell eggs treatments combinations resulted in two main denaturation peaks, *i.e.*, endotherms, in albumen samples (Table 4.7). Endotherms were observed in treated samples at 66.8 and 79.9°C as a result of denaturation of conalbumin (ovotransfrerrin) and ovalbumin respectively (Ferreira *et al.*, 1997; Serrano *et al.*, 1997)(Table 4.7). Selected treatments I and II did not significantly ($p > 0.05$) affect protein denaturation patterns in albumen, when compared to endotherms in untreated controls (Table 4.7). Therefore, it could be assumed that albumen proteins were unaffected as a result of selected treatments.

Increase in malonaldehyde concentration, a naturally occurring compound in yolk of fresh eggs, is a suitable indicator of lipid oxidation (Marshall *et al.*, 1994; Botsoglou *et al.*, 1997; Pegg, 2001; Wong and Kitts, 2003). In the present study, a slight but not significant ($p > 0.05$) increase in malonaldehyde was observed after selected treatment I (0.043 mg/kg yolk) with respect to that determined in untreated control (0.035 mg/kg yolk)(Table 4.8). As a result, it could be assumed that no lipid oxidation occurred after this treatment. Nonetheless, selected treatment II resulted in significantly ($p < 0.05$) higher malonaldehyde (0.047 mg/kg yolk) than that observed in untreated control (Table 4.8). Ozone, or its decomposition products, may have reached the yolk inside intact shell eggs during this treatment and caused lipid oxidation. Malonaldehyde concentration was not significantly different ($p > 0.05$) in eggs treated by combinations I and II (Table 4.8). Furthermore, malonaldehyde in untreated control, and in eggs treated by both selected combinations (0.043-0.047 mg/kg yolk) was significantly lower ($p < 0.05$) than that observed in gamma-irradiated eggs (0.08 mg/kg yolk)(Table 4.8).

Results in this study indicate that treatment of shell eggs by combinations of mild heat, vacuum, and pressurized gaseous ozone synergistically inactivates *Salmonella* Enteritidis inside shell eggs by ≥ 5 $\log_{10}$. Furthermore, combination treatments required to achieve such microbial reduction did not drastically affect interior egg quality. Therefore, technology based on combinations of heat and ozone should be considered in the egg industry for future applications to produce *Salmonella*-free shell eggs.

REFERENCES

Achen, M., and A.E. Yousef. 2001. Efficacy of ozone against *Escherichia coli* O157:H7 on apples. J. Food Sci. 66: 1380-1384.

Bailey, J. S., R. J. Buhr, N. A. Cox, and M. E. Berrang. 1996. Effect of hatching cabinet sanitation treatments on *Salmonella* cross-contamination and hatchability of broiler eggs. Poult. Sci. 75: 191-196.

Botsoglou, N.A., A.L. Yannakopoulus, D.J. Fletouris, A. S. Tserveni-Goussi, and P.D. Fortomaris. 1997. Effect of dietary thyme on the oxidative stability of egg yolk. J. Agric. Food Chem. 45: 3711-3716.

Board, R.G., and H.S. Tranter. 1995. The microbiology of eggs, p. 81-104. *In* W.J. Stadelman, and O.J. Coterill (ed.), Egg science and technology, The Haworth Press Inc., Binghamton, NY.

Buzby, J.C. 2002. Older adults at risk of complications from microbial foodborne illness. USDA Economic Research Service. FoodRev. 25: 30-35.

Chantarapanont, W., L. Slutsker, R.V. Tauxe, and L.R. Beauchat. 2000. Factors influencing inactivation of *Salmonella* Enteritidis in hard-cooked eggs. J. Food Prot. 63: 36-43.

CFR. 2000b. Irradiation in the production, processing, and handling of food. Vol. 65. No. 141. Office Federal Register, Washington, D.C.

CFR. 2001. Secondary direct food additives permitted in food for human consumption. Vol. 66. No. 123. Office Federal Register, Washington, D.C.

Cox, J.P., J.M. Cox, and R.W. Cox. July 1995. Hyperpasteurization of food. U.S. patent 5,431,939.

Cox, N.A., M.E. Berrang, R.J. Buhr, and J.S. Bailey. 2000. Bactericidal treatment of hatching eggs IV. Hydrogen peroxide applied with vacuum and a surfactant to eliminate *Salmonella* from hatching eggs. J. Appl. Poultr. Res. 9: 530-534.

Cunningham, F.E. 1995. Egg product pasteurization, p. 289-321. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Dave, S. 1999. Efficacy of ozone against *Salmonella* Enteritidis in aqueous suspensions and on poultry meat. [M.Sc. thesis]. Columbus, Ohio, U.S.A.: Ohio State University. p. 26-68. The Ohio State University, Columbus, Ohio.

Davey, K.R. 1992. A terminology for models in predictive microbiology. Food Microbiol. 9: 353-356.

Diaz, M.E., S.E. Law, and J.F. Frank. 2001. Control of pathogenic microorganisms and turbidity in poultry-processing chiller water using UV-enhanced ozonation. Oz. Sci. Eng. 23: 53-64.

Ferreira, M., C. Hofer, and A. Raemy. 1997. A calorimetric study of egg white proteins. J. Thermal Anal. 48: 683-690.

Fleischman, G.J., C.L. Napier, D. Stewart, and S.A. Palumbo. 2003. Effect of temperature on the growth response of *Salmonella* Enteritidis inoculated onto the vitelline membranes of fresh eggs. 66: 1368-1373.

Frenzen, P.D., J.C. Buzby, and T. Roberts. 1999. An updated estimate of the economic costs of human illness due to foodborne *Salmonella* in the Unites States. Proceedings of the 3rd. international symposium on the epidemiology and control of *Salmonella* in pork, August, Washington, D.C.

Froix, M.F., J. Pochan, A. Goedde, and D. Hinman. 1977. A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (*Gallus*/*gallus* (L.)). Polymer. 18: 1213-1218.

Funk, E.M. 1948. The relation of the yolk index determined in natural position to the yolk as determined after separating the yolk from the albumen. Poult. Sci. 27: 367.

Greer, G.G., and S.D.M. Jones. 1989. Effects of ozone on beef carcass shrinkage, muscle quality and bacterial spoilage. Can. Inst. Food Sci. Technol. J. 22: 156-160.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchell, and W.H. Andrews. 1993. Growth of *Salmonella* Enteritidis in grade A eggs during prolonged storage. Poult. Sci. 72:373-377.

Haugh, R.R. 1937. The Haugh unit for measuring egg quality. U.S. Egg and Poultry Magazine. 43: 553-555, 572-573.

Hou, H., R.K. Singh, P.M. Muriana, and W.J. Stadelman. 1996. Pasteurization of intact shell eggs. Food Microbiol. 13: 93-101.

Ito, H., H. Nakatani, H. Bamba, and T. Hayashi. 1999. Influence of the hatchability of Japanese quail eggs (hatching eggs) and sterilization effect for *Salmonella* by ozone gas sterilization. Res. Bull. Aichi-ken Agric. Res. Cent. 31: 305-310.

Khadre, M.A., A.E. Yousef, and J.-G. Kim. 2001. Microbiological aspects of ozone applications in food: A review. J. Food Sci. 66: 1242-1252.

Khuri, A.I., and J.A. Cornell. 1996. Response surfaces: designs and analyses. Marcel Dekker, Inc. New York.

Kim, J.-G., A.E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Prot. 62: 1071-1087.

Kim, J-G., A.E. Yousef, and M.A. Khadre. 2003. Ozone and its current and future application in the food industry. Adv. Food Nutr. Res. 45: 167-218.

King, A.D., and C.W. Nagel. 1975. Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa*. J. Food Sci. 40: 362-366.

Kira, S., and M. Ogata. 1988. Effect of ozone on denaturation and antigenicity of egg albumin. Okayama Igakkai Zasshi (J. Okayama Med. Assoc.). 100: 1-7

Koidis, P., M. Bori, and K. Vareltzis. 2000. Efficacy of ozone treatment to eliminate *Salmonella* Enteritidis from eggshell surface. Arch. Lebensmittelhyg. 51: 4-6.

Krivopishin, I.P. 1970. Permeability of the shell of chicken eggs for ozone. Sb. Rab. Molodykh Uch., Vses. Nauch.-Issled. Tekhnol. Inst. Ptitsevodstva. 12: 88-91.

Labbe, R.G., and M. Kinsley. 2001. Limitations in the use of ozone to disinfect maple sap. 64: 104-107.

Ma, C.-Y. 1996. Effects of gamma irradiation on physicochemical and functional properties of eggs and egg products. Radiat. Phys. Chem. 48: 375.

Ma, C.-Y., M.R. Sahasrabudhe, L.M. Poste, V.R. Harwalkar, J.R. Chambers, and K.P.J. O'Hara. 1994. Gamma irradiation and physicochemical properties of eggs and egg products, p. 283-300. *In* J.S. Sim, and S. Nakai (ed.), Egg uses and processing technologies, CAB International, Oxon, UK.

Marshall, A.C., A.R. Sams, and M.E. Van Elswyk. Oxidative stability and sensory quality of stored eggs from hens fed 1.5% menhaden oil. J. Food Sci. 59: 561-563.

McMeekin, T.A., J.N. Olley, T. Ross, and D.A. Ratkowsky. 1993. Predictive microbiology: theory and application. John Wiley and Sons Inc. New York.

Mitsuda, H., M.J.A.H. Ominami, and A. Yamamoto. 1990. Synergistic effect of ozone and carbon dioxide gases for sterilizing food. Proc. Japan Acad. 66: 68-72.

Neter, J., M. Kutner, C.J. Nachtsheim, and W. Wasserman. 1996. Applied linear regression models. Times Mirror Higher Education Group, Inc., Chicago.

Novak, J.S., and J.T.C. Yuan. 2003. Viability of *Clostridium perfringens*, *Escherichia coli*, and *Listeria monocytogenes* surviving mild heat or aqueous ozone treatment on beef followed by heat, alkali, or salt stress. J. Food Prot. 66: 382-389.

Padron, M. 1995. Egg dipping in hydrogen peroxide solution to eliminate *Salmonella* Typhimurium from eggshell membranes. Avian. Dis. 39: 627-630.

Pegg, R.B. 2001. Spectrophotometric measurement of secondary lipid oxidation products, p. D2.4.1-D2.4.18. *In* Wrolstad, R.E., T.E. Acree, H. An, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, and P. Sporns (ed.), Current protocols in food analytical chemistry, vol.1. John Wiley & Sons Inc., Hoboken, N.J.

Pflug, I.J., R.G. Holcomb, and M.M. Gomez. 2001. Principles of thermal destruction of microorganisms, p. 79-129. *In* S.S. Block (ed.), Disinfection, sterilization and preservation, Lippincott Williams & Wilkins, Philadelphia, PA.

SAS Institute. 2001. JMP IN® version 4.0.4. SAS Institute Inc., Cary, NC.

Scott, V.N. 1989. Interaction of factors to control microbial spoilage of refrigerated foods. J. Food Prot. 52: 431-435.

Serrano, L.E., E.A. Murano, K. Shenoy, and D.G. Olson. 1997. D values of *Salmonella* Enteritidis isolates and quality attributes of shell eggs and liquid whole eggs with irradiation. Poult. Sci. 76: 202-205.

Schuman, J.D., B.W. Sheldon, J. Vandepopuliere, and H.R. Ball Jr. 1997. Immersion heat treatments for inactivation of *Salmonella* Enteritidis with intact eggs. J. Appl. Microbiol. 83: 438-444.

Shimada, K., and S. Matsushita. 1980. Thermal coagulation of egg albumin. J. Agric. Food Chem. 28: 409-412.

Stadelman, W.J. 1995b. Quality identification of shell eggs, p. 39-66. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Stadelman, W.J., R.K. Singh, P.M. Muriana, and H. Hou. 1996. Pasteurization of eggs in the shell. Poult. Sci. 75: 1122-1125.

Tellez, I.G., R.M. Trejo, R.E. Sanchez, R.M. Ceniceros, Q.P. Luna, P. Zazua, and B.M. Hargis. 1995. Effect of gamma irradiation on commercial eggs experimentally inoculated with *Salmonella* Enteritidis. Radiat. Phys. Chem. 46: 789-792.

Unal, R., J-G. Kim, and A.E. Yousef. 2001. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulse electric field. J. Food Prot. 64: 777-782.

USDA, Agricultural Marketing Service. 1997. Pasteurized shell eggs (pasteurized in-shell eggs). Vol. 162. No. 185: 49955-57. Fed. Regist. Washington, DC.

Vandepopuliere, J.M., and O.J. Cotterill. December 1999. Method of controlling *Salmonella* in shell eggs. U.S. patent 6,004,603.

Whistler, P.E., and B.W. Sheldon. 1989. Bactericidal activity, eggshell conductance, and hatchability effects of ozone versus formaldehyde disinfection. Poult. Sci**.** 68:1074-1077.

Wong, P.Y.Y., and D.D. Kitts. 2003. Physicochemical and functional properties of shell eggs following electron beam irradiation. J. Sci. Food Agric. 83: 44-52.

Zeidler, G. 2001b. Shell egg quality and preservation, p. 1199-1217. *In* D.D. Bell, and D. Weaver Jr. (ed.), Commercial chicken meat and egg production, Kluwer Academic Publishers, Norwell, MA.

Experiment	Heating temperature (°C)	Heating time (min)	O_3 or O_3/CO_2 time (min)	O_3 partial pressure (psig)	CO_2 partial pressure (psig)	Total gas pressure (psig)
1	57	40	40	-	-	-
2	57	40	40	10	10	20
3[a]	57	40	-	-	10	10
4[a]	57	40	-	20	-	20
5	57	20	20	10	5	15
6	58	20	20	10	5	15
7	58	20	20	10	10	20
8[b]	58	20	20	-	5	5
9	58	20	20	15	5	15
10	58	20	20	10	-	10
11	58	40	20	10	5	15
12[a]	58	20	-	10	5	15
13	58	20	40	10	5	15
14	59	40	40	20	-	20
15[a]	59	40	-	10	10	20
16	59	40	-	-	-	-
17[b]	59	40	40	-	10	10
18	59	20	20	10	5	15
19[a]	-	-	-	10	10	20
20	-	-	40	20	-	20
21	-	-	20	10	5	15
22[a]	-	-	-	-	10	10
23[a]	-	-	-	20	-	20
24	-	-	40	-	-	-
25	-	-	40	10	10	20

[a] Experiments in which the total gas pressure was reached in the treatment vessel and gases were immediately released.

[b] Experiments in which the CO_2 partial pressure was reached in the treatment vessel, followed by gas release and its substitution for O_3

Table 4.1: Experimental conditions used to test the effect of treatment factors and their combinations on the inactivation of *Salmonella* Enteritidis within shell eggs.

Statistical estimates	Probability (p)[c]
Response surface model[a]	< 0.0001
Standard error of mean[b] = 0.5	
Individual parameters	
Temperature (57-59°C)	< 0.0001
Heating time (5-40 min)	< 0.0001
Ozonation time (5-40 min)	< 0.0001
Pressurized O_3 (up to 20 psig/138 kPa)	0.05
Pressurized CO_2 (up to10 psig/69 kPa)	0.13
Interactions	
Temperature and heating time	0.37
Temperature and ozonation time	0.55
Heating time and ozonation time	0.02
Temperature and Pressurized O_3	0.02
Heating time and Pressurized O_3	0.07
Temperature and Pressurized CO_2	0.67
Heating time and Pressurized CO_2	0.69
Ozonation time and Pressurized CO_2	0.35
Pressurized O_3 and CO_2	0.07

[a] n = 112
[b] Log_{10} CFU/g egg
[c] Significance level = 0.05

Table 4.2: Analyses of treatment factors for inactivation of *Salmonella* Enteritidis inside shell eggs using heat, pressurized ozone, pressurized carbon dioxide, and their combinations.

Log_{10} inactivation ratio[a] = 5.03 + (-0.15) X_1 + (-2.82) X_2 + (-0.32) X_3 + (-0.11) X_4 + (0.05) X_5 + (-0.06) X_1X_2 + (0.14) X_1X_3 + (-0.20) X_2X_3 + (0.12) X_1X_4 + (-0.02) X_2X_4+ (0.002) X_3X_4 + (0.004) X_1X_5 + (0.008) X_2X_5 + (-0.01) X_3X_5 + (-0.14) X_4X_5 + (0.04) X_1^2 + (1.6) X_2^2 + (-0.54) X_3^2 + (0.24) X_4^2 + (0.51) $X_5^2 \pm 0.5$

Where:

$X_1 = °T^b - 58$
$X_2 = (0.05 * HT^c) - 1$
$X_3 = (0.05 * O_{3time}{}^d) - 1$
$X_4 = (0.10 * O_{3psig}{}^e) - 1$
$X_5 = (0.20 * CO_{2psig}{}^f) - 1$

$r^2 = 0.97^g$

[a] *Salmonella* Enteritidis inactivated in $\log_{10}$ CFU/g egg
[b] (°T) = Temperature (57-59°C)
[c] (HT) = Heating time (5-40 min)
[d] (O_{3time}) = Ozonation time (5-40 min)
[e] (O_{3psig}) = Ozone pressure at 0-20 psig (0-138 kPa)
[f] (CO_{2psig}) = Carbon dioxide pressure at 0-10 psig (0-69 kPa)
[g] Obtained from actual versus predicted data relationship

Table 4.3: Response surface model for *Salmonella* Enteritidis inactivation inside shell eggs by combination of heat and pressurized O_3 or mixes of pressurized O_3-CO_2

Treatment			Log_{10} CFU/g egg ± SD	Decrease in log_{10} count	
Factor	Conditions	Time (min)		Observed	Predicted
Control	Inoculated non-treated	0	6.9 ± 0.1^{a}	0	0
O_3	12-14% wt/wt; 10 psig	40	6.6 ± 0.3^{a}	0.3	0.5
Heat	57°C ambient	20	2.5 ± 0.2^{b}	4.4	4.1
Selected treatment I	57°C heating (20 min) + O_3 12-14% wt/wt; 10 psig (40 min)	60	1.2 ± 0.5 est.^{c}	≥ 5.7	5.4

Table 4.4: Inactivation of *Salmonella* Enteritidis in shell eggs by selected treatment I, and comparison of observed and model predicted data for microbial inactivation. Data with similar superscripts are not significantly different ($p \geq 0.05$); est.: estimate count.

Treatment			Log_{10} CFU/g egg ± SD	Decrease in log_{10} count	
Factor	Conditions	Time (min)		Observed	Predicted
Control	Inoculated non-treated	0	6.9 ± 0.1^{a}	0	0
O_3	12-14% wt/wt; 10 psig	40	6.6 ± 0.3^{a}	0.3	0.5
Heat	57°C ambient	25	1.7 ± 0.2^{b}	≥ 5.2	4.7
Selected treatment II	57°C heating (25 min) + O_3 12-14% wt/wt; 10 psig (40 min)	65	0.6 ± 0.5 est.^{c}	≥ 6.3	6.0

Table 4.5: Inactivation of *Salmonella* Enteritidis in shell eggs by selected treatment II, and comparison of observed and model predicted data for microbial inactivation. Data with similar superscripts are not significantly different ($p \geq 0.05$); est.: estimate count.

Treatments[a]	Quality Parameters[b]				
	Haugh Units	Yolk Index	Albumen Turbidity[c]	pH	
				Albumen	Yolk
Control	72.5 ± 4.8A	0.39 ± 0.01A	0.03 ± 0.01A	8.99 ± 0.02A	5.99 ± 0.05A
Treatment I	80.8 ± 3.5B	0.40 ± 0.01A	0.07 ± 0.01B	8.87 ± 0.02B	6.15 ± 0.13A
Treatment II	84.0 ± 3.2B	0.40 ± 0.01A	0.09 ± 0.01B	8.85 ± 0.03B	6.15 ± 0.16A

[a] Control, untreated shell eggs; Treatment I, combination of immersion treatment of eggs at ambient 57°C (20 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (60 min total treatment time); Treatment II, combination of immersion treatment at ambient 57°C (25 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (65 min total treatment time). During ozonation eggs inside vessel were agitated at 200 rpm in both selected treatments.

[b] Data presented as Mean ± S.D. in experiments performed in triplicate with 6 eggs per condition. Means within columns not followed by the same letter differ significantly ($p < 0.05$).

[c] Measured as absorbance at 600 nm.

Table 4.6. Interior egg quality of shell eggs treated by selected combinations of heat and ozone to achieve ≥ 5-$\log_{10}$ reduction of *Salmonella* Enteritidis.

Treatments[a]	Denaturation peak temperature (°C)[b]	
	Conalbumin (Endotherm 1)	Ovalbumin (Endotherm 2)
Control	66.80 ± 0.20	79.90 ± 0.17
Treatment I	66.95 ± 0.25	79.90 ± 0.05
Treatment II	66.63 ± 0.40	80.01 ± 0.30

[a] Control, untreated shell eggs; Treatment I, combination of immersion treatment of eggs at ambient 57°C (20 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (60 min total treatment time); Treatment II, combination of immersion treatment at ambient 57°C (25 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (65 min total treatment time). During ozonation eggs inside vessel were agitated at 200 rpm in both selected treatments.

[b] Values presented as Mean ± S.D. in experiments performed with four replicates per trial.

Table 4.7: Differential scanning calorimetry (DSC) endotherms of albumen proteins from shell eggs treated by selected combinations of heat and ozone to achieve ≥ 5-$\log_{10}$ reduction of *Salmonella* Enteritidis.

Treatments[a]	Malonaldehyde concentration (mg/kg yolk)[b]
Control	0.035 ± 0.009A
Treatment I	0.043 ± 0.009AB
Treatment II	0.047 ± 0.006B
Gamma radiation	0.080 ± 0.006C

[a] Control, negative lipid oxidation control as untreated shell eggs; Gamma radiation, positive lipid oxidation control consisting on exposure of eggs to Cobalt-60 source (6 kGy); Treatment I, combination of immersion treatment of eggs at ambient 57°C (20 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (60 min total treatment time). Treatment II, combination of immersion treatment at ambient 57°C (25 min), followed by gaseous O_3 treatment (12-14% wt/wt; 10 psig/69 kPa) with venting for 40 min (65 min total treatment time). During ozonation eggs inside vessel were agitated at 200 rpm in both selected treatments.

[b] Values presented as Mean ± S.D. in measurements performed with six independent repeats for control and selected treatments, and three repeats for irradiated controls. Means within columns not followed by the same letter differ significantly ($p < 0.05$)

Table 4.8: Lipid oxidation in yolk of shell eggs treated by selected combinations of heat and ozone to achieve ≥ 5-$\log_{10}$ reduction of *Salmonella* Enteritidis.

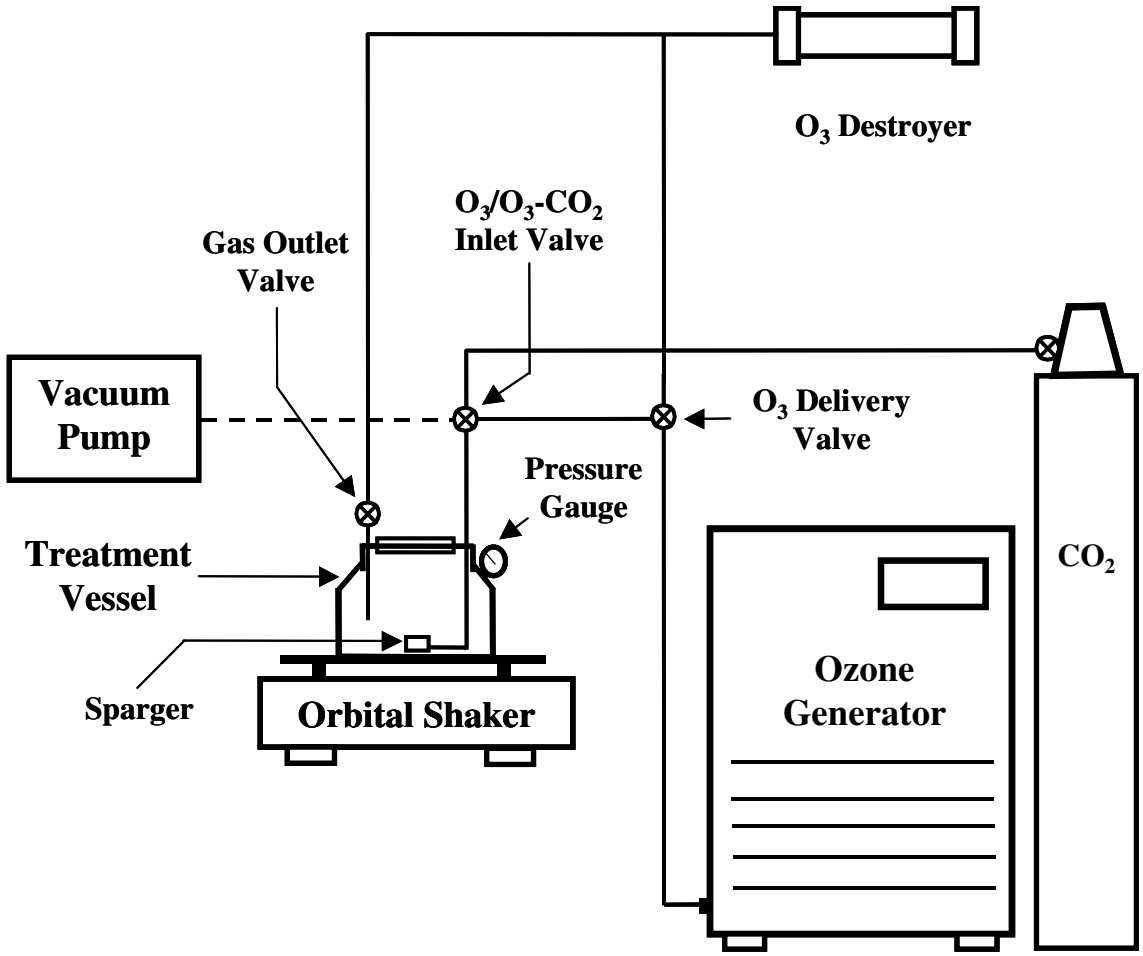

Figure 4.1: Experimental setup for treatment of shell eggs with pressurized O_3 and mixes of pressurized O_3 and CO_2

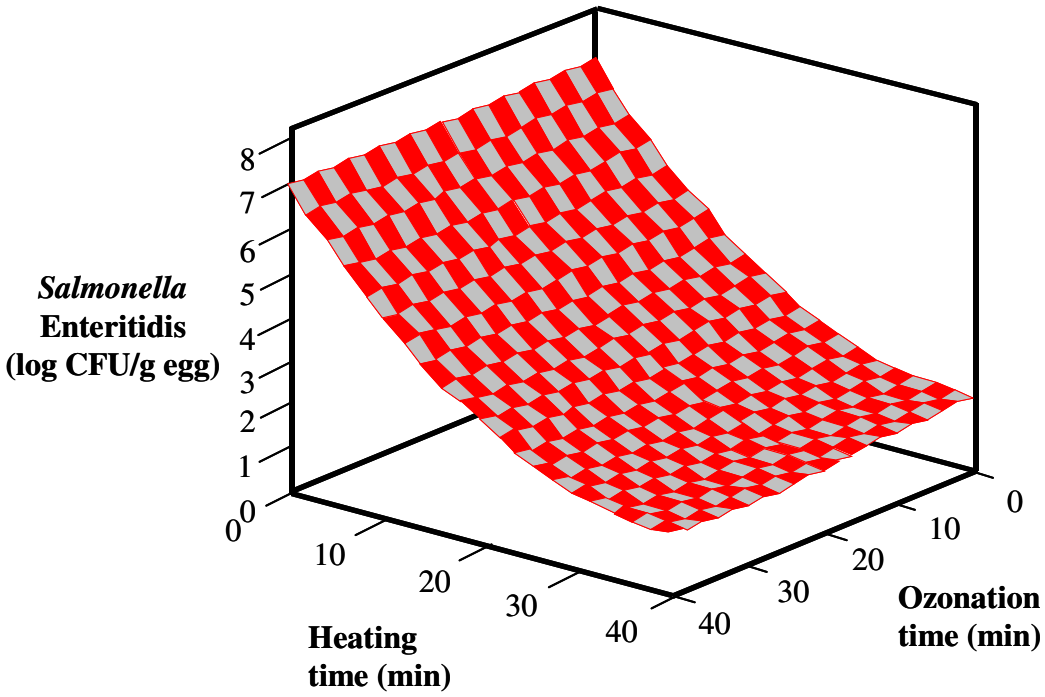

Figure 4.2: Three-dimensional representation of interaction between heating time at ambient 57°C, and ozonation time (12-14% wt/wt O_3; 10 psig/69 kPa) in the elimination of *Salmonella* Enteritidis inside shell eggs. Microbial inactivation data shown were obtained from response surface model predictions (Modified from JMP IN, SAS Institute).

APPENDIX

PENETRATION OF OZONE ACROSS THE SHELL OF HEN'S EGGS

ABSTRACT

Ozone effectively inactivates microorganisms on the surface of shell eggs, and some evidence suggests that when ozone is combined with other treatments, such as heat, it could inactivate *Salmonella* spp. in egg contents. There are limited reports on the factors that affect the penetration of gaseous ozone through eggshells. In this study, ozone penetration across shells of hen's eggs was investigated by a colorimetric procedure. Ozone was determined qualitatively inside shells by its reaction with the redox dye, potassium indigo trisulfonate. Shells, filled with redox dye, were treated with ozone at 0-10 psig for ≤ 40 min. In other experiments, shells, which were previously heated at ambient 57°C for 0-30 min, were treated with ozone (0-10 psig for ≤ 40 min). Shells, containing redox dye, which were untreated, previously heated, and treated with pressurized air over time, were used as controls. Results indicated that ozonation time (≤ 40 min) was highly significant ($p < 0.0001$) for ozone penetration across eggshells. Previous heating of shells (57ºC for 30 min), followed by use of pressurized ozone (10 psig) for ≤ 40 min, significantly ($p \leq 0.05$) increased penetration of gas across shells.

INTRODUCTION

Sanitizers are able to effectively inactivate microorganisms on the porous eggshell surface. However, there is little information on the penetration of antimicrobial agents through eggshell pores. It is assumed that in order to achieve microbial inactivation inside eggs, an active form of the sanitizer should penetrate the shell and go deep into the egg to reach located microorganisms (Cox *et al.*, 2000). The eggshell is a protective matrix formed of fibrous proteins and calcium carbonate crystals, which form a spongy structure that contains approximately ten thousand pores (Li-Chan *et al.*, 1995). Eggshell pores are involved in the exchange of oxygen (O_2), carbon dioxide (CO_2), and water vapor between the external environment and the egg interior by passive diffusion (movement of molecules from an area of high to low concentration)(Rahn *et al.*, 1979). Gas diffusion through the eggshell is mainly dependant on the pore area, and on the difference in concentration of gases moving across the shell (Paganelli *et al.*, 1975). A previous estimation indicates that shell pores in hen eggs have a total area of 2.3 mm^2 and an individual average diameter of 17 μm (Wangesteen and Rahn, 1970/71). In addition, it has been observed that when the inner eggshell membrane is removed, diffusion of gases through the shell increases (Romanoff and Romanoff, 1949).

Ozone (O_3) is an antimicrobial agent that is able to effectively inactivate microorganisms, including *Salmonella* spp., on the surface of eggs (Koidis, 2000). Furthermore, a previous report suggested that use of gaseous ozone in combination with thermal treatments could inactivate *Salmonella* spp. inside shell eggs (Cox *et al.*, 1995).

It is known that gaseous ozone is more stable than its aqueous phase, and that application of sanitizers under pressure could increase penetration of chemicals deep into the eggshell, with the resulting improvement in their efficacy (Padron, 1995; Kim *et al.*, 2003). In addition, previous reports suggest that heat and pressure could induce changes on eggshell membranes, and consequently modify shell permeability to gases (Romanoff and Romanoff, 1949; Froix *et al.*, 1977; Board and Tranter, 1995). Therefore, use of pressurized gaseous ozone in heated eggs could result in increased gas penetrability across the shell with the resulting enhancement in microbial inactivation in egg contents (Cox *et al.*, 1995). Krivopishin (1970) measured the penetrability of ozone through the shell of hen's eggs using a vacuum device, and very low gaseous ozone concentrations. Nonetheless, there is no information available on the penetration of ozone through shells previously heated, or on the penetrability of the gas under pressure at high concentration. Therefore, the objective of this study is to investigate the penetration of gaseous ozone under pressure through eggshells, using a redox dye detection method, and to determine the effect of prior heating of shells on ozone penetration. Pre-heating of shells was performed at ambient 57°C for 30 min, a temperature-time combination previously reported by Stadelman *et al.* (1996) as critical to reduce microorganisms in eggs without affecting their quality.

MATERIALS AND METHODS

Shell preparation

Unfertilized, non-washed fresh shell eggs (63 ± 4 g/egg) were obtained from Ohio Poultry Association (Columbus, OH). Selected eggs stored at 4°C were warmed to approximately 21°C. Shell eggs were washed with tap water (22-25ºC), scrubbed with a plastic brush, and subsequently immersed in ethanol (70% vol/vol) for 30 min as described by Hammack *et al.* (1993). Sanitized eggs were permitted to dry at ambient temperature for ~ 40 min before preparation of shells.

Eggshells of individual eggs were perforated twice in opposite sites of the approximate center of the egg width, using a 2.54-cm/18-gauge needle coupled to a 1-ml disposable syringe (Becton Dickinson & Co., Franklin Lakes, NJ). Perforations were broadened to 2-mm diam, using a 3.2-cm length needle with appropriate diameter, and yolk was punctured through perforations. Mixed albumen and yolk were carefully extracted from eggs with a 5-ml pipettor tip (Eppendorf®, Brinkman Instruments, Inc., Westbury, NY), and subsequently discarded. Individual shells were internally washed ~ 7 times with distilled water injected and extracted with the pipettor tip. Wash-water turbidity was monitored in spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY) until it reached ~ 0.03 optical density at 600 nm (OD_{600}). Eggshells were externally rinsed with distilled water at ambient temperature and gently wiped dry with soft paper tissue (Kimwipes®, Kimberly-Clark, Co. Roswell, GA). Shells were placed in desiccator (Nalgene™, Nalge Nunc Int., Rochester, NY), containing calcium sulfate

mineral absorbent (Drierite™, W.A. Hammond Drierite Co, Ltd., Xenia, OH), for 18-24 h at ambient temperature. Interior of individual dried shells was visually inspected on egg candler (Virglas model 8-200; Virtis, SP Industries Co., Gardiner, NY) to determine integrity of air sac.

Shell-filling with redox dye

Perforations on one side of individual dried shells were sealed with approximately 1 cm^2 of electrical tape (Intertape®, Intertape Polymer Group, Inc., Sarasota, FL). Covered perforations were additionally sealed with ~ 1.3 cm^2 ozone-resistant polytetrafluoroethylene (PTFE) films with silicone adhesive backing (Cole-Parmer Instrument Co., Vernon, IL). A solution of the redox dye (potassium indigo trisulfonate; Aldrich Chem. Co., Milwaukee, WI) was prepared as described by Bader and Hoigné (1981). The indigo stock solution was adjusted to ~ 1.0 OD_{600} with distilled water. Prepared dye solution (50 ± 3 ml) was poured into a 60-ml disposable syringe (B & D, NJ), and carefully injected into second perforation of one-side-sealed dried eggshells using a 2.54-cm/18-gauge needle attached to the syringe. Eggshell filling with dye solution was monitored on egg candler (Virtis, NY). Area surrounding second eggshell perforation was wiped with soft paper tissue moistened in absolute ethanol, and permitted to dry for approximately 2 min. The second perforations on individual eggshells were sealed with double tape as previously described. Filled eggshells were kept at ambient 25ºC for ~ 10 min before treatments.

Ozone Generation

Gaseous ozone (O_3) was produced in electrochemical generator at 12-14% wt/wt ozone in oxygen mixture (LT 1 Model, Lynntech, Inc., College Station, TX). Experimental setup was located in a Siemens chemical fume hood (Siemens Building Technologies, Inc., Buffalo Grove, IL), and ozone in treatment area was monitored with a portable ozone detector (Model EZ-1X; Eco Sensors®, Eco Sensors, Inc., Santa Fe, NM). Excessive ozone was destroyed in heated catalyst (Lynntech, TX).

Heat treatment of shells

Preliminary experiments indicated that heat alone affected the color of indigo dye inside shells heated at ambient 57ºC for ≤ 30 min. Therefore, whole shell eggs were first heated, and then used to prepare eggshells containing indigo dye as described before. Heat treatment of eggs was performed in a water bath with circulating pump (Model 260, Precision®, Precision Scientific, Inc., Chicago, IL) containing distilled water at ambient 57 ± 0.2ºC. Shell eggs were immersed in water for 30 min, dried, and permitted to cool at ambient 22ºC for ~ 40 min before shell preparation.

Ozonation of shells

One shell, containing indigo dye, per experimental condition was placed in a modified gasket-sealed stainless-steel vessel (4000 ml, 21.6 cm diam × 15.5 cm height; Alloy Products Corp., Waukesha, WI) adapted with a 30-psig pressure gauge (Ashcroft®, Dresser Inc., Stratford, CT). Eggshells were treated in triplicate with gaseous ozone

for ≤ 40 min under 0-10 psig. After treatments, ozone under pressure was slowly released from vessel (~ 3 min releasing time). Eggshells containing indigo dye, treated at different time periods, were used to measure ozone penetration as described later. Compressed air was used as pressure control in selected experiments.

Ozone detection

Ozone-treated eggshells containing indigo dye were immediately transferred to clean carton trays and sprayed with ozone neutralizer (0.01% sodium thiosulfate, $Na_2S_2O_3$; J.T. Baker Chemical Co., Phillipsburg, NJ). Shells were gently dried with soft paper tissue. Eggshells were carefully broken on the rim of clean 500-ml glass beaker. Indigo dye solution of individual eggshells was recovered into clean 250-ml beakers. Recovered indigo dye solution was mixed with magnetic bar using mild agitation on stirring plate (Corning, Model PC-320, Corning, Inc., Corning, NY) for approximately 1 min under dark conditions. Aliquots (0.5 ml) of indigo dye were diluted 1:4 with distilled water. Absorbance of diluted indigo dye solutions was measured in a spectrophotometer (Milton Roy Co., NY). Fresh indigo dye solution, prepared as previously described, was used to calibrate spectrophotometer. Corrected OD_{600} was determined as follows: optical densities of indigo dye obtained from treated and control eggshells were multiplied by dilution factor. Subsequently 0.03 OD_{600} of last rinse wash water was subtracted from original measurements.

Corrected measurements were transformed to percentage of transmittance (%T) for statistical analysis. Increase in %T of indigo dye after treatments was used as indicator of increase in decoloration of indigo dye as a result of its reaction with ozone.

Statistical analysis

Forty-eight eggshells were used to study ozone penetration. Statistical estimations consisted on analysis of variance with 0.05 significance level, and comparison of means by Tukey ($\alpha = 0.05$). Data analysis was performed in JMP IN® software version 4.0.4 (SAS Institute, 2001, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Factors affecting ozone penetration across eggshells

Results indicated that ozonation time (≤ 40 min) was highly significant ($p = 0.0001$) for penetration of gaseous ozone across shells (Table A.1). Application of ozone under 10 psig (69 kPa) or heating alone was not significant ($p < 0.05$) for gas penetration (Table A.1). However, an interaction between shell heating and use of pressurized ozone was marginally significant ($p = 0.05$) for gas penetration (Table A.1).

Previous studies have reported that diffusion is involved in the penetration of gasses through microscopic eggshells pores (Wangensteen, 1970/71; Rahn *et al.*, 1987). It is known that diffusion of gas molecules to the interior of shells requires the formation

of a gradient, in which there is a higher concentration of the gas on the outside of the shell than that in its inside (Rahn *et al.*, 1979). Relative gas penetrability across hen's eggshells, usually reported with respect to air, follows this descending order: hydrogen (H_2) > carbon dioxide (CO_2) > Nitrogen (N_2) > Oxygen (O_2) > Ozone (O_3)(Table A.2)(Romanoff and Romanoff, 1949; Krivopishin, 1970). Ozone (MW = 48), a heavier gas than air (MW ~ 28.9), has a relative shell penetrability of 68.1% (Table A.2)(Krivopishin, 1970). It is assumed that the lower the molecular weight of a gas, the higher its degree of penetration across shells (Table A.2). However, CO_2, a relatively heavy gas (MW = 44), shows a higher diffusion rate across shells than O_2 (MW = 32), a lighter gas (Table A.2). Romanoff and Romanoff (1949) indicated that difference in O_2-CO_2 diffusion is regularly observed in animal membranes. In this study, use of pressurized ozone (10 psig/69 kPa), with the subsequent increase in ozone concentration was ineffective to increase gas penetration (Table A.1). Krivopishin (1970) reported that the degree of ozone penetrability across shells was not correlated with gas concentration in the environment, and indicated that ozone penetrated in higher proportion across the obtuse eggshell end than through its pointing end. This is in agreement with previous reports indicating that the eggshell's blunt end has a high number of pores (Romanoff and Romanoff, 1949; Li-Chan *et al.*, 1995). Gas penetration across eggshells involves the movement of gas molecules through shell pores and membranes. Eggshell membranes are protective structures, formed by protein fibers, located underneath the shell (Li-Chan *et al.*, 1995). Romanoff and Romanoff (1949) indicated that removal of the inner shell membrane increased gas diffusion across shells. In addition, previous reports have

suggested that heat and pressure could modify eggshell membranes to produce internal openings in the pores (Fig. A.2)(Froix *et al.*, 1977; Board and Tranter, 1995). Therefore, it could be assumed that use of pressurized ozone in previously heated shells could increase ozone penetrability. Results in this study indicated that interaction between shell heating and pressurized ozone was marginally significant ($p = 0.05$) for penetration of the gas (Table A.1).

Ozone penetration across shells over time

Gaseous ozone diffused over time across shells at ambient atmospheric pressure (0 psig), after 20 and 40 min treatment, and produced significant ($p < 0.05$) increase in redox dye transmittance by 23 and 28%, respectively, when compared to untreated controls (16.7%) for the same time periods (Fig. A.1). A similar trend was observed when pressurized ozone (10 psig) was used in non-previously treated shells, or in shells previously heated at 57ºC for 30 min (Fig. A.1). The color of the dye inside eggs did not significantly ($p > 0.05$) vary over time (40 min) in untreated controls (Fig A.1). Similarly, additional control using compressed air (10 psig for 40 min) did not significantly ($p > 0.05$) change dye color over time (data not shown). It is possible that ozone reaction with eggshell materials could limit its detection inside shells. However, calcium carbonate, the main eggshell component, has a limited reactivity with ozone (Alebić-Juretić *et al.*, 2000). Nonetheless, organic material and proteins on the shell and its membranes could react with ozone and reduce its availability to reach the egg interior (Koidis *et al.*, 2000).

Results in this study indicate that penetration of gaseous ozone across whole eggshells could be determined qualitatively using shells filled with a redox dye. Exposure of shells to ozone resulted in gas penetration over time (≤ 40 min) regardless of previous shell heating (57ºC for 30 min) or use of pressurized ozone (10 psig/69 kPa). However heating shells at higher temperatures and longer times, or ozonation at increased pressure-time combinations could result in incremented ozone penetration.

REFERENCES

Alebić-Juretić, A., T. Cvitaš, and L. Klasinc. 2000. Kinetics of heterogeneous ozone reactions. 2000. Chemosphere. 41: 667-670.

Bader, H., and J. Hoigné. 1981. Determination of ozone in water by the indigo method. Water Res. 15: 449-456.

Board, R.G., and H.S. Tranter. 1995. The microbiology of eggs, p. 81-104. *In* W.J. Stadelman, and O.J. Coterill (ed.), Egg science and technology, The Haworth Press Inc., Binghamton, NY.

Cox, J.P., J.M. Cox, and R.W. Cox. July 1995. Hyperpasteurization of food. U.S. patent 5,431,939.

Cox, N.A., M.E. Berrang, R.J. Buhr, and J.S. Bailey. 2000. Bactericidal treatment of hatching eggs IV. Hydrogen peroxide applied with vacuum and surfactant to eliminate *Salmonella* from hatching eggs. J. Appl. Poult. Res. 9: 530-534.

Froix, M.F., J. Pochan, A. Goedde, and D. Hinman. 1977. A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (*Gallus*/*gallus* (L.)). Polymer. 18: 1213-1218.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchell, and W.H. Andrews. 1993. Growth of *Salmonella* Enteritidis in grade A eggs during prolonged storage. Poult. Sci. 72:373-377.

Kim, J-G., A.E. Yousef, and M.A. Khadre. 2003. Ozone and its current and future application in the food industry. Adv. Food Nutr. Res. 45: 167-218.

Koidis, P., M. Bori, and K. Vareltzis. 2000. Efficacy of ozone treatment to eliminate *Salmonella* Enteritidis from eggshell surface. Arch. Lebensmittelhyg. 51: 4-6.

Krivopishin, I.P. 1970. Permeability of the shell of chicken eggs for ozone. Sb. Rab. Molodykh Uch., Vses. Nauch.-Issled. Tekhnol. Inst. Ptitsevodstva. 12: 88-91.

Li-Chan, E.C. Y., W.D. Powrie, and S. Nakai. 1995. The chemistry of eggs and egg products, p. 105-175. *In* W. J. Stadelman and O. J. Cotterill (ed.), Egg science and technology, The Haworth Press Inc., Binghamton, NY.

Padron, M. 1995. Egg dipping in hydrogen peroxide solution to eliminate *Salmonella* Typhimurium from eggshell membranes. Avian Dis. 39: 627-630.

Paganelli, C.V., A. Ar, H. Rahn, and O.D. Wangensteen. 1975. Diffusion in the gas phase: the effects of ambient pressure and gas composition. Respir. Physiol. 25: 247-258.

Rahn, H., A. Ar, and C.V. Paganelli. 1979. How bird eggs breathe. Sci. Amer. 240: 45-55.

Rahn, H., C.V. Paganelli, and A. Ar. 1987. Pores and gas exchange of avian eggs: a review. J. Exp. Zool. Suppl. 1: 165-172.

Romanoff, A.L., and A.J. Romanoff. 1949. The avian egg. John Wiley & Sons, Inc., New York.

SAS Institute. 2001. JMP IN® version 4.0.4. SAS Institute Inc., Cary, NC.

Stadelman, W.J., R.K. Singh, P.M. Muriana, and H. Hou. 1996. Pasteurization of eggs in the shell. Poult. Sci. 75: 1122-1125.

Wangensteen, O.D., D. Wilson, and H. Rahn. 1970/71. Diffusion of gases across the shell of the hen's egg. Respir. Physiol. 11: 16-30.

Statistical estimates	Probability (p)[a]
Individual parameters	
Shell heating[b]	0.41
Pressurized O_3 (10 psig)	0.62
Ozonation time (≤ 40 min)	< 0.0001
Interactions	
Shell heating and pressurized O_3	0.05
Shell heating and ozonation time	0.69
Pressurized O_3 and ozonation time	0.67

[a] Significance level = 0.05

[b] Heat treatment at ambient 57 ± 0.2°C for 30 min

Table A.1: Analysis of factors affecting ozone penetration across eggshells (n = 48)

Gas	Molecular weight (MW)	Relative penetration across eggshells (%)[a]
Hydrogen (H_2)	2.0	140.98[b]
Carbon dioxide (CO_2)	44.0	103.19[b]
Nitrogen (N_2)	28.0	100.86[b]
Oxygen (O_2)	32.0	92.85[b]
Ozone (O_3)	48.0	68.1[c]

[a] Relative to penetration of air (MW ~ 28.9) across eggshells = 100%
[b] Romanoff and Romanoff, 1949.
[c] Krivopishin, 1970.

Table A.2: Relative penetration of selected gasses across the shell of hen's eggs (Modified from Romanoff and Romanoff, 1949).

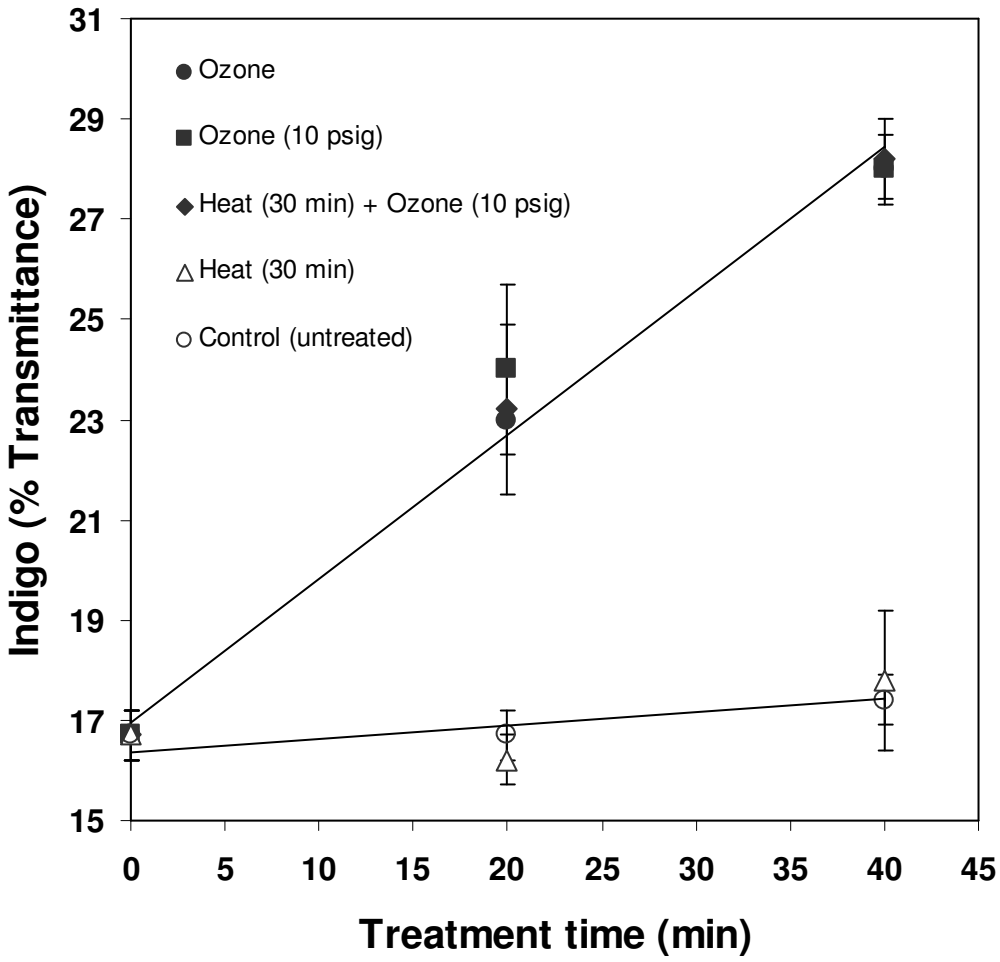

Figure A.1: Penetration of ozone across the shell of hen's eggs. Points represent the mean of measurements in experiments performed in triplicate. Error bars indicate standard deviation.

LIST OF REFERENCES

Achen, M., and A.E. Yousef. 2001. Efficacy of ozone against *Escherichia coli* O157:H7 on apples. J. Food Sci. 66: 1380-1384.

Adler. M.G., and G.R. Hill. 1950. The kinetics and mechanism of hydroxide iron catalyzed ozone decomposition in aqueous solution.

Alebić-Juretić, A., T. Cvitaš, and L. Klasnic. 2000. Kinetics of heterogeneous ozone reactions. Chemosphere. 41: 667-670.

American Egg Board. 2003. U.S. egg industry fact sheet. Available at http://www.aeb.org/eii/facts/industry-facts-10-2003.htm. Accessed 20 February, 2003.

American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. Standard methods for the examination of water and wastewater, 19th edition, Washington, DC.

Anderson, K.E., P.A. Curtis, and F.T. Jones. December 1995. Rapid chilling of shell eggs using cryogenic gases. U.S. patent 5,474,794.

Andrews, C.C., and O.J. Murphy. 2002. Generation and delivery device for ozone gas. U.S. patent 6,461,487.

Anellis, A., J. Lubas, and M.M. Rayman. 1954. Heat resistance in liquid eggs of some strains of the genus *Salmonella*. Food Res. 19: 377-395.

Angulo, F.J., and D.L. Swerdlow. 1999. Epidemiology of human *Salmonella enterica* serovar Enteritidis infections in the United States. pp. 33-41. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Bader, H., and J. Hoigné. 1981. Determination of ozone in water by the indigo method. Water Res. 15: 449-456.

Bailey, J.S., R.J. Buhr, N.A. Cox, and M.E. Berrang. 1996. Effect of hatching cabinet sanitation treatments on *Salmonella* cross-contamination and hatchability of broiler eggs. Poult. Sci. 75: 191-196.

Bartlett, F.M., J.M. Laird, C.L. Addison, and R.C. Mckellar. 1993. The analysis of egg wash water for the rapid assessment of microbial quality. Poult. Sci. 72:1584-1591.

Bell, C., and A. Kyrikiades. 2002. *Salmonella*. Blackwell Science Ltd., London.

Berrang, M.E., N.A. Cox, J.S. Bailey, and L.C. Blankenship. 1991. Methods for inoculation and recovery of *Salmonella* from chicken eggs. Poult. Sci. 70: 2267-2270.

Berrang, M.E., N.A. Cox, J.S. Bailey, and R.J. Buhr. 1995. Efficacy of ultra violet light treatment for elimination of *Salmonella* on hatching eggs. Poult. Sci. 74:50 (abstr.).

Bintsis, T., E. Litopoulou-Tzanetaki, and R.K. Robinson. 2000. Existing and potential applications of ultraviolet light in the food industry - a critical review. J. Sci. Food Agric. 80: 637-645.

Blatchley III, E.R., and M.M. Peel. 2001. Disinfection by ultraviolet irradiation, p. 823-851. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Board, R.G., J.C. Ayres, A.A. Kraft, and R.H. Forsythe. 1964. The microbial contamination of eggshells and egg packing materials. Poult. Sci. 43: 584-595.

Board, R.G., and H.S. Tranter. 1995. The microbiology of eggs, p. 81-104. *In* W.J. Stadelman, and O.J. Coterill (ed.), Egg science and technology, The Haworth Press Inc., Binghamton, NY.

Botsoglou, N.A., A.L. Yannakopoulus, D.J. Fletouris, A. S. Tserveni-Goussi, and P.D. Fortomaris. 1997. Effect of dietary thyme on the oxidative stability of egg yolk. J. Agric. Food Chem. 45: 3711-3716.

Brackett, R. 2000. Commercial processing technologies. Presented on the 6th annual symposia series on food safety in the 21st century: Shell eggs and egg products – safety issues. Inst. Food Technologists, Chicago, IL, May 18.

Brackett, R.E., J.D. Schuman, H.R. Ball, and J. Scouten. 2001. Thermal inactivation kinetics of *Salmonella* spp. within intact eggs heated using humidity-controlled air. J. Food Prot. 64: 934-938.

Brady, J.E., and G.E. Humiston. 1978. General Chemistry Principles and Structure. John Wiley & Sons. New York.

Braun, P., and K. Felhaber. 1995. Migration of *Salmonella* Enteritidis from the albumen into the egg yolk. Int. J. Food Microbiol. 25: 95-99.

Bruce, J., and E.M. Drysdale. 1994. Trans-shell transmission, p. 63-91. *In* R.G. Board and R. Fuller (ed.), Microbiology of the avian egg. Chapman and Hall, New York.

Buzby, J.C. 2002. Older adults at risk of complications from microbial foodborne illness. USDA Economic Research Service. FoodRev. 25: 30-35.

Catalano, C.R. and S.J. Knabel. 1994b. Destruction of *Salmonella* Enteritidis by high pH and rapid chilling during simulated commercial egg processing. J. Food Prot. 57:592-595, 603.

CDC. 1988. Update: *Salmonella* Enteritidis infections and *Salmonella* Enteritidis grade A shell eggs-United States. Morbid. Mortal. Weekly Rep. 37: August 19, 1988.

CDC. 1990. Epidemiologic notes and reports update: *Salmonella* Enteritidis infections and grade A shell eggs-United States, 1989. Morbid. Mortal. Weekly Rep. 38: 877-880.

CDC. 1993. Outbreaks of *Salmonella* Enteritidis gastroenteritis-California, 1993. Morbid. Mortal. Weekly Rep. 42: October 22, 1993.

CDC. 1996. Outbreaks of *Salmonella* serotype Enteritidis infection associated with consumption of raw eggs-United States, 1994-1995. Morbid. Mortal. Weekly Rep. 45: August 30, 1996.

CDC. 2003. Outbreaks of *Salmonella* serotype Enteritidis infection associated with eating shell eggs-United States, 1999-2001. Morbid. Mortal. Weekly Rep. 51: 1149-1152.

CFR. 1998. Refrigeration and labeling requirements for shell-eggs. Vol. 63. No. 166. Office Federal Register, Washington, DC.

CFR. 2000a. Food labeling, safe handling statements, labeling of shell eggs; refrigeration of shell eggs held for retail distribution. Vol. 65. No. 234. Office Federal Register, Washington, DC.

CFR. 2000b. Irradiation in the production, processing and handling of food. Vol. 65. No. 141. Office Federal Register, Washington, DC.

CFR. 2001. Secondary direct food additives permitted in food for human consumption. Vol. 66. No.123. Office Federal Register, Washington, DC.

Chantarapanont, W., L. Slutsker, R.V. Tauxe, and L.R. Beauchat. 2000. Factors influencing inactivation of *Salmonella* Enteritidis in hard-cooked eggs. J. Food Prot. 63: 36-43.

Cogan, T.A., G. Domingue, H.M. Lappin-Scott, C.E. Benson, M.J. Woodward, and T.J. Humphrey. 2001. Growth of *Salmonella* Enteritidis in artificially contaminated eggs: the effects of inoculum size and suspending media. Int. J. Food Microbiol. 70: 131-141.

Cohen, M.L., and R.V. Tauxe. 1986. Drug-resistant *Salmonella* in the United States: an epidemiologic perspective. Science. 234: 964-969.

Cottee, J., P. Kunstadt, and F. Fraser. 1995. Consumer acceptance of irradiated chicken and produce in the U.S.A. Radiat. Phys. Chem. 46: 673-676.

Cotterill, O.J. 1968. Equivalent pasteurization temperatures to kill *Salmonella* in liquid egg white at various pH levels. Poult. Sci. 72: 1584-1591.

Cox, J.P., J.M. Cox, and R.W. Cox. July 1995. Hyperpasteurization of food. U.S. patent 5,431,939.

Cox, N.A., M.E. Berrang, R.J. Buhr, and J.S. Bailey. 2000. Bactericidal treatment of hatching eggs IV. Hydrogen peroxide applied with vacuum and surfactant to eliminate *Salmonella* from hatching eggs. J. Appl. Poult. Res. 9: 530-534.

Cunningham, F.E. 1995. Egg product pasteurization, p. 289-321. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

D'Aoust, J.-Y. 1989. *Salmonella*, p.327-445. *In* M.P. Doyle (ed.), Foodborne bacterial pathogens, Marcel Dekker, New York.

D'Aoust, J.-Y. 2001. *Salmonella*, p.163-191. *In* R.G. Labbé, and S. Garcia (ed.), Guide to foodborne pathogens, Wiley-Interscience, New York.

Dave, S. 1999. Efficacy of ozone against *Salmonella* Enteritidis in aqueous suspensions and on poultry meat. [M.Sc. thesis]. Columbus, Ohio, U.S.A.: Ohio State University. p. 26-68. The Ohio State University, Columbus, Ohio.

Davey, K.R. 1992. A terminology for models in predictive microbiology. Food Microbiol. 9: 353-356.

Davies, R.H., and M. Breslin. 2003. Investigations into possible alternative decontamination methods for *Salmonella* Enteritidis on the surface of table eggs. J. Vet. Med. 50: 38-41.

Diaz, M.E., S.E. Law, and J.F. Frank 2001. Control of pathogenic microorganisms and turbidity in poultry-processing chiller water using UV-enhanced ozonation. Oz. Sci. Eng. 23: 53-64.

Dillon, D., R. Combes, M. McConville, and E. Zeiger. 1992. Ozone is mutagenic in *Salmonella*. Environ. Mol. Mutagen. 19: 331-337.

Dunn, J. 1996. Pulsed light and pulsed electric field for foods and eggs. Poult. Sci. 75: 1133-1136.

Dunn, J. 2001. Pulsed-light disinfection of water and sterilization of blow/fill/seal manufactured aseptic pharmaceutical products, p. 765-793. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Ebel E. and W. Schlosser. 2000. Estimating the annual fraction of eggs contaminated with *Salmonella enteritidis* in the United States. Int. J. Food Microbiol. 61: 51-62.

Ewell, A.W. 1946. Recent ozone investigation. J. Appl. Physics. 17: 908-911.

Fajardo, T.A., R.C. Ananthesawaran, V.M. Puri, and S.J. Knabel. 1995. Penetration of *Salmonella* Enteritidis into eggs subjected to rapid cooling. J. Food Prot. 58: 473-477.

Ferreira, M., C. Hofer, and A. Raemy. 1997. A calorimetric study of egg white proteins. J. Thermal Anal. 48: 683-690.

Finke, M., P. Shillam, T. McGivern, X. Wang, D. Vugia, S. Segler, D. Gerber, S. Stenzel, S. Zanski, M. Kennedy, R. Marcus, and the EIP FoodNet working group. Hospitalizations among cases with the most common serotypes of *Salmonella*: FoodNet, 1996-2000. International Conference on Emerging Infectious Diseases. Atlanta, GA, March 2002.

Fleischman, G.J., C.L. Napier, D. Stewart, and S.A. Palumbo. 2003. Effect of temperature on the growth response of *Salmonella Enteritidis* inoculated onto the vitelline membrane of fresh eggs. J. Food Prot. 66: 1368-1373.

Frenzen, P.D., J.C. Buzby, and T. Roberts. 1999. An updated estimate of the economic costs of human illness due to foodborne *Salmonella* in the Unites States. Proceedings of the 3rd. international symposium on the epidemiology and control of *Salmonella* in pork, August, Washington, D.C.

Froix, M.F., J. Pochan, A. Goedde, and D. Hinman. 1977. A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (*Gallus*/*gallus* (L.)). Polymer. 18: 1213-1218.

Funk, E.M. 1948. The relation of the yolk index determined in natural position to the yolk as determined after separating the yolk from the albumen. Poult. Sci. 27: 367.

Gast, R.K. 1993. Immersion in boiling water to disinfect egg shells before culturing egg contents for *Salmonella* Enteritidis. J. Food Prot. 56: 533-535.

Gast, R.K. and C.W. Beard. 1990. Production of *Salmonella* Enteritidis contaminated eggs by experimentally infected hens. Avian Dis. 34: 438-446.

Gast, R.K. and C.W. Beard. 1992. Detection and enumeration of *Salmonella* Enteritidis in fresh and stored eggs laid by experimentally infected hens. J. Food Prot. 55: 152-156.

Goerzen, P.R., and T.A. Scott. 1995. Ultraviolet light sanitation for broiler hatching eggs. Poult. Sci. 74: 83 (abstr.).

Gomez, T.M., Y. Molatjemi, S. Miyagawa, F.K. Kaferstein, and K. Stohr. 1997. Foodborne salmonellosis. World Health Stat. Q. 50: 81-89.

Gordon, G., W.J. Cooper, R.G. Rice, and G.E. Pacey. 1988. Methods for measuring disinfectant residuals. J. Am. Water Works Assoc. 80: 94-108.

Graham, D.M. 1997. Use of ozone for food processing. Food Technol. 51: 72-75.

Greer, G.G., and S.D.M. Jones. 1989. Effects of ozone on beef carcass shrinkage, muscle quality and bacterial spoilage. Can. Inst. Food Sci. Technol. J. 22: 156-160.

Grimes, H.D., K.K. Perkins, and W.F. Boss. 1983. Ozone degrades into hydroxyl radical under physiological conditions. Plant Physiol. 72: 1016-1020.

Grimont, P.A.D., P. Bouvet, F. Grimont, and J.-C. Desenclos. 1999. *Salmonella enterica* serovar Enteritidis in France: epidemiology, prevention, and control. pp. 43-49. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Guard-Petter, J. 2001. The chicken, the egg and *Salmonella* Enteritidis. Environ. Microbiol. 3: 421-430.

Guthrie, R.K. 1992. *Salmonella*, CRC, Boca Raton, FL.

Hammack, T.S., P.S. Sherrod, V.R. Bruce, G.A. June, F.B. Satchel, and W.H. Andrews. 1993. Growth of *Salmonella* Enterirtids in grade A eggs during prolonged storage. Poult. Sci. 72: 373-377.

Hansen, J.M., and H.L. Shaffer. 2001. Sterilization and preservation by radiation sterilization, p. 729-746. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Haugh, R.R. 1937. The Haugh unit for measuring egg quality. U.S. Egg and Poultry Magazine. 43: 553-555, 572-573.

Hennessy, T.W., C.W. Hedberg, L. Slutsker, K.E. White, J.M. Besser-Wiek, M.E. Moen, J. Feldman, W.W. Coleman, L.M. Edmonson, K.L. MacDonald, and M.T. Osterholm.1996. A national outbreak of *Salmonella* Enteritidis infections from ice cream. New Eng. J. Med. 334: 1281-1286.

Himathongkham, S., H. Riemann, and R. Ernst. 1999. Efficacy of disinfection of shell eggs externally contaminated with *Salmonella* Enteritidis. Implications for egg testing. Int. J. Food Microbiol. 49: 161-167.

Hoigné, J., and H. Bader. 1975. Ozonation of water; role of hydroxyl radicals as oxidizing intermediates. Science. 190: 782-784.

Holley, R.A. and M. Proloux. 1986. Use of egg washwater pH to prevent survival of *Salmonella* at moderate temperatures. Poult. Sci. 65: 922-928.

Horváth, M., L. Bilitzky, and J. Huttner. 1985. Ozone. Elsevier, New York.

Hou, H., R.K. Singh, P.M. Muriana, and W.J. Stadelman. 1996. Pasteurization of intact shell eggs. Food Microbiol. 13: 93-101.

Humphrey, T.J. 1994. Contamination of eggs with potential human pathogens, p. 93-116. *In* R.G. Board and R. Fuller (ed.), Microbiology of the avian egg. Chapman and Hall, New York.

Humphrey, T.J. 1999. Contamination of eggs and poultry meat with *Salmonella enterica* serovar Enteritidis, p. 183-192. *In* A.M. Saeed, R.K. Gast, M.E. Potter, and P.G. Wall (ed.), *Salmonella enterica* serovar Enteritidis in humans and animals: epidemiology, pathogenesis, and control, Iowa State University Press, Ames, IA.

Humphrey, T.J., A. Baskerville, S.L. Mawer, B. Rowe, and S. Hopper. 1989. *Salmonella* Enteritidis PT4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. 103: 415-423.

Humphrey, T.J., P.A. Chapman, B. Rowe, and R.J. Gilbert. 1990. A comparative study of the heat resistance of salmonellas in homogenized whole egg, egg yolk or albumen. Epidemiol. Infect. 104: 237-241.

Humphrey, T.J., A. Whitehead, A.H.L. Gawler, A. Henley, and B. Rowe. 1991. Numbers of *Salmonella* Enteritidis in the contents of naturally contaminated hen's eggs. Epidemiol. Infect. 106: 489-496.

Hunt, N.K., and B.J. Marinas. 1999. Inactivation of *Escherichia coli* with ozone: chemical and inactivation kinetics. Wat. Res. 33: 2633-2641.

Hunter, C. 2000. Changing attitudes to irradiation throughout the food chain. Radiat. Phys. Chem. 57: 239-243.

Hutchison, M.L., J. Gittins, A. Walker, A. Moore, C. Burton, and N. Sparks. 2003. Washing table eggs: a review of the scientific and engineering issues. World's Poult. Sci. J. 59: 233-248.

Hutchison, M.L., J. Gittins, A. Walker, N. Sparks, T.J. Humphrey, C. Burton, and A. Moore. 2004. An assessment of the microbiological risks involved with egg washing under commercial conditions. J. Food Prot. 67: 4-11.

Ito, H., H. Nakatani, H. Bamba, and T. Hayashi. 1999. Influence of the hatchability of Japanese quail eggs (hatching eggs) and sterilization effect for *Salmonella* by ozone gas sterilization. Res. Bull. Aichi Agric. Res. 31: 305-310.

Jacobs, P.T., and S-M. Lin 2001. Sterilization process utilizing low-temperature plasma, p. 747-763. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Jay, J.M. 1996. Modern food microbiology. Chapman & Hall, New York.

Jones, F.T., D.V. Rives, and J.B. Carey. 1995. *Salmonella* contamination in commercial eggs and an egg production facility. Poult. Sci. 74: 753-757.

JMP IN. 2001. JMP IN statistical software for Windows, 4.0.4. version. SAS Institute Inc., Cary, NC.

Juneja, V.K., B.S. Elben, and H.M. Marks. 2001. Modeling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. Int. J. Food Microbiol. 70: 37-51.

Juneja, V.K., and J.S. Novak. 2003. Adaptation of foodborne pathogens to stress from exposure to physical intervention strategies, p. 31-53. *In* A. E. Yousef and V. K. Juneja (ed.), Microbial stress adaptation and food safety, CRC Press, Boca Raton, FL.

Khadre, M.A., A. E. Yousef, and J.-G. Kim. 2001. Microbiological aspects of ozone applications in food: A review. J. Food Sci. 66: 1242-1252.

Khuri, A.I., and J.A. Cornell. 1996. Response surfaces: designs and analyses. Marcel Dekker, Inc. New York.

Kim, J-G. 1998. Ozone as an antimicrobial agent in minimally processed foods. Ph.D. Thesis. The Ohio State University, Columbus, OH.

Kim, J.-W and M.F. Slavik. 1996. Changes in eggshell surface microstructure after washing with cetylpyridinium chloride or trisodium phosphate. J. Food Prot. 59: 859-863.

Kim, J-G., A.E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Prot. 62: 1071-1087.

Kim, J.-G., A.E. Yousef, and M.A. Khadre. 2003. Ozone and its current and future application in the food industry. Adv. Food Nutr. Res. 45: 167-218.

King, A.D., and C.W. Nagel. 1975. Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa*. J. Food Sci. 40: 362-366.

Kinner, J.A. and W.A. Moats. 1981. Effect of temperature, pH, and detergent on the survival of bacteria associated with shell eggs. Poult. Sci. 60: 761-767.

Kira, S., and M. Ogata. 1988. Effect of ozone on denaturation and antigenicity of egg albumin. Okayama Igakkai Zasshi (J. Okayama Med. Assoc.). 100: 1-7

Koidis, P., M. Bori, and K. Vareltzis. 2000. Efficacy of ozone treatment to eliminate *Salmonella* Enteritidis from eggshell surface. Arch. Lebensmittelhyg. 51: 4-6.

Koike, K., G. Inoue, M. Suzuki, J. Saida, and I. Komatsu. 1998. Decomposition characteristics of concentrated ozone. J. Chem. Eng. Jap. 31: 195-200.

Komanapalli, I.R., and B.H.S. Lau. 1996. Ozone-induced damage of *Escherichia coli* K-12. Appl. Microbiol. Biotechnol. 46: 610-614.

Knape, K.D., J.B. Carey, R.P. Burgess, Y.M. Kwon, and S.C. Ricke. 1999. Comparison of chlorine with an iodine-based compound on eggshell surface microbial populations in a commercial egg washer. J. Food Safety. 19: 185-194.

Knape, K.D., J.B. Carey, and S.C. Ricke. 2001. Response of foodborne *Salmonella* spp. marker strains inoculated on egg shell surfaces to disinfectants in a commercial egg washer. J. Environ. Sci. Health. 36: 219-227.

Krivopishin, I.P. 1970. Permeability of the shell of chicken eggs for ozone. Sb. Rab. Molodykh Uch., Vses. Nauch.-Issled. Tekhnol. Inst. Ptitsevodstva. 12: 88-91.

Kuo, F.L., J.B. Carey, and S.C. Ricke. 1997a. UV irradiation of shell eggs: effect on populations of anaerobes, molds, and inoculated *Salmonella* Typhimurium. J. Food Prot. 60: 639-643.

Kuo, F.L., S.C. Ricke, and J.B. Carey. 1997b. Shell egg sanitation: UV radiation and egg rotation to effectively reduce populations of aerobes, yeasts, and molds. J. Food Prot. 60: 694-697.

Labbe, R.G., and M. Kinsley. 2001. Limitations in the use of ozone to disinfect maple sap. 64: 104-107.

Latala, A. and Z. Dobrzanski. 1989. Effect of ultraviolet radiation on the microbiological contamination of the shells of eggs for human consumption. Med. Wet. 45: 420-422.

Li-Chan, E.C.Y., W.D. Powrie, and S. Nakai. 1995. The chemistry of eggs and egg products. p. 105-175. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Lith, L.A.J.T. van, F.F. Putirulan, and R.W.A.W Mulder. 1995. Pasteurization of table eggs to eliminate *Salmonellae*. Arch. Geflügelk. 59: 157-160.

Lynntech, 1998. LT 1 Electrochemical ozone generator instruction's manual. College Station , TX.

Ma, C.Y. 1996. Effects of gamma irradiation on physicochemical and functional properties of eggs and egg products. Radiat. Phys. Chem. 48: 375.

Ma, C.-Y., M.R. Sahasrabudhe, L.M. Poste, V.R. Harwalkar, J.R. Chambers, and K.P.J. O'Hara. 1994. Gamma irradiation and physicochemical properties of eggs and egg products, p. 283-300. *In* J.S. Sim, and S. Nakai (ed.), Egg uses and processing technologies, CAB International, Oxon, UK.

Marshall, A.C., A.R. Sams, and M.E. Van Elswyk. Oxidative stability and sensory quality of stored eggs from hens fed 1.5% menhaden oil. J. Food Sci. 59: 561-563.

McMeekin, T.A., J.N. Olley, T. Ross, and D.A. Ratkowsky. 1993. Predictive microbiology: theory and application. John Wiley and Sons Inc. New York.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5: 607-625.

Merck Index, 1989. 11th Edition. Budavari, Merck & Co., New Jersey.

Mermelstein, N.H. 2000. Cryogenic system rapidly cools eggs. Food Technol. 54: 100-103.

Mermelstein, N.H. 2001. Pasteurization of shell eggs. Food Technol. 55: 72-73, 79.

Michalski, C.B., R.E. Brackett, Y.-C. Hung, and G.O.I. Ezeike. 1999. Use of capillary tubes and plate exchanger to validate U.S. department of agriculture pasteurization protocols for elimination of *Salmonella* Enteritidis from liquid egg products. J. Food Prot. 62: 112-117.

Mitsuda, H., M.J.A.H. Ominami, and A. Yamamoto. 1990. Synergistic effect of ozone and carbon dioxide gases for sterilizing food. Proc. Japan Acad. 66: 68-72.

Moats, W.A. 1978. Egg washing-a review. J. Food Prot. 41: 919-925.

Moon, S., and K.B. Song. 2000. Effect of high dose γ-irradiation on the physicochemical properties of shell eggs during storage. Han'guk Nonghwa Hakhoechi. 43: 260-265.

Murray, R.G., S. Pamela, and H.E. Elson. 1965. Location of mucopeptide of selection of the cell wall of *E. coli* and other gram-negative bacteria. Can. J. Microbiol. 11: 547-560.

Neter, J., M. Kutner, C.J. Nachtsheim, and W. Wasserman. 1996. Applied linear regression models. Times Mirror Higher Education Group, Chicago.

Novak, J. S., and J. T. C. Yuan. 2003. Viability of *Clostridium perfringens*, *Escherichia coli*, and *Listeria monocytogenes* surviving mild heat or aqueous ozone treatment on beef followed by heat, alkali, or salt stress. J. Food Prot. 66: 382-389.

Okubo, T., S. Akachi, and H. Hatta. 1997. Structure of hen eggs and physiology of egg laying, p. 1-12. *In* T. Yamamoto, L.R. Juneja, H. Hatta, and M. Kim (ed.), Hen eggs. CRC Press, Boca Raton.

Padron, M.N. 1990. *Salmonella* Typhimurium penetration through the eggshell of hatching eggs. Avian Dis. 34: 463-465.

Padron, M. 1995. Egg dipping in hydrogen peroxide solution to eliminate *Salmonella* Typhimurium from eggshell membranes. Avian Dis. 39: 627-639.

Paganelli, C.V., A. Ar, H. Rahn, and O.D. Wangensteen. 1975. Diffusion in the gas phase: the effects of ambient pressure and gas composition. Respir. Physiol. 25: 247-258.

Pegg, R.B. 2001. Spectrophotometric measurement of secondary lipid oxidation products, p. D2.4.1-D2.4.18. *In* Wrolstad, R.E., T.E. Acree, H. An, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, and P. Sporns (ed.), Current protocols in food analytical chemistry, vol.1. John Wiley & Sons Inc., Hoboken, N.J.

Pflug, I.J., R.G. Holcomb, and M.M. Gomez. 2001. Principles of thermal destruction of microorganisms, p. 79-129. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams & Wilkins, Philadelphia, PA.

Popoff, M.Y., J. Bockemül, and F.W. Brenner. 1998. Supplement 1997 (no. 41) to the Kauffmann-White scheme. Res. Microbiol. 149: 601-604.

Poppe, C. 1999. Epidemiology of *Salmonella enterica* serovar Enteritidis. pp. 3-18. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

President's Council on Food Safety, 1999. Egg safety from production to consumption: an action plan to eliminate *Salmonella* Enteritidis illnesses due to eggs. USDA, HHS, EPA, Dept. of Commerce.

Rahn, H., A. Ar, and C.V. Paganelli. 1979. How bird eggs breathe. Sci. Amer. 240: 45-55.

Rahn, H., C.V. Paganelli, and A. Ar. 1987. Pores and gas exchange of avian eggs: a review. J. Exp. Zool. Suppl. 1: 165-172.

Rice, R.G. 1986. Analytical aspects of ozone: treatment of water and wastewater. Lewis Publishers, Chelsea, MI.

Rizk, S.S., J.C. Ayres, and A.A. Craft. 1966. Effect of holding condition on the development of salmonellae in artificially inoculated hens' eggs. Poult. Sci. 45: 823-829.

Romanoff, A.L., and A.J. Romanoff. 1949. The avian egg. John Wiley & Sons, New York.

Rusell, S.M. 2003. The effect of electrolyzed oxidative water applied using electrostatic spraying on pathogenic and indicator bacteria on the surface of eggs. Poult. Sci. 82: 158-162.

SAS Institute. 2001. JMP IN® version 4.0.4. SAS Institute Inc., Cary, NC.

Schuman, J.D., B.W. Sheldon, J.M. Vandepopuliere, and H.R. Ball. 1997. Immersion heat treatments for inactivation of *Salmonella* Enteritidis with intact eggs. J. Appl. Microbiol. 83: 438-444.

Schuman, J.D. 2000. Methods for thermal processing of shell eggs. Presented in the 6th annual symposia series on Food Safety in the 21st century: Shell eggs and egg products – safety issues. Inst. Food Technologists, Chicago, IL, May 18.

Scott, V.N. 1989. Interaction of factors to control microbial spoilage of refrigerated foods. J. Food Prot. 52: 431-435.

Scott, D.B.M., and E.C. Lesher. 1963. Effect of ozone on survival and permeability of *Escherichia coli*. J. Bacteriol. 85:567-576.

Serrano, L.E., E.A. Murano, K. Shenoy, and D.G. Olson. 1997. D values of *Salmonella* Enteritidis isolates and quality attributes of shell eggs and liquid whole eggs treated with irradiation. Poult. Sci. 76: 202-205.

Shama, G. 2000. Ultraviolet light. pp. 2208-2214. In R.K. Robinson, C. Batt, and P. Patel (ed.) Encyclopedia of Food Microbiology. Academic Press, London.

Sharma, R. R., A. Demirici, L. R. Beuchat, and W. F. Fett. 2003. Application of ozone for inactivation of *Escherichia coli* O157:H7 on inoculated alfalfa sprouts. J. Food Process. Preserv. 27: 51-64.

Shechter, H. 1973. Spectrophotometric method for determination of ozone in aqueous solutions. Water Res. 7: 729-739.

Shimada, K., and S. Matsushita. 1980. Thermal coagulation of egg albumin. J. Agric. Food Chem. 28: 409-412.

Shivaprasad, H.L., J.F. Timoney, S. Morales, B. Lucio, and R.C. Baker. 1990. Pathogenesis of *Salmonella* Enteritidis infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serological responses. Avian Dis. 34: 548-557.

Stadelman, W.J. 1995a. The egg industry, p. 1-37. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Stadelman, W.J. 1995b. Quality identification of shell eggs, p. 39-66. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, Inc., Binghamton, NY.

Stadelman, W.J. 1995c. The preservation of quality in shell eggs, p. 67-79. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Stadelman, W.J., R.K. Singh, P.M. Muriana, and H. Hou. 1996. Pasteurization of eggs in the shell. Poult. Sci. 75: 1122-1125.

Tellez, I.G., R.M. Trejo, R.E. Sanchez, R.M. Ceniceros, Q.P. Luna, P. Zazua, and B.M. Hargis. 1995. Effect of gamma irradiation on commercial eggs experimentally inoculated with *Salmonella* Enteritidis. Radiat. Phys. Chem. 46: 789-792.

Thiagarajan, D. 1995. Studies on the mechanism of transovarian transmission of *Salmonella* Enteritidis in laying hens. Ph.D. thesis. Purdue University, West Lafayette.

Thompson, W.S., F.F. Busta, D.R. Thomson, and C.E. Allen. 1979. Inactivation of *Salmonellae* in autoclaved ground beef exposed to constantly rising temperatures. 42: 410-415.

Timoney, J.F., H.L. Shivaprasad, R.C. Baker, and B. Row. 1989. Egg transmission after infection of hens with *Salmonella* Enteritidis phage type 4. Vet. Rec.125: 600-601.

Todd, E.C.D. 1996. Risk assessment of use of cracked eggs in Canada. Int. J. Food Microbiol. 30: 125-143.

Tschäpe, H., A. Liesegang, B. Gericke, R. Prager, W. Rabasch, and R. Helmuth 1999. Ups and downs of *Salmonella enterica* serovar Enteritidis in Germany. pp. 51-61. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Unal, R., J-G. Kim, and A.E. Yousef. 2001. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulse electric field. J. Food Prot. 64: 777-782.

USDA. 1966. Improved methods, techniques and equipment for cleaning eggs. Marketing Research Report No. 757.

USDA, Agricultural Marketing Service. 1997. Pasteurized shell eggs (pasteurized in-shell eggs). Vol. 162. No. 185: 49955-57. Fed. Regist. Washington, DC.

USDA. 2003. Egg-grading manual. Handbook no. 75. Available at http://www.ams.usda.gov/poultry/pdfs/EggGrading manual.pdf. Accessed 7 February, 2003.

Vandepopuliere, J.M., and O.J. Cotterill. December 1999. Method of controlling *Salmonella* in shell eggs. U.S. patent 6,004,603.

Wall, P.G., and L.R. Ward. 1999. Epidemiology of *Salmonella enterica* serovar Enteritidis phage type 4 in England and Wales. pp. 19-25. *In* A.M. Saeed, P.K. Gast, M.E. Potter, and P.G. Wall (ed.) *Salmonella enterica* serovar Enteritidis in humans and animals. Iowa State University Press. Ames, IA.

Wangensteen, O.D., D. Wilson, and H. Rahn. 1970/71. Diffusion of gases across the shell of the hen's egg. Respir. Physiol. 11: 16-30.

Watkins, B.A. 1995. The nutritive value of the egg, p. 177-194. *In* W.J. Stadelman, and O.J. Cotterill (ed.), Egg science and technology. The Haworth Press, New York.

Weavers, L.K., and G.B. Wickramanayake. 2001. Disinfection and sterilization using ozone, p. 205-214. *In* S.S. Block (ed.), Disinfection, sterilization, and preservation, Lippincott Williams and Wilkins, Philadelphia, PA.

Whistler, P.E., and B.W. Sheldon. 1989. Bactericidal activity, eggshell conductance, and hatchability effects of ozone versus formaldehyde disinfection. Poult. Sci. 68:1074-1077.

Wickramanayake, G.B. 1984. Kinetics and mechanism of ozone inactivation of protozoan cysts. Ph.D. thesis. The Ohio State University, Columbus.

Wojctowicz, J.A. 1996. Ozone, p. 953-994. *In* R.E. Kirk, and D.E. Othmer (ed.), Encyclopedia of Chemical Technology, vol. 17. Wiley-Interscience, New York.

Wong, E., R.H. Linton, and D.E. Gerrad. 1998. Reduction of *Escherichia coli* and *Salmonella* Senftenberg on pork skin and pork muscle using ultraviolet light. Food Microbiol. 15: 415-423.

Wong, P.Y.Y., and D.D. Kitts. 2003. Physicochemical and functional properties of shell eggs following electron beam irradiation. J. Sci. Food Agric. 83: 44-52.

Worley, B.S., W.B. Wheatley, S.D. Lauten, D.E. Williams, E.C. Mora, and S.D. Worley. 1992. Inactivation of *Salmonella* Enteritidis on shell eggs by novel N-halamine biocidal compounds. J. Ind. Microbiol. 11: 37-42.

Wynn, C.S., B.S. Kirk, and R. McNabey. 1973. Pilot plant for tertiary treatment of wastewater with ozone. EPA report R2-73-146, US EPA, Municipal Environ. Res. Lab., Cincinnati, OH.

Yamasaki, M., N. Takahasi, and M. Hirose. 2003. Crystal structure of s-ovalbumin as a non-loop-inserted thermostabilized serpin form. J. Biol. Chem. 278: 35524-35530.

Zeidler, G. 2001a. Processing and packaging shell eggs, p. 1129-1161. *In* D.D. Bell, and D. Weaver Jr. (ed.), Commercial chicken meat and egg production, Kluwer Academic Publishers, Norwell, MA.

Zeidler, G. 2001b. Shell egg quality and preservation, p. 1199-1217. *In* D.D. Bell, and D. Weaver Jr. (ed.), Commercial chicken meat and egg production, Kluwer Academic Publishers, Norwell, MA.