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## Application of ozonated water technology for improving quality and safety of peeled shrimp meat

Amrish Suresh Chawla  
*Louisiana State University and Agricultural and Mechanical College*

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**APPLICATION OF OZONATED WATER TECHNOLOGY FOR  
IMPROVING QUALITY AND SAFETY OF PEELED SHRIMP MEAT**

**A Thesis**

**Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science**

**in**

**The Department of Food Science**

**by**

**Amrish Suresh Chawla  
Bachelor of Technology in Dairy Technology  
Gujarat Agricultural Univeristy, India, 2002  
August 2006**

## **DEDICATION**

To my parents Meena and Suresh Kumar Chawla and my sister Shuchi Chawla for their love, support and encouragement.

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Most useful work is not accomplished by a single person or by chance. Much effort and devotion from many people has been put into this research to get it published. I would like to thank all those to whom I owe even a little time, material and support from my heart.

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## ABSTRACT

Ozone is an effective sanitizing agent against a broad spectrum of pathogenic and spoilage organisms. Optimization of treatment applications of ozonated water is needed for increased use in the food industry.

An experimental apparatus and process has been developed to digitally measure ozone concentrations in processing water at the point of product application. Two application methods were evaluated. Ozone concentrations were measured rapidly at the point of product application. Shrimp samples were either sprayed or soaked for 20, 40 or 60 seconds with similar volumes of water with dissolved ozone levels of 1, 2, or 3 ppm. Microbial destruction using aerobic plate counts (APC), and lipid oxidation using the TBARS test, was measured to determine an optimal treatment.

Lowering the water temperature to 10°C facilitated the production of elevated levels of dissolved ozone ( $dO_3$ ), whereas high chlorine levels reduced  $dO_3$ . The soaking treatment resulted in greater bacterial reduction than the spray treatment of peeled shrimp, and application time had little effect at low concentrations of  $dO_3$ .

Well handled shrimp samples were then treated within 24 h of harvest using the optimal treatment of soaking in 3 ppm for 60 s. Peeled shrimp were sampled at two day intervals to evaluate APC and rancidity and at 3 day intervals for bioamines (putrescine and cadaverine) using gas chromatography (GC). Sensory quality changes were evaluated using consumer sensory testing. A *Listeria monocytogenes* inoculation study was also conducted.

Treated shrimp took 16 days to reach bacterial loads of  $10^7$  CFU/g as compared to untreated shrimp which showed these levels at day 12. Day 12 and day 15 untreated shrimp were rejected by a majority of the consumer panel and treated shrimp were not, based on their odors of decomposition. These rejected untreated shrimp showed  $>2.6$  ppm putrescine and  $>1.5$  ppm cadaverine. Untreated shrimp reached spoilage levels of  $10^7$  CFU/g 4 days before treated samples during iced storage (12 vs. 16 days). Shrimp inoculated with *L. monocytogenes* serotype (1/2a) and *L. monocytogenes* serotype (4b) resulted in a  $>10^4$  CFU/g reduction after treatment. As expected oxidative rancidity did not increase in any of these studies.

## **1. INTRODUCTION**

Shrimp has surpassed canned tuna as the most popular seafood consumed in the U.S.: however, most of this shrimp is imported. The state of Louisiana leads the nation in shrimp production at over 210 million pounds a year, and is the second highest producing state of finfish at 1.3 billion pounds annually (NMFS, 2006). High product quality is a critical priority for seafood processors. Improvements in product quality, safety, and shelf life result in increased product reception and consumption, decreased discards and loss, and protection from regulatory actions. Shrimp is the most economically valuable fishery in Louisiana, and mechanically peeled and frozen shrimp accounts for the major product form processed in the state and the Gulf of Mexico region (Schwab, personal communication, 2005). However the viability of the domestic shrimp industry is under constant economic pressure from the high volumes of hand peeled and low cost imported shrimp (Anon, 2004)

Improved product quality is necessary for the domestic industry to compete with the imported product and support national shrimp industry marketing initiatives (DeSantis, 2003). The wild shrimp industry is also under constant threat from aquacultured shrimp as it is of a high quality and readily available. The high volumes of shrimp landings in the Gulf of Mexico waters has resulted in the development of high volume mechanical peeling operators. Due to the variable pre and post harvest conditions of the wild shrimp fisheries, wild shrimp can develop increased levels of spoilage bacteria. Using ozonated water in the peeling operations presents an opportunity to reduce

bacterial levels and improve the quality and shelf life of peeled shrimp. This high quality shrimp will present the shrimp industry better chances to compete with the low cost imports.

Ozone is a USFDA and USDA approved antibacterial agent that can be applied to food products (FDA, 1982; USDA, 1997). Ozone has seen use in the food processing industry as gaseous ozone and dissolved in water as ozonated water. Both have been used as a bactericide on a wide range of food products including meat, poultry, eggs, raw fruits and vegetables, seafood and fruit juices, as well as sanitation of product contact surfaces (Guzel-Seydim et al., 2003; Anon, 2005). Ozone has many advantages as a sanitizer. Gaseous ozone and ozonated water can be generated on site, at the facility of use. Chemical reaction of ozone with organic material occurs at very rapid rates and short reaction times, which effectively prevents microorganisms from developing tolerance to ozone (Kim, 1998). Many studies have reported the advantages and superior bactericidal properties of ozone as compared to chlorine (Green et al., 1993; Kim and Yousef, 1999; Klaiber et al., 2004). Ozone has a short half life at ambient temperature and does not leave behind residues unlike other commonly used sanitizers. Ozone is effective against a wide range of bacteria, viruses, yeast, molds and protozoa (Anon, 2005; Guzel-Seydim et al., 2003). The effectiveness and susceptibility of microorganisms to  $\text{dO}_3$  depends upon the water temperature and pH, and the presence of dissolved compounds (salt, sugar, minerals and surfactants) and suspended organic matter (Kim and Yousef, 1999).

Despite these advantages, the use of ozonated water technology has not been widely adopted in the seafood industry. Recent studies of ozonated water on salmon fillets and roe found increased oxidation during frozen storage of these high lipid products (Carpo et al., 2004). In addition to incurred capital and operational costs, seafood processors have received limited understanding of  $dO_3$  solubility, including the effects of temperature and water quality. These inputs and off-gassing often result in the incorrect measurement and knowledge of actual ozone concentrations at the point of product application (Chawla et al., 2006a). Optimized ozonated water time-concentration treatments have shown significant reduction in aerobic and spoilage bacterial (Chawla et al., 2006b).

These results suggest that ozonated water technology can be successfully used as a germicidal agent in seafood processing to extend the shelf life and quality of wild shrimp in a time when efforts are being made to eliminate the use of commonly used chlorine due to its ability to form potential carcinogens on reacting with organic matter (Graham, 1997; Anon, 2005)

## **2. LITERATURE REVIEW**

### **2.1. U.S. Shrimp Species**

U.S. shrimp landings consist of cold and warm-water shrimp (Haby et al., 2002). Due to variation in the water quality of coastal bays and estuaries that are home to juvenile shrimp a wide variation is seen in the domestic landings (DeSantis, 2003). Cold-water shrimp are landed primarily off the northwest and northeast coasts of the U.S. and usually account for less than 20% of the total annual landings of all shrimp in the U.S. However, the majority of domestic landings are warm-water shrimp, which are landed primarily in the Gulf of Mexico and South Atlantic (GSA) region (Haby et al., 2002). The primary species landed in the GSA region include pink shrimp (*Farfantepenaeus duorarum*), white shrimp (*Litopenaeus setiferus*), and brown shrimp (*F. aztecus*). Smaller quantities of other species are landed, including rock shrimp (*Sicyonia brevirostris*), royal reds (*Pleoticus robustus*), and seabobs (*Xiphopenaeus kroyeri*). The warm-water shrimp harvesting industry in the (GSA) region represents the most economically important component of all of the domestic commercial seafood harvesting sectors in the United States. The shrimp industry contributes to local coastal economies on several levels. Shrimp are offloaded by shore-side handling facilities, which then set in motion a myriad of economic activities associated with processing, packing, wholesale distribution and consumer expenditures.

### **2.2. Ozone**

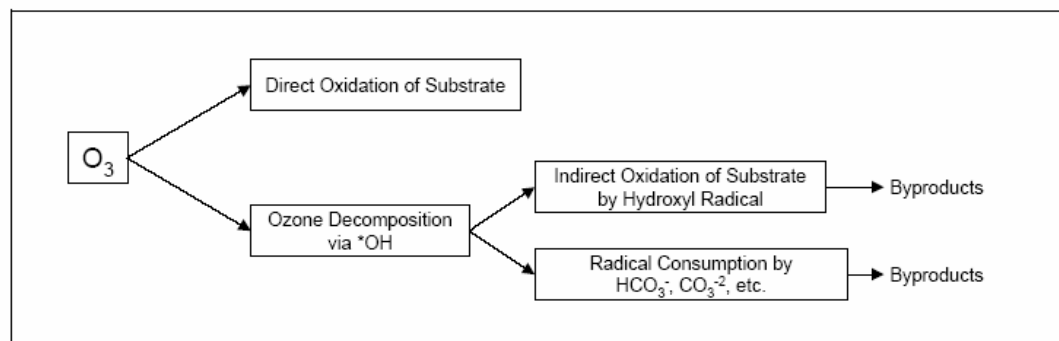
Ozone (O<sub>3</sub>) is an unstable form of elemental oxygen (O<sub>2</sub>) and was discovered in 1839 by a European researcher C.F. Schonbein (Guzel Seydim et



al., 2003). It derives its name from the Greek words oxus (acid) and gennan (generate). Ultra Violet (UV) radiation emitted by the sun having wavelengths less than 240 nm reacts with molecular ozone in the stratosphere and produces ozone. It is also formed by the lightening discharge during a thunderstorm, and is the clean smell attributed to air. It is also produced on the troposphere as an irritant from the internal combustion engines, where nitrous oxide produced from combustion of fossil fuels combines with oxygen and produces ozone.

### 2.2.a. Reaction Mechanism

Ozone has been shown to decompose in water and produce hydroxyl free radicals (Hoigné and Bader, 1983a, 1983b; Glaze et al., 1987). As shown in Figure 1.2, ozone can oxidize compounds by the following two methods in aqueous solutions: direct reaction with molecular ozone or reaction with hydroxyl free radicals produced during ozone decomposition (Hoigné and Bader, 1977).



**Figure 2.1. Oxidation Reactions of Compounds during Ozonation of Water (Source EPA Guidance Manual, 1999)**

Both of these oxidation pathways are inherently different and compete with each other for the oxidation of the substrate. Aqueous ozone occurs in a higher concentration than hydroxyl free radicals, under normal conditions of

oxidation. However, direct oxidation with aqueous ozone is slow compared to oxidation with hydroxyl free radicals, which have a faster reaction rate. Direct oxidation with ozone is important under acidic conditions; oxidation is caused by hydroxyl free radicals under conditions of high pH, presence of UV radiation or hydrogen peroxide addition (Hoigné and Bader, 1977). This latter mechanism is used in advanced oxidation processes such as peroxone, to increase the oxidation rates of substrates. The spontaneous decomposition of ozone occurs through a series of steps. The exact mechanism and reactions associated have not been established, but mechanistic models have been proposed (Hoigné and Bader, 1983a, 1983b; Glaze, 1987).

Ozone decomposition in water proceeds in a step wise manner producing the hydroperoxyl ( $\bullet\text{OH}_2$ ), hydroxyl ( $\bullet\text{OH}$ ) and superoxide( $\bullet\text{O}^-$ ) radicals (Adler and Hill, 1984; Grimes et al., 1983; Hoigne and Bader, 1975). It is believed that hydroxyl radicals form as one of the intermediate products, and can directly react with compounds in the water. The decomposition of ozone in pure water proceeds with hydroxyl free radicals produced as an intermediate product of ozone decomposition, resulting in the net production of 1.5 mole hydroxyl free radicals per mole ozone. Ozone forms hydroxyl radicals and reacts with many compounds naturally occurring in water such as natural organic matter, organic oxidation by-products, synthetic organic compounds bicarbonates and carbonate ions. Bicarbonate or carbonate ions, commonly measured as alkalinity, will scavenge the hydroxyl radicals and form carbonate radicals (Staehelin et al., 1984; Glaze and Kang, 1988). The dissociation rate constants of many of these compounds are high, and hence

they will react with the hydroxyl radicals before interacting with dispersed particles such as microorganisms. This stepwise breakdown reaction of ozone is extremely rapid, leading to the suggestion that the antimicrobial effect of ozone is only a surface phenomenon (Hoigne and Bader, 1975).

### **2.2.b. Ozone Measurement**

Many methods are available to measure ozone in both gaseous and aqueous forms. These methods can be classified as physical, chemical, and physiochemical. The physical methods measure responses such as adsorption of radiation in the visible, UV or infrared (IR) regions. Adsorption spectra at 552 nm measures reactions with chemical agents such as Bis-TerpyridineIron(II) in dilute hydrochloric acid solution (Tomiyasu and Gordon, 1984). Spectrophotometric measurement of ozone bleaching of acid chrome violet K (ACVK) can also be used (Masschelein, 1977). Chemical methods measure formation of reaction products when ozone reacts with chemical reagents such as potassium iodide (KI) or hydrogen iodide (HI). The physiochemical methods measure the physical effects of ozone reaction with different reagents such as chemiluminescence and the heat of reaction.

The two most commonly used methods for ozone measurement are UV adsorption and Indigo colorimetry.

Gaseous ozone absorbs light in the short UV wavelength region with a maximum absorbance at 253.7 nm (Gordon et al., 1992). In general, the instrument measures the amount of light absorbed when no ozone is present and the amount of light absorbed when ozone is present. The meter output is the difference of the two readings, which is directly related to the actual

amount of ozone present. The International Ozone Association (IOA) has accepted this procedure (IOA, 1989).

The indigo colorimetric method developed by Bader and Hogine (1981) is the standard method for measuring ozone concentrations in researches with ozone. The indigo colorimetric method is sensitive, precise, fast, and more selective for ozone than other methods. Ozone binds across the carbon-carbon double bond of a sulphonated indigo dye and decolorizes it. There are two indigo colorimetric methods: spectrophotometric and visual. For the spectrophotometric procedure the lower limit of detection is 2 mg/L, while for the visual procedure the detection limit is 10 mg/L. Hydrogen peroxide, chlorine, manganese ions, ozone decomposition products, and the products of organic ozonation exhibit less interference with the indigo colorimetric method than any of the other methods (Langlais et al., 1991).

### **2.2.c. Antimicrobial Effect of Ozone**

Ozone is a potent oxidizing agent that can be used for disinfection in the food industry (Rice, 1996). Low concentrations of ozone and shorter contact times are necessary compared to other weaker oxidizers such as chlorine, mono-chloramine and chlorine dioxide (DeMers and Renner, 1992). Since ozone is highly unstable, it does not maintain a high residual level and can be only used as a primary disinfectant (Bader and Hoigen, 1981). This often requires the use of other disinfectants like UV radiation, pulsed electric fields or other chemical agents like chlorine, chloramines or chlorine dioxide in combination with ozone to attain sufficient levels of disinfection in food products (Kim and Yousef, 1999) A comprehensive review published in 2001

has shown the effectiveness of ozone as a germicidal agent against a wide range of pathogenic organisms like bacteria, virus's protozoa, fungal and bacterial spores (Kim and Yousef, 1999). According to one study, the disinfection efficiency of ozone is not affected by pH. However, as the rate of decay of the hydroxyl free radicals is faster at higher pH, more ozone is needed to maintain concentration (Morris, 1975). Ozone decomposition is a complex function of pH, temperature, concentration of inorganic and organic matter and humidity (Hoigne and Bader, 1975, 1976)

pH has little effect on the ability of  $\text{dO}_3$  residuals to inactivate acid fast bacteria such as *Actinomyces* and *Mycobacterium* (Farooq et al., 1976). The virucidal efficacy of ozone decreased only slightly in one study as pH was lowered (Roy, 1979). Several different types of oxidants are formed by the decomposition of ozone at high pH and they have different reactivities (Langlais et al., 1991) These secondary oxidants like hydroperoxyl ( $\bullet\text{OH}_2$ ), hydroxyl ( $\bullet\text{OH}$ ) and superoxide ( $\bullet\text{O}^-$ ) radicals, with their different reaction rates are responsible for changes in the disinfection efficacy of ozone. The degree of microbial inactivation remained unchanged when inactivation studies were carried out at a constant residual ozone concentration and different pH (5.7–10.1) (Farooq, 1977). Studies conducted with poliovirus 1 and with rotaviruses SA-11 and Wa have shown decreased virus inactivation by ozone at alkaline pH (Harakeh and Butler, 1984; Vaughn et al, 1987). Ozone resulted in increased inactivation of *Giardia muris* cysts when the pH was increased from 7-9 (Wickramanayake, 1984a). It is believed that changes in cyst chemistry facilitated ozone interaction with cyst constituents at higher pH

values. However, inactivation of *Naegleria gruberi* cyst in the same study showed slower inactivation at pH 9 than at low pH levels, indicating that inactivation at different pH levels are organism specific.

The solubility of  $dO_3$  and its stability decreases as water temperature increases (Katzenelson, 1974). However, an increase in temperature does not affect the chemical oxidation and disinfection rates of ozone. While ozone decomposition increases and its solubility are significantly reduced when water temperature is raised from 0 to 30°C, there is virtually no effect on rates of bacterial disinfection (Kinman, 1975). Ozonated water treatment of contaminated apples showed no significant reduction in surface *E. coli* counts at 4°C, 22°C and 40°C, although the highest concentration of ozone was recorded at 4°C (Achen and Yousef, 2001).

Ozone treatments of river waters heavily polluted with organic matter were investigated. Bacterial levels in such waters were found to increase. It is suggested that ozonation of organic material in river waters produced small organic fragments that were readily metabolized by microorganisms (Trojan and Hanson, 1989). Food systems are rich in organic matter which will compete with microorganisms for ozone demand, and thus reduce its disinfection efficiency. The addition of 20ppm soluble starch did not significantly affect the destruction of gram positive organisms (including *L. monocytogenes*, *S. aureus*) and gram negative organisms (including *E. coli* and *Salmonella*). However a significant reduction in the bacteria was found in the presence of 20 ppm bovine serum albumin (Restanio et al., 1995). Residual levels of ozone in water containing bovine serum albumin were lower than

those in water with soluble starch and deionized water. Pure cell suspensions of *P. fluorescens*, *E. coli* O157:H7, *L. mesentroids*, and *L. monocytogenes* showed a  $10^2$  to  $10^3$  log reduction when subjected to 1 ppm of ozone gas for less than 10s(Kim, 1998).

Dehydrated cells resulting from low relative humidity (RH) showed increased resistance to gaseous ozone (Guerin, 1963). Studies conducted with low ozone levels at variable RH levels have found that at an RH level of 45%, ozone showed no germicidal potential (Elford and Eude, 1942). Low ozone concentrations (0.1mg/l), when used at high humidity levels, resulted in substantial bacterial reductions (Ewell, 1946). Kim and Yousef (1999) found that 200 ppm ozone produced no effect on the microbial load at  $a_w$  of 0.85 while upto  $10^5$  CFU/g were observed at  $a_w$  of 0.95.

All bacteria are not present on the surface of foods or in free suspensions in food systems. Association of bacteria with suspended particles or sub cellular components may reduce the effectiveness of the ozone treatment applied to the food product. Studies conducted by Langlais et al. (1978) indicated that it is necessary to consider criteria such as degree of agitation and mass transfer to establish the efficacy of ozone as a disinfectant. The antimicrobial effect of ozone was increased substantially upon application of sonication to break down clumps of microorganisms (Berg et al., 1964). However, such an effect was not found in the treatment of fresh lettuce with ozone by Kim and Yousef (1999).

#### **2.2.d. Mechanism of Action**

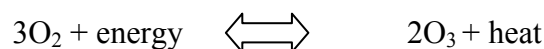
Oxidation reactions are responsible for inactivation of bacteria treated with ozone (Bringmann, 1954; Chang, 1971). The first point of contact between ozone and bacteria is the bacterial cell membrane, via oxidation of amino acids like tryptophan, or by oxidation of cell wall glycoproteins and glycolipids (Scott and Lesher, 1963; Goldstein and Mcdonagh, 1975). Dave (1999) found that ozone disrupted the cell walls of *Salmonella enteritidis*. Ozone may also disrupt the normal functioning of the cell by reacting with the slufhydryl groups of enzymes important to the cell metabolism, particularly the cystein residues (Ingram and Haines, 1949; Chang, 1971). Ozone has been found to interact with both the purine and pyrimidine bases of nucleic acids and modify them with thymine being more sensitive than uracil or cytosine (Giese and Christensen, 1954; Scott and Lesher, 1963; Ishizaki et al, 1981). More recent work has shown that ozone treatment does not destroy spores by causing DNA damage, but affects spore germination by damaging the inner membrane of the spore coat (Young, 2000). Ozone is shown to have produced single and double-strand breaks in plasmid DNA and to open up circular plasmid DNA (Hamelin, 1985). Ozone treatment also decreased transcription activity of plasmid DNA (Mura and Chung, 1990). Ozone has also been shown to cause mutation in *E. coli.*, however ozone was considered to be a weak mutagen (Dubeau and Chung, 1982). Ozone has been shown to interact and modify proteins in the virus capsid, which are used by the virus to attach to the cell surface (Cronholm et al., 1976; Riesser, 1976). Kim et al. (1980) studied the inactivation of bacteriophage f2 upon reaction with ozone, and suggested



that ozone damaged the phage. This damage resulted in the release of ribonucleic acid (RNA) and disrupted adsorption to the host pili. Ribonucleic acid not protected by the phage coat is further susceptible to oxidation by ozone. A similar mode of action was proposed for phage inactivation and release of deoxyribonucleic acid (DNA) in studies conducted on bacteriophage T4 (Sproul and Kim, 1982). Studies conducted with the tobacco mosaic virus (TMV) have shown that ozone attacks both the capsid and RNA. Damaged RNA forms cross links with the capsid subunits to cause a loss of the viruses infectivity (Yoshizaki et al., 1988). It was suggested that ozone causes partial destruction or complete removal of plugs in *Naegleria gruberi* cysts (Langlais and Perrine, 1984). Ozone may increase the permeability of *Giardia muris* cysts and subsequently damage the plasma membranes and eventually the other cellular components (Wickramanayake, 1984c)

### **2.3. Ozone Generation**

High transmission UV lamps emitting radiation at wavelengths of 185 nm produce low concentrations (0.03 ppm) of ozone (Ewell, 1946). Ozone is generally formed by combining an oxygen atom with an oxygen molecule. This reaction is endothermic and requires a considerable input of energy.



Ozone is very unstable and decomposes quickly to elemental oxygen (Horvath et al, 1985). Due to its unstable nature, ozone is commonly produced at the point of application. Synthetic ozone was first produced by Schönbein by electrolyzing sulphuric acid (Langlais et al., 1991). However, the corona discharge method is the most popular method for generating ozone. Several

commercial ozone generators are currently available that produce both gaseous and ozone dissolved in water. Ozone can also be generated by chemical, thermonuclear and electrolytic reactions (Rice 1996; Hovarth et al., 1985).

In many ways the corona discharge method replicates the action of lightening in a thunderstorm in a closed system. Dry air or pure oxygen gas is passed through a small gap between two dielectric electrodes. These electrodes can be either concentric circles or parallel plates. When an electric potential is applied across the electrodes, electrons flow through the narrow gap and transfer their energy to the oxygen atoms to facilitate ozone formation, as shown in Figure 1.2.

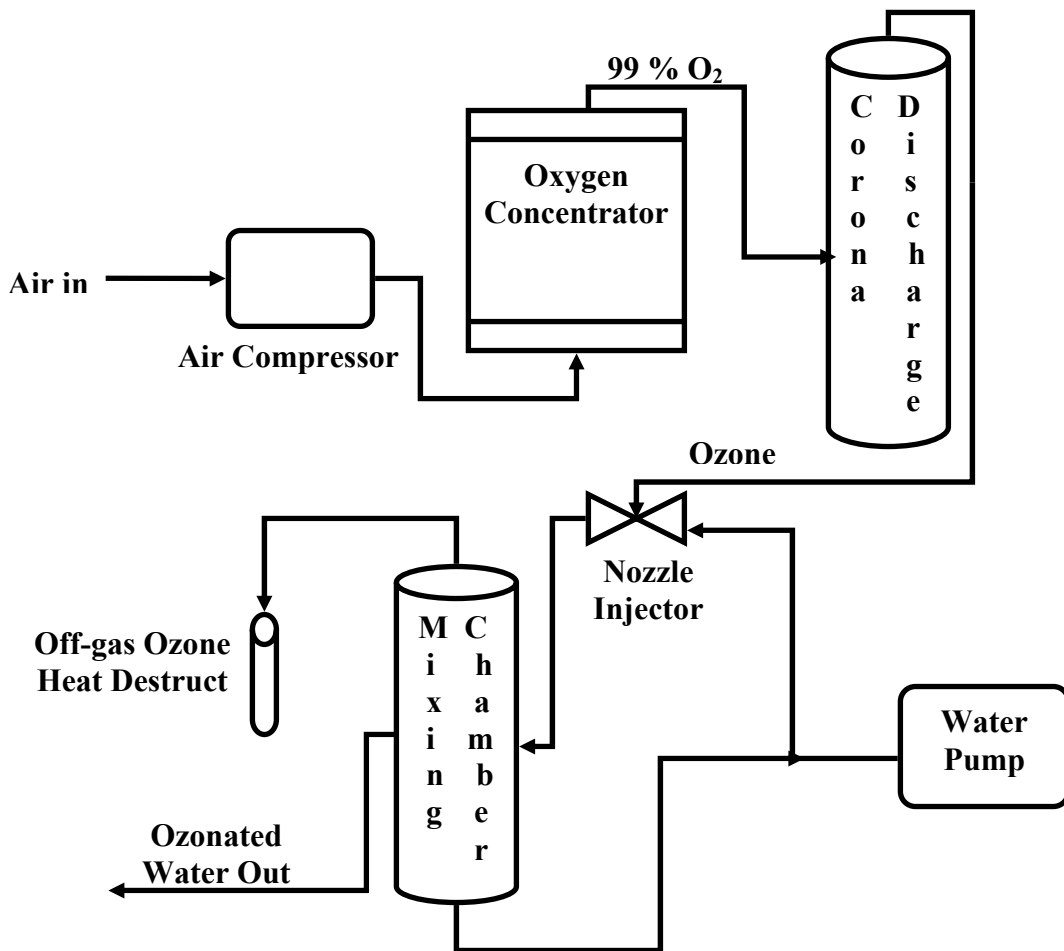


Figure 2.2 Basic Ozone Generator

Ozone production in the gap varies with the voltage applied, frequency of current, the dielectric properties of the materials, thickness of the material, absolute pressure within the system and the size of the gap between the electrodes. Ozone production is accompanied by heat generation which results in losses of about 85 percent of the electrical energy input into the system (Rice, 1996). An effective cooling system is required in such generators to improve the efficiency of ozone generation. Moisture present in the feed gas can lead to the formation of nitric acid and results in corrosion. Moisture removal from the feed gas is critical to the ozone generation system (Rosen, 1972). The other important components of a modern generator are the gas feed system, ozone contactor and the off-gas destruct system.

### **2.3.a. Gas Feed Systems**

Ozone generators use high purity oxygen, air or a mixture of the two as feed gas. High purity oxygen is either supplied through an oxygen tank or is generated within the system. Large systems use cryogenic generation with pressure swing adsorption (PSA) or vacuum swing adsorption (VSA). Pressure swing adsorption is a process wherein air at high pressure is pumped through a molecular sieve that selectively removes carbon dioxide, nitrogen, water vapor and moisture from the air and can generate 85-90 % pure oxygen. A heating system coupled to the sieve helps in drying the sieve (DelOzone, 2004).

### **2.3.b. Ozone Contactors**

Ozone is dissolved in water using different dissolution methods. In order to produce efficient disinfection by ozone, transfer efficiencies of greater than 80 percent are typically required (DeMers and Renner, 1992). The

common ozone dissolution methods include; injectors, bubble diffuser contactors and turbine mixers.

### **2.3.c. Injector Dissolution**

Injector dissolution is commonly used in Europe, Canada and the United States. It involves dissolution of ozone in water under a vacuum that is produced in the system by a venturi section along with a high pressure recirculating water pump. The resulting turbulence enhances ozone dissolution.

#### **2.3.c.i. Bubble Diffuser Contactors**

Bubble diffuser contactors are one of the most common ozone dissolution methods used world wide (Langlais et al., 1991). It offers the advantages of no moving parts, high ozone transfer rates, operational simplicity, process flexibility and no additional input energy. However this method requires the use of large mixing containers and wear and tear of gaskets in the system.

#### **2.3.c.ii. Turbine Mixers**

These systems used either a submerged turbine and motor or a turbine with external motor for dissolving ozone gas into water. Ozone transfer is enhanced due to high turbulence resulting in small bubble size (Langlais et al., 1991).

### **2.3.d. Off Gas Destruct Unit**

Ozone that does not dissolve in water is released as off-gas and is toxic. This excess ozone is destroyed by directing it through an ozone destruct unit. These destruct units can be thermal which heats the gas above 350°C, catalytic which operate at 100°C and prevents moisture buildup, or a combination of the

two. The off-gas destruct unit is designed to reduce the concentration to 0.1 ppm of ozone by volume, the current limit set by OSHA for worker exposure in an eight hour shift.

#### **2.4. Developing Technologies**

Ozone is used in combination with freezing to decrease the amount of antimicrobial agent used and increase microbial inactivation (Giacobbe and Yuan, 2005; Take and Skhirtladze, 2006). Yuan and Steiner (2005) have developed a novel way to sanitize and cool food products using ozone and nitrogen. Ozone has been used in combination with microwaves for the destruction of prions (Klaptchuk, 2005). In order to achieve higher disinfection rates, a food sanitation tunnel system has been developed that uses gaseous ozone in combination with UV radiation, hydro peroxides and super oxides (Fink et al., 2004). Ozone has been combined with a surfactant to improve contact with the surface being sanitized (Smith et al., 2002). Ozone has produce similar reductions of human rotavirus (8-9 log CFU/g) as other non-thermal processing techniques like high pressure processing (HPP) and pulsed electric fields (PEF) (Khadre and Yousef, 2002). Increased disinfection efficiencies to reduce microbial loads, were reported when ozone was mechanically mixed with hydrogen peroxide ( $H_2O_2$ ), chlorine dioxide ( $ClO_2$ ) and chlorite ions ( $ClO_2^-$ ) (Son et al., 2005).

#### **2.5. Ozone vs Chlorine**

Widespread use of chlorine as a sanitizer is being reconsidered because chlorine reacts with natural organic matter (NOM) and bromine to form total halo methanes (THM) and brominated THM (Sorlini and Collivignarelli,

2005). These residual compounds are potential carcinogens. Microorganisms are known to develop resistance to chlorine (Clark, 2003; Guzel-Seydim et al., 2003). Ozone and chloramines are being used for water treatment to reduce the formation of undesirable chloral hydrates, THM and haloaceticacids (Guay et al., 2005; Janda et al., 2004).

## **2.6. Ozone Application in Foods**

### **2.6.a. Fruits and Vegetables**

Achen et al. (2001) found that bubbling of ozone was more effective in reducing counts of *Escherichia coli* O157:H7 inoculated on whole apples.

Koseki and Isobe (2006) have shown that ozone can be effectively used in washing iceberg lettuce to produce greater kills in bacterial levels. Ozone has been suggested as an alternative to traditional chlorine in processing fresh cut lettuce without adversely affecting the sensory quality and antioxidant levels (Beltran, 2005a). A shelf life study conducted by Zhang et al. (2005) showed that treatment with low concentrations of ozonated water at 0.08 ppm

decreased bacterial loads in fresh cut celery and improved sensory scores over the period of the study. Ozone has been suggested as an alternative to thermal

pasteurization in the processing of apple cider and orange juice to produce reductions to the order of  $10^5$  in *Escherichia coli* O157:H7 and *Salmonella* populations (Williams et al., 2005; Steenstrup and Floros, 2004). Ozone was found to effectively reduce the inoculated populations of *Listeria*

*monocytogenes* NCTC 7973 and *Escherichia coli* P36 in watercress, spinach, coriander, lettuce and celery seeds (Warriner et al., 2005). Ozone treatment

was shown to produce a 3.3 log reduction in fresh cut potato strips that were

vacuum packaged (Beltran, 2005b). A comparative study to determine inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in fresh produce using ozone, chlorine dioxide, chlorinated trisodium phosphate (CTP) and peroxyacetic acid; found 3ppm ozone treatment for 15 s to be most effective against the pathogens (Rodgers, 2004). Ozone was found to be less effective as a sanitizer in reducing microbial loads as compared to washing fresh uncut carrots with chlorine (Klaiber et al., 2004). Enterobacteriaceae species showed higher reduction as compared to mesophiles and *Pseudomonas* upon washing lettuce head and shredded lettuce with ozonated water (Baur et al., 2004).

#### **2.6.b. Meat and meat products**

A study on beef that was heated showed decreased resistance of vegetative cells and spores of *C. perfringens* to ozone treatment (Novak and Yuan, 2004). Novak and Yuan (2003) indicated that microorganisms surviving ozone treatment were less likely to endanger food safety as compared to the organisms surviving sublethal heat treatments.

#### **2.6.c. Poultry**

Raw shell eggs when exposed to UV radiation followed by ozone treatment produced > 4.6 log reduction in *Salmonella enteritidis* populations (Rodriguez, 2005). Chicken skin inoculated with *Salmonella infantis* and *P. aeruginosa*. showed reductions in initial counts when treated to greater than 2000 ppm gaseous ozone but the indigenous coliforms were not affected by the treatment (Al-Haddad et al., 2005). Koidis et al. (2000) suggested the use of

ozonated water treatment to reduce populations of *Salmonella enteritidis* on egg shell surfaces.

#### **2.6.d. Cereal and Grains**

Kottapalli et al., (2005) indicated significant reduction in *Fusarium* survival rates upon treatment with gaseous ozone. An inoculated study of alfalfa seeds with *Salmonella* serotypes found that ozone alone was not sufficient to reduce bacterial levels and suggested use of a secondary sanitizer following ozone treatment (Rajowski and Rice, 2004). Another study that used continuous sparging of alfalfa sprouts with ozonated water found significant reduction in natural microflora and *L.monocytogenes* (Wade et al., 2003). Sharma et al (2002) showed that significant reduction of *Escherichia coli* O157:H7 in alfalfa sprouts was obtained by increasing the ozone concentrations longer contact times did not significantly change bacterial destruction. Significant reduction in pesticide residues in wheat was achieved upon treatment with ozone (Zhanggui et al., 2003). Ozone has been shown to be an effective and permanent method in reducing aflatoxin AFB1, and the mutagenic potential of AFB1-contaminated corn (Prudente et al., 2002).

#### **2.6.e. Seafood**

Crapo et al. (2004) in their studies with Alaska salmon fish fillets and roe found chlorine to be more effective for controlling bacteria as compared to ozone, however ozone was more effective as a sanitizing agent for fish processing surfaces. Intermittent washing and pumping of fish from ship holds using ozonated water was shown to extend refrigerated shelf life (Koettters et



al., 1997). Treating fresh scad with gaseous ozone showed reduced bacterial levels and improved sensory scores (Silva et al., 1998).

#### **2.6.f. Dairy**

Serra et al. (2003) found that ozone was effective in reducing aerial fungal loads in cheese ripening rooms but not on the cheese surface.

### **2.7. Biogenic Amine Analysis**

#### **2.7.a. Background:**

The Food and Drug Administration (FDA) Defect Action Level Handbook, describes decomposition as the bacterial breakdown of the normal product tissues and the subsequent enzyme induced chemical changes. These changes are manifested by abnormal odors, taste, texture, color, etc. (FDA, 1998). Such changes in the chemical and sensory attributes of the product can be used as quality indicators for commerce and regulation.

Proper handling and storage throughout handling and distribution is required to maintain the quality and safety of shrimp, a highly perishable muscle protein product. The quality preservation of shrimp is primarily carried out by temperature control by refrigeration or freezing on ice. The type of bacteria that multiply and produce enzymes that degrade shrimp, have been shown to differ as a function of storage and temperature (Benner et al., 2003).

Spoilage of meats and seafood stored at low temperature is usually accompanied by production of off-flavor compounds such as ammonia and amines. These chemicals can be used as quantitative indicators to evaluate the quality of meats and seafood. The microbial flora, their quantity and storage temperature play a significant role in determining the quantity of these

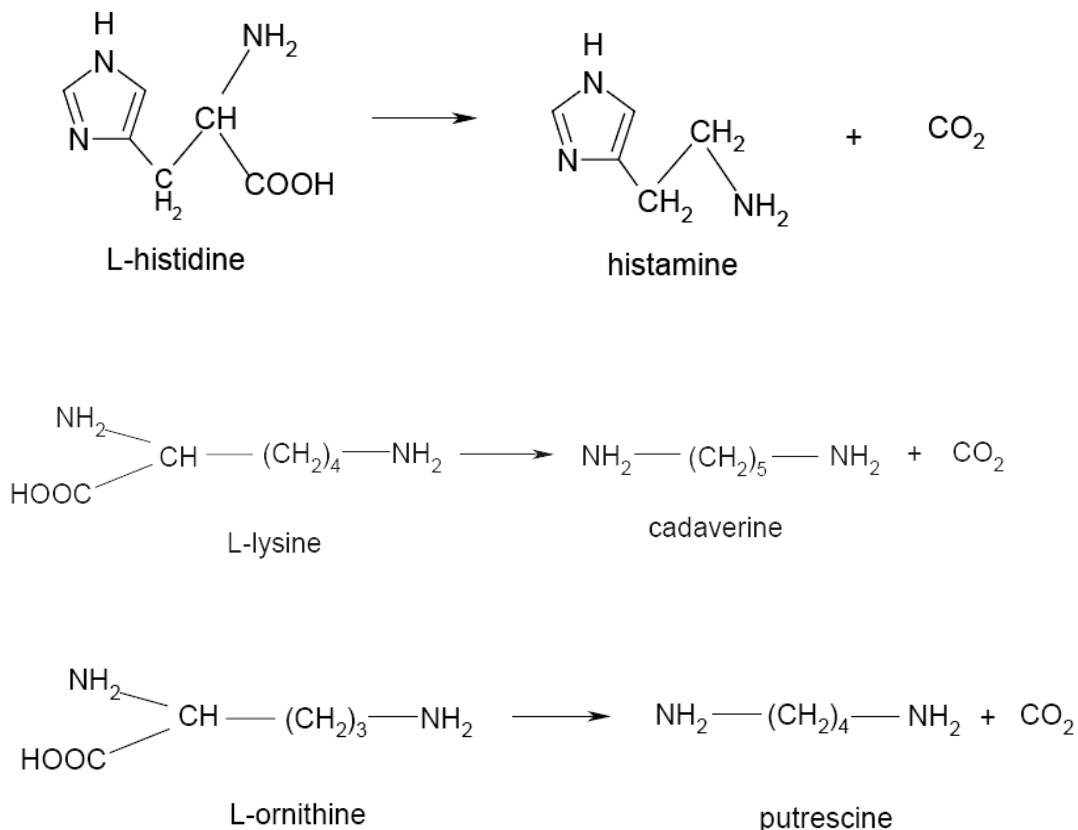
decomposition metabolites. Determining the relative quantities of these breakdown products can give an estimation of degree of spoilage of the meat or seafood product. These compounds may also be used in commerce and regulation to grade and reject the product (Rawles et al., 1996).

Historically, indole concentrations above 25ug/100g has been used to confirm decomposition in shrimp (FDA, 1996). However, indole is produced when shrimp decomposes at high temperatures and may not be produced in shrimp stored at low temperatures. Lower levels of indole alone did not ascertain that the shrimp are acceptable. Although indole is a good chemical indicator of decomposition at higher temperatures, deterioration at low temperatures may result in shrimp with less than 25ug/100g of indole that fails sensory examination and is clearly decomposed. The difference in spoilage pathways due to thermal exposure presents the need to investigate alternative chemical indicators for shrimp decomposition. Diamines, putrescine and cadaverine, formed in shrimp under conditions of both high and low temperature spoilage have been studied as spoilage indicators. Studies using a modified method of extraction and column conditions for shrimp, have shown that canned and raw shrimp failing sensory evaluation contained greater than 4.8 ppm putrescine and greater than 1.3 ppm cadaverine (Rogers et al., 2003).

#### **2.7.b. Bioamine Production**

Different biogenic amines, as shown in Figure 1.3, are typically produced by the decarboxylation of amino acids (Rice, 1976). The decarboxylation reactions can proceed through endogenous(naturally occurring) or exogenous(microbial enzymes) pathways (Rawles et al., 1996).

However, research has shown that the main source of bioamines is through microbial decomposition (Wendakkoon et al., 1992).



**Figure 2.3. Generation of histamine, putrescine, and cadaverine from their corresponding decarboxylated amino acids.**

The term biogenic amines refers to the nonvolatile amines such as cadaverine, putrescine, spermidine, spermine, tyramine, tryptamine, and histamine produced post mortem in fish and shellfish products facilitated by growth of bacteria in fish (Maga, 1978; Coutts et al, 1986; Yen et al.,1991; Rawles et al., 1996). Biogenic amines are low molecular weight organic bases with either aliphatic, alicyclic or heterocyclic structures (Davidek and Davidek, 1995). The decarboxilation of the amino acid histidine found in high levels in the tissues of scombroid fishes produces histamine (Frank, 1985). Histamine

acting synergistically with putrescine and cadaverine is believed to cause scombroid poisoning ( Bjeldanes et al., 1978). Histamine content is used as a marker to indicate degradation of fish (Mietz et al., 1978). Consumption of decomposed scombroid fish can lead to histamine fish poisoning (Arnold et al., 1978) and hence the detection of biogenic amines is important to prevent the sale of decomposed fish.

### **2.7.c. Gas Chromatography and Amine Analysis**

Gas chromatography (GC) has been one of the most widely investigated and applied techniques in the field of analytical chemistry since the early 1970's (McNair, 1998; Grob, 2004). Gas chromatography is a versatile investigative tool because it is simple, fast, reproducible and relatively inexpensive as compared to other methods of investigation (McNair, 1998; Grob, 2004). Gas chromatography is widely used to separate organic compounds that are volatile and semi-volatile in nature. It is used to analyze a wide range of products including pesticide residues, flavors, colors, pharmaceutical drugs, etc. (Grob, 2004).

GC has three main components: a carrier gas (mobile phase), a separating column (stationary phase) and the analyte. The carrier gas is usually inert hydrogen or helium that is used to carry the analyte through the stationary column, heat is used to vaporize the analyte compound to facilitate travel through the column, where it interacts with the liquid stationary phase (McNair, 1998; Grob, 2004). The analyte separates and elutes from the stationary phase depending on its solubility relative to the stationary phase.

The eluting analyte enters a detector that produces an electrical response. The

electrical signal is processed by a data system, usually a computer that then generates an image displaying the analyte peaks which is called a chromatogram. The computer can then determine the peak height or area under the peak, which is then used to quantify the amount of the analyte (McNair, 1998; Grob, 2004).

Compounds that contain nitrogen (primary, secondary tertiary and aliphatic amines) are difficult to analyze directly by GC, because a significant amount of these basic amines is usually adsorbed onto the acidic columns. Decomposition of the amines in the systems also of concern (Kataoka, 1996). These difficulties in biogenic amine detection can be reduced if they are derivitized with a suitable agent (Kataoka, 1996). The derivitization step offers several advantages, as it improves the volatility of amines that facilitates GC. Analysis increases the selectivity and sensitivity of detection, enhances the overall separation (AOAC, 1999) and reduces tailing thus improving the peak shape (Kataoka, 1996; Yen et al., 1991). Kataoka (1996) has published a comprehensive review describing several methods that can be used to derivitize biogenic amines such as acylation, silylation, and carbamate formation.

The use of G.C. for the detection analysis and quantification of biogenic amines is of particular interest in the food industry.

## **2.8. Sensory Analysis**

Ranking is one of the simple discriminative difference tests commonly used in sensory evaluation of foods. It is of ordinal type scale and used to compare several samples on the basis of a single or specific character (IFT,

1981) such as overall preference. It is used to screen one or two of the best samples from a group of samples. It is also useful in sorting or reducing the number of test samples to manageable number when large numbers of products are to be tested. Another application of ranking test is its usage in recruiting panelist for descriptive analysis which requires ascertaining the ability of panelists to discriminate four basic tastes. Ranking can be used for evaluating the influence of changes in ingredients, processing techniques, packaging, etc. on food sensory properties (Petrukhina and Kriukova, 2003).

Simplicity, rapidity and provision of testing several samples at once are merits of ranking tests. However, results of this test itself yield only ordinal data and it lacks equality in distance. Thus the results of the rank test exhibit only the direction of the differences and not the size of the differences between samples. Further, statistical analysis of rank tests results in very complicated as it does not fit into normal distribution.

The rank data obtained from the test can be analyzed with Kramer Test (Kramer et al.,1974), Friedman non-parametric statistical test with Randomized Block Design (RBD) and Fishers Least Significant Difference (LSD) technique and Wilcoxon Rank Sum test.

## **2.9. Thio-barbituric Acid Reacting Substances (TBARS) Test**

Lipid oxidation in fish results in the formation of aldehydes, ketones and fatty acids and is known to cause losses in quality due to formation of off flavors (Pearson et al., 1983). Lipid oxidation in fish is based on several factors such as the fat content, type of fatty acids present, distribution of fat in the body, degree of unsaturation of fat, external factors like exposure to light and

heat, ultraviolet radiation and presence of chemical accelerators (Khayat and Schwall, 1983). Addition of a highly reactive molecule such as ozone is of concern as it would be expected to increase the rate at which this oxidation would occur. The Thio-Barbituric Acid test (Tarladgis et al., 1960) is commonly used in the food industry to detect lipid oxidation. A modified method by Lemon (1975) has been used in this study.

### **2.10. *Listeria* in Foods**

*L. monocytogenes* is a Gram positive food borne pathogen that can grow over wide temperature ranges from 1 to 45°C. It can grow in high salt environments and can tolerate up to 10 % NaCl. The current US. Regulatory policy requires absence of *L. monocytogenes* in 25 g of a Ready to Eat food sample. *L. monocytogenes* is wide spread in the environment and is found in soil, water, and sewage and decaying vegetation. Humans, pets, raw agricultural commodities, vegetables, fish, dairy products are common carriers of this organism (Ryser and Marth, 1999). It has been isolated from a wide variety of seafood processing environments (Dillon and Patel, 1992). *Listeria* is found in a wide variety of food processing environments like poultry, meats, vegetables, dairy products and fish (Eklund et al., 1995; NACMCF, 1991). *L. monocytogenes* is able to survive at refrigeration temperatures and post processing contamination with this organism is a serious concern in the food industry (Dillon and Patel, 1992; Eklund et al., 1995; Jahncke et al., 2004)

Ozone has been successfully used to sublethally damage *L. monocytogenes* cells in an inoculated study (Lee D, Martin Se et al. 1998). Ozonated water has been shown to significantly reduce *L. monocytogenes*

levels (Restaino et al., 1995). Planktonic and biofilm cells of *L. monocytogenes* were found to be completely destroyed upon exposure to ozone (Robbins et al., 2005).

### **2.11. Summary of Ozone**

Ozone has been used effectively as a sanitizing agent in the food processing industry both as gaseous ozone and dissolved in water to reduce microorganisms on a wide range of food products and contact surfaces (Rice, 1982; Kim and Yousef, 1999; Guzel-Seydim et al., 2003). In recent years ozone in combination with other sanitizers and treatments has been used to target a broader range of microorganisms having food safety concerns. Some of the industries where ozone has been used are.



**Table 2.1 Summary of the properties of ozone.**

Advantages	<ul style="list-style-type: none"><li>• More effective than chlorine, chlorine dioxide and chloramines for inactivation of viruses, bacteria, protozoa, bacterial spores and cysts and fungi</li><li>• Requires short contact times</li><li>• Decomposition by product is oxygen</li></ul>
Disadvantages	<ul style="list-style-type: none"><li>• Is less effective in mediums that have high pH, temperature and organic solids</li><li>• Ozone generation systems are expensive</li><li>• Leaves no residuals and hence must be used with a secondary sanitizer for effective and long term disinfections</li><li>• Is highly corrosive and needs special operating conditions</li></ul>
Safety	<ul style="list-style-type: none"><li>• Several agencies have fixed the maximum exposure to ozone no more than 0.1 mg/l by volume in an eight hour work shift (OSHA, ANSI/ASTM, ACGIH, AIHA)</li><li>• No person should be exposed to a concentration of 0.3mg/l by volume for more than 10 minutes (ANSI/ASTM)</li><li>• No person should be exposed to a concentration of 0.3 mg/l by volume for up to 15 minutes (ACGIH)</li></ul>

## **CHAPTER 3**

### **DEVELOPMENT OF A PROCESS TO MEASURE OZONE CONCENTRATIONS IN PROCESSING WATER AT THE POINT OF PRODUCT APPLICATION**

#### **3.1. Introduction**

Sanitizers are used in food processing operations to decontaminate product contact surfaces and to reduce the number of spoilage and pathogenic microorganisms. Commonly used sanitizers include quaternary ammonium, iodine or bromine, and chlorine or chlorine compounds. Although effective when used properly, a main concern with abundant use of chlorine is the accumulation of residues in the environment (Green et al., 1993). Chlorine can react with naturally occurring aquatic humus compounds in streams and rivers to form potentially carcinogenic compounds such as trihalomethanes (THM) and haloacetic acids (HAA) (Garcia et al., 2003; Guzel-Seydim et al., 2003). High concentrations of chlorine can be effective against viruses, but bacteria can build up resistance to repeated use of lower concentrations of commonly used sanitizers (Green et al., 1993).

Gaseous ozone and ozonated water has been used in the food industry for sanitation of product contact surfaces, as well as for the treatment of milk, meat products, gelatin and other food products. Ozone is also used for artificial ageing of alcoholic beverages, cider manufacture, odor control and medical therapy (Kim, 1998).

Ozone has many advantages as a sanitizer. Gaseous ozone and ozonated water can be generated on site, at the facility of use. Chemical reaction of ozone with organic material occurs at very rapid rates and short

reaction times, which effectively prevents microorganisms from developing tolerance to ozone (Kim, 1998). Many studies have reported the advantages and superior bactericidal properties of ozone as compared to chlorine (Green et al., 1993; Kim and Yousef, 1999; Klaiber et al., 2004). The effectiveness and susceptibility of microorganisms to  $dO_3$  depends upon the water temperature and pH, and the presence of dissolved compounds (salt, sugar, minerals and surfactants) and suspended organic matter (Kim and Yousef, 1999).

Despite these advantages, ozonated water is not widely used to sanitize processing and food surfaces in the seafood industry. Ozone is a powerful oxidant and an effective sanitizer, but can be toxic and cause human health concerns with improper use. Ozone toxicity in humans is expressed as irritation in the eyes, nose and throat. Off-gassing, the release of gaseous ozone from the gas-water mixture, at higher temperatures can be a human health concern. This off-gassing can also result in reduced ozone levels in the water actually applied to product and surfaces during processing. Off-gassing can lead to errors in quantifying the amount of ozone used in bacterial destruction studies.

The objective of this research is to develop a process to measure  $dO_3$  concentration in processing water at the point of product application, as opposed to within the ozone generation system itself. This experimental design will then facilitate further investigation and optimization of microbial destruction and changes in quality of peeled shrimp meat processed with ozonated water.

## **3.2. Materials and Methods**

### **3.2.a. Ozone Generation**

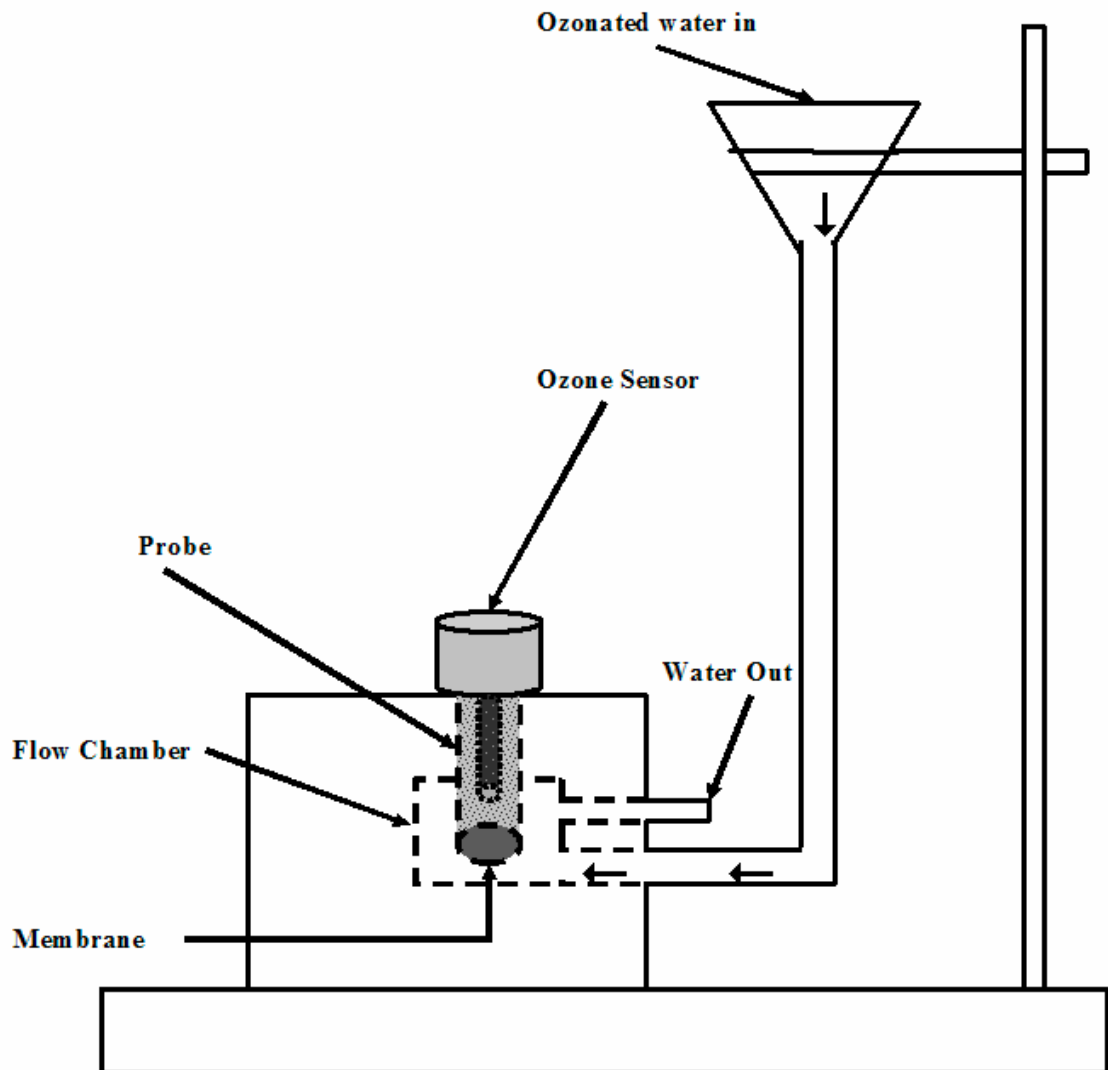
Ozonated water was generated using a DelOzone (San Luis Obispo, CA, 93401 USA)-AirLiquide (Houston, TX, 77056 USA) Infinity series corona discharge  $dO_3$  generator (Model AGW 1500SL). This system uses concentrated oxygen (90-95% pure) as the input gas and a nozzle injection system (Mazzi injectors) for mixing ozone with water. The system produces a water flow rate of 12.6 lpm (liters per minute) and has an ozone output range of 10-25 g/hr (grams per hour).

### **3.2.b. Ozone Measurement**

Dissolved ozone concentrations were measured with a flow-through process, using an Analytical Technology, Inc. (Model A 15/64) (Collegeville, PA, 19456 USA)  $dO_3$  sensor (Figure. 3.1).

The sensor was inserted in an acrylic flow cell assembly to maintain constant flow rate and pressure across the sensor membrane and continuously measure  $dO_3$  concentrations. The sensor has two operating ranges, and the higher range setting (0–20.00 ppm  $dO_3$ ) was selected for our studies. The  $dO_3$  sensor utilizes a polarographic membrane element. The sensor measures ozone concentrations directly on the principle of a Clark oxygen electrode. The electrode consists of two half-cells separated by a salt bridge. A platinum and a silver electrode are separated by an insulating material placed in a concentrated solution of potassium chloride. Ozone concentrations are displayed digitally on a backlit liquid crystal display.

The ozone sensor requires a constant pressure over the polarographic membrane to accurately measure  $dO_3$  concentrations. This constant pressure was produced by enclosing the sensor in the flow cell assembly connected to the funnel.



**Figure 3.1: Dissolved Ozone ( $dO_3$ ) Measurement System Using a Flow Cell and  $dO_3$  Sensor**

Water collected in the funnel is directed through the flow cell and past the sensor at the rate of 20 ml/s (milliliters per second). The sensor has a response

time of 90% in 45 seconds and an automatic temperature compensation function from -1.7°C to 49°C.

The sensor was calibrated daily with High Range Hach Acuvac Kits (Hach Indigo Method 8311) (Loveland, CO, 80539 USA) for lower concentrations (0–1.5 ppm dO<sub>3</sub>), and with an indigo blue colorimetric method (Bader et al., 1982) for higher concentrations (1.5–3 ppm dO<sub>3</sub>).

Ozone gas escaping into the atmosphere from the gas water mixture was observed using an Ecosensor Ozone Monitor (Model A-21ZX) (Santa Fe, NM, 87505 USA). This is a HMOS (heated metal oxide semiconductor) sensor that quickly measures ambient ozone levels from 0.02-9.99 ppm.

### **3.2.c. Water Temperature and Quality Parameters**

Water temperature was measured using an EXTECH Instruments Big Digit, Type K Single Input Thermometer (Model 421501) (Waltham, MA, 02451 USA). Total dissolved solids (TDS) were measured using a OAKTON Instruments TDS tester (Vernon Hills, IL, 60061 USA). Free chlorine was measured using Hach DPD Acuvac® free chlorine test kits (0–2.5 mg/l free chlorine). The nitrate and nitrite content was measured using the Hach nitrate–nitrite test kit (Model NI–12). The iron and manganese content was measured using a Simultaneous ICP - OES (Inductively Coupled Plasma-Optical Emission Spectrometer) (Model Vista-MTX) (Varian Inc., Palo Alto, CA, 94598 USA).

### **3.2.d. Statistical Analysis**

One way ANOVA and Student's t values were calculated using the JMP<sub>in</sub>® software, Copyright © 1989-2000 SAS Institute Inc. (Cary, NC, 27513 USA).

### **3.3. Results and Discussion**

Ozone concentration was digitally measured at the point of product application using the flow-through sensor and flow-cell (Figure. 3.1). Identical processing water samples were measured for ozone concentrations using chemical methods. The sensor readings were verified with Acuvac Hach kits and the indigo-blue colorimetric method. The mean values of ozone concentrations compared using ANOVA and student's t values from all methods of measurement did not differ significantly at the 95% confidence level (Table 3.1).

The effect of water temperature on dO<sub>3</sub> concentrations was measured using a single pass design (Figure. 3.2). Water temperature in a 450 liter polyethylene tank was controlled with ice. dO<sub>3</sub> in water (0°C-35°C) was measured after a single pass through the ozone generator (Figure. 3.3). dO<sub>3</sub> concentration increased with decreasing water temperature. This increase was greatest when the processing water was chilled to 10°C. This result suggests that chilling the processing water to 10°C offers a practical and realistic opportunity for improved performance of dO<sub>3</sub> generation systems in seafood processing plants. In addition, off-gassing of ozone into the atmosphere during

**Table 3.1: Verification of Dissolved Ozone Results from the Flow-through Sensor with Chemical Measurement Methods**

\* Accu Vac Hach kits could measure ozone concentrations only up to 1.5 ppm.

Water Temp	Ozone Concentration (ppm)		
	ATI-Sensor	Hach-Kit	Indigo-Blue
°C	Digital	Chemical	Chemical
0	1.61	NA*	1.64
5	1.35	1.35	1.41
10	1.19	1.15	1.21
15	0.64	0.65	0.64
20	0.52	0.50	0.53
25	0.46	0.50	0.48
30	0.38	0.35	0.39
35	0.14	0.15	0.14



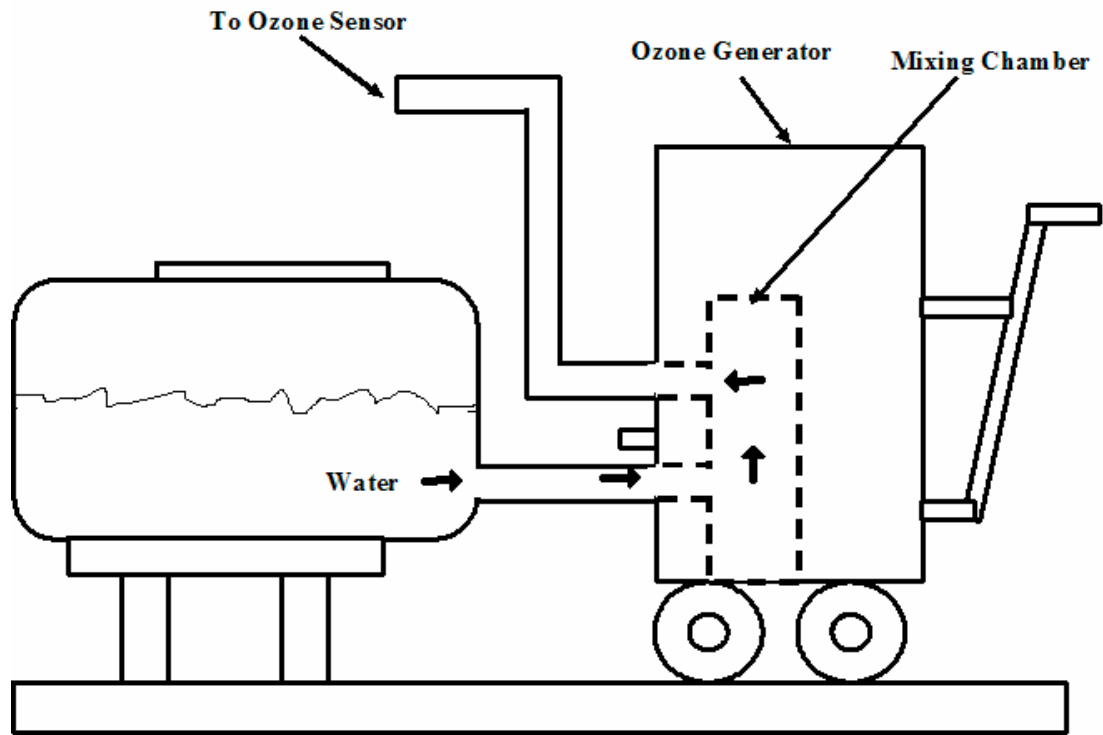


Figure 3.2: Single-pass Design to Produce Ozonated Water

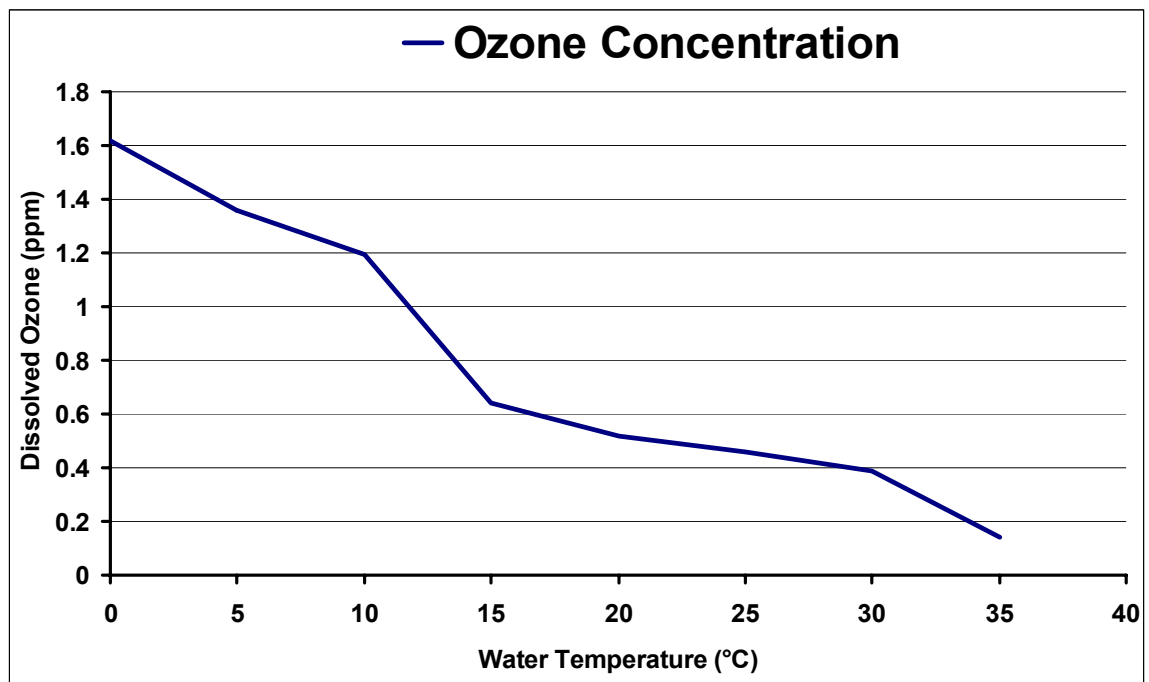
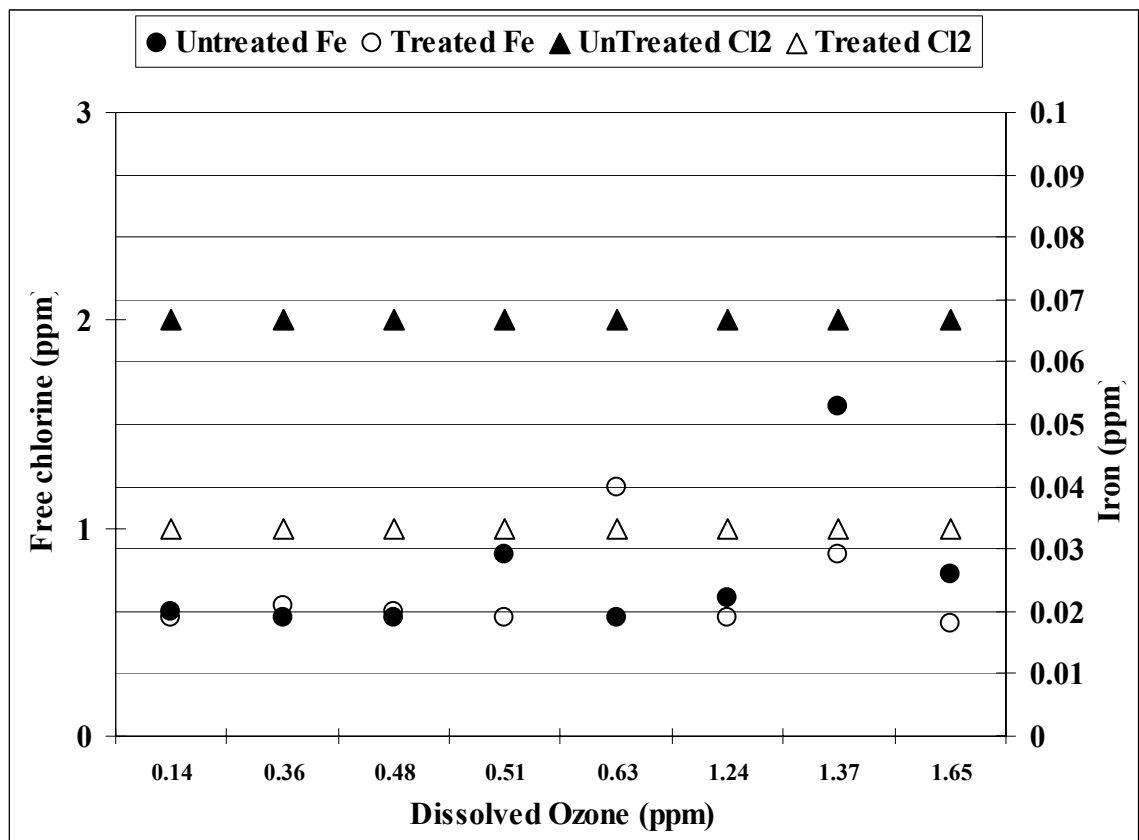


Figure 3.3: Ozone Concentration and Water Temperature Leaving Single Pass Design.

these trials was more readily observed at the higher water temperatures while monitoring the processing area with the Ecosensor.

The single pass design was also used to evaluate the effect of water quality parameters on  $dO_3$  concentration. Water quality parameters measured included pH, TDS, nitrates, nitrites, free chlorine, iron and manganese content. Increased ozone concentrations did not affect the water pH, and very little change was seen in the manganese, nitrites and TDS content (data not shown). There was a substantial decrease in the free chlorine content of water after ozonation (Figure. 3.4).



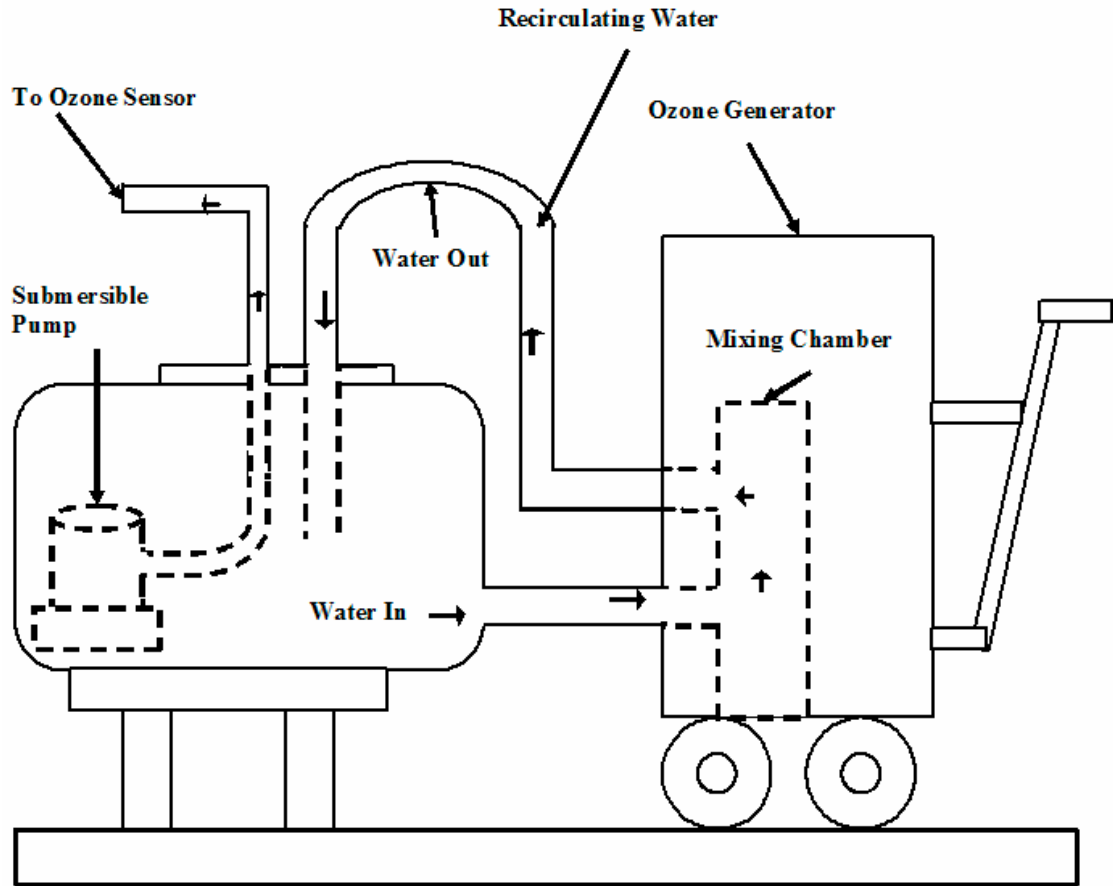
**Figure 3.4: Changes in free chlorine and iron content of ozone treated water**

Changes in iron content at higher ozone levels was also indicated. These ionic species participate in the initiation stage of ozone decomposition. The soluble

ferrous ions are oxidized by ozone to form the insoluble ferric ions. Similarly, the hypochlorite ions are oxidized to form chlorates and chlorides. These ions are involved in the formation of free radicals, such as the superoxide radical ion ( $O_2^-$ ) and the hydroperoxide radical ( $^+HO_2$ ). These free radicals lead to formation of a hydroxyl ( $\cdot OH$ ) ion which is highly reactive and consumes an ozone ( $O_3$ ) molecule, thus reducing the concentration of  $dO_3$  in water (Stahelin and Hoigene, 1985).

Dissolved ozone concentration was measured in the water exiting the system after a single pass through the ozone generator. The highest ozone concentration produced was 1.6 ppm in water at 1°C. A recirculation system was then designed to generate ozone concentrations of 3 ppm (Figure. 3.5). Water temperature of 10°C was maintained using ice. The ozone concentration was increased via recirculation of the water through the ozone generator. Initial measurements of  $dO_3$  concentration were performed by immersing the  $dO_3$  sensor, enclosed in a protective PVC tube, into the recirculation tank. However, these measurements were found to be highly variable in different areas of the tank. The sensor requires water flow and a constant pressure across the sensing membrane. Insufficient water movement and difference in water levels in different tank locations resulted in the varied sensor measurements.

A process to transport the ozonated water past the sensor in the flow cell was designed to measure  $dO_3$  in water at the point of product application (Figure. 3.5). A 0.5 hp submersible pump was placed in the recirculation tank to



**Figure 3.5: Recirculation design to produce water with increased ozone concentration**

deliver the ozonated water to the flow cell. The turbulence created by the submersible pump decreased the ozone concentration in the water being pumped out of the tank. This reduction emphasized the importance of measuring ozone concentration in the water at the point of application, and not in the recirculation system itself. Continuous recirculation of the water through the ozone generator produced elevated  $dO_3$  levels in the water.

Two different product application treatments will be studied, soaking and spraying. For the soak treatment, ozonated water was pumped from the recirculation tank into containers, and samples will be immersed in these containers for different time intervals. For the spray treatment, a low pressure

high volume nozzle is fitted to the discharge end of the submersible pump for spraying of ozonated water on the product surface.

An ozone concentration of 3 ppm was selected as the upper level for eventual product treatment. This 3 ppm concentration was selected based on practical constraints encountered in seafood processing plants, including recirculation time requirements, water quality limitations and off-gassing concerns. The time required to achieve 3 ppm  $dO_3$  concentration was 90 minutes in 10°C water. Longer concentration times would prove impractical in most seafood processing facilities. Higher ozone concentrations may produce detrimental quality effects in the treated food product. High ambient water temperatures may lead to increased off-gassing, which would create human health and safety concerns. Off-gassing was observed for both initial spray and the soak treatments, and continued experiments with shrimp meat samples will be conducted in a controlled environment. Greater off-gassing was observed for the spray application.

### **3.4. Conclusion**

Dissolved ozone concentration in processing water at the point of product application was quantitatively and rapidly measured using a flow-through sensor. This system will be used to optimize ozonated water treatment of peeled shrimp meat.  $dO_3$  is commonly measured within the generator system during industrial application. These measurements are valid for the ozone dissolved in water within the generator, but will indicate higher concentrations than for the water that is actually applied to the product and

process surfaces. Colder processing water temperature resulted in increased  $dO_3$  levels and decreased ozone off-gassing from the water.

Achieving increased ozone levels in processing water at the point of application presents an opportunity for improved product sanitation during seafood processing. The use of higher ozone levels will require active control of off-gassing from processing water. To optimize and increase the use of ozonated water in the seafood industry, water should be pre-chilled and off-gassing must be contained. This control should be designed into the entire processing and application system.

## **CHAPTER 4**

### **PROCESS OPTIMIZATION FOR APPLICATION OF OZONATED WATER IN SHRIMP PROCESSING**

#### **4.1. Introduction**

Consumption of fish and shellfish in the U.S. has steadily increased to 16.6 pounds per person in 2004. Imports accounts for over 80 percent of the seafood consumed in the U.S., and shrimp reached a record 4.2 pounds of shrimp per person in 2004 to surpass canned tuna as the most consumed seafood type (NMFS, 2006). Annual domestic landings of shrimp have remained mostly steady over the past decade, near the 200 million pounds harvested in 2004, while shrimp exports have grown to exceed 1 billion pounds (NMFS, 2006).

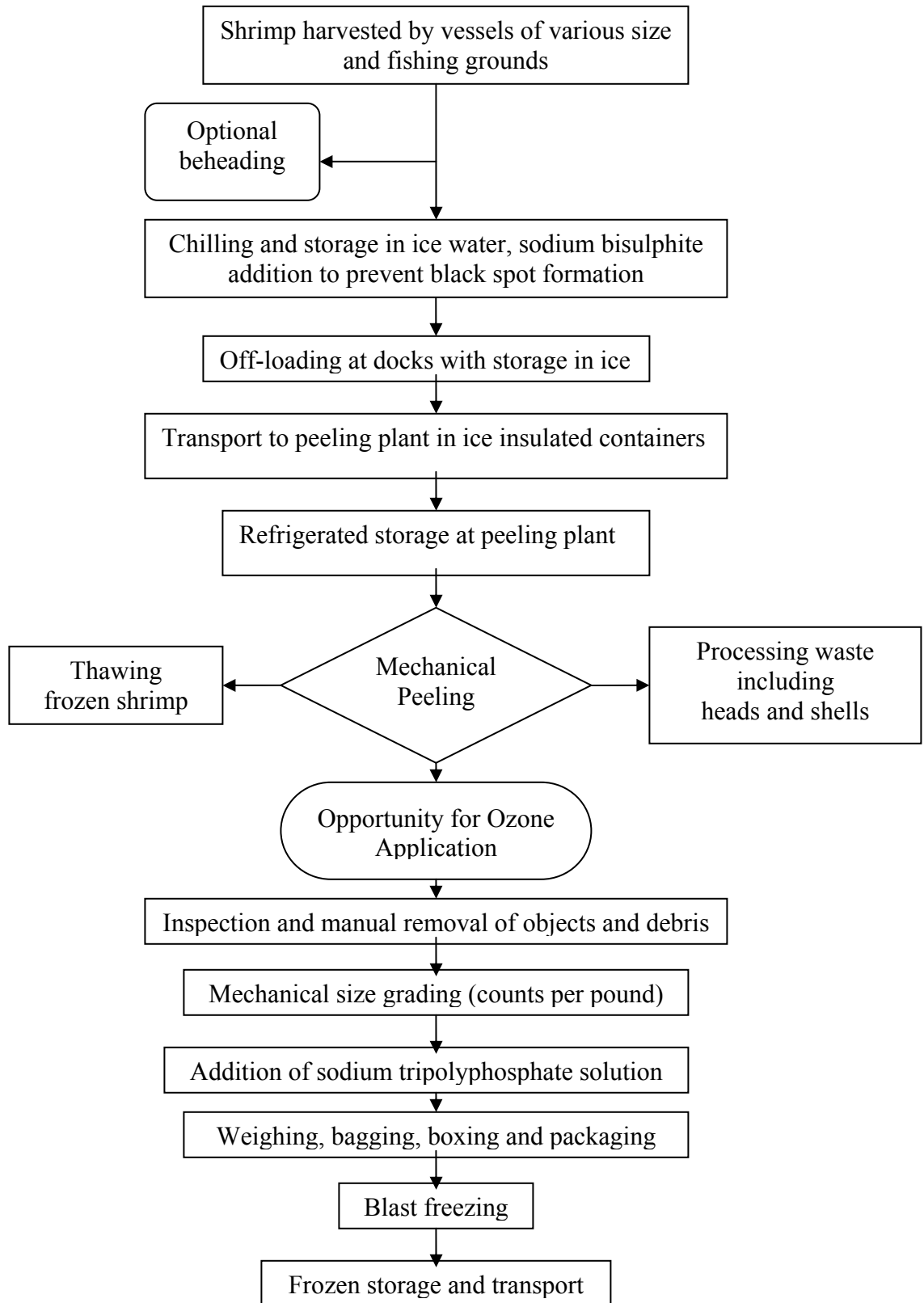
The majority of shrimp in the U.S. are harvested from Gulf of Mexico and South Atlantic (GSA) waters. Louisiana fishermen account for more than 40% of domestic shrimp landings, reaching 133 million pounds with an estimated worth of 1.6 billion dollars in 2004 (NMFS, 2006). The majority of these shrimp are mechanically peeled and frozen in Louisiana, with additional processors located in Alabama, Texas, and Mississippi. Shrimp generate the greatest economic value of all domestic commercial fisheries in the U. S. (NMFS, 2006) and the industry contributes to many components of local coastal economies.

Shrimp are harvested primarily by trawl gear on vessels with either ice storage holds or brine freezing equipment. In Louisiana, the majority of shrimp are harvested by trawling in-shore and near-shore waters, and are chilled and stored on board in ice. These trips range from overnight to 10-14 days. Significant volumes of shrimp are also brine frozen on board off-shore vessels. Iced and frozen shrimp are unloaded at shore-side dock facilities, and transported to processing plants for

mechanical peeling, packing, and primarily wholesale distribution (Figure 1). Additional volumes are packaged as headless with shells on and without peeling. This traditional wild harvested and mechanically processed shrimp industry is competing with the growing volume of farm-raised and hand peeled shrimp imports, which are now typically manually processed and frozen within 24 hours of harvest. Application of practical technologies to improve product quality and shelf life is a critical need and key component of the domestic, wild harvest shrimp industry efforts to compete with imported, farm-raised shrimp.

The successful application of ozone technology provides an opportunity to improve product quality for the mechanically peeled shrimp industry. The USFDA and USDA have amended food additive regulations to provide for the safe use of ozone in gaseous and aqueous forms as an antimicrobial agent on food, including seafood (FDA, 1982; USDA, 1997). Ozonated water has been used in a range of food processing facilities as a surface sanitizer (Guzel-Sydim, 1996). Although chemical sanitizers, including quaternary ammonium, iodine, and chlorine, are commonly used to sanitize food processing surfaces, they are not approved for direct application to seafood products. While chlorine has been the sanitizer of choice for seafood processors, there is a growing concern of adverse environmental effects with the widespread use of chlorine and a preference to gradually phase out chlorine use from food processing plants (Birks, 2003; Garcia et al., 2003).





**Figure 4.1: Flow Diagram of Peeled Shrimp Processing in Louisiana**

Ozone ( $O_3$ ) is a form of elemental oxygen ( $O_2$ ) discovered in 1839 by the European researcher C.F. Schonbein (Guzel–Seydim et al., 2003). Ozone is very unstable at atmospheric conditions and decomposes quickly to elemental oxygen (Horvath et al., 1985). Ozone is a powerful oxidizer and ranks fifth in thermodynamic oxidation potential behind elemental fluorine. Ozone has been shown to deactivate a large number of organisms, including bacteria, fungi, yeast, parasites and viruses, and can also oxidize natural organic compounds as well as synthetic substances, such as detergents, herbicides and composite pesticides (Graham, 1997; Guzel-Seydim et al., 2003). Ozone has been used in the food processing industry both as gaseous ozone and dissolved in water to reduce bacteria on a wide range of food products and contact surfaces, as well as converting green tea to black tea (Crapo et al., 2004; Kim and Yousef, 1999; Guzel-Seydim et al., 2003).

Ozone has been studied at various stages of seafood processing. Use of ozonated ice aboard fishing vessels has been shown to extend the shelf life of whole fish by 3-5 days (Rice et al., 1982). Ozonated water produced a substantial reduction in bacterial populations on stainless steel contact surfaces (Dosti and Guzel-Seydim, 2005). Intermittent washing and pumping of fish from ship holds using ozonated water was shown to extend refrigerated shelf life (Koettters et al., 1997). Treating fresh scad with gaseous ozone showed reduced bacterial levels and improved sensory scores (Silva et al., 1998). However, recent studies with ozonated water on salmon fillets and roe have indicated a decrease in frozen shelf life due to oxidation of fatty acids during storage (Crapo et al., 2004). Systems to generate ozonated water are commercially available. These turn-key systems now

contain features to destroy excess ozone for improved safety, and can be easily fitted to available water and electrical sources for ozonated water generation and application (DelOzone, 2004).

Despite these known advantages, the use of ozonated water has not been widely adopted in the seafood industry. Quality and temperature of the water available to the seafood processor can have a significant impact on the ozone levels produced by these ozonated water systems. Impurities in water are known to promote decomposition of dissolved ozone ( $dO_3$ ) and reduce its efficiency (Stahelin and Hoigne, 1985). Water temperature greatly influences the solubility of ozone in water, which increases with decreasing temperature. Recent work in our laboratory has shown the need to reduce the water temperature of ambient processing water in southern Louisiana to produce increased levels of  $dO_3$  (Chawla et al., 2006).

Most commercial systems measure  $dO_3$  levels in the water within the ozone generating system. Off-gassing of this  $dO_3$  from the water exiting the system, and before reaching the food product, can greatly reduce the amount of  $dO_3$  that actually contacts the product surface. An experimental measurement system had been developed to determine  $dO_3$  concentration in processing water at the point product application and contact (Chawla et al., 2006).

The goal of this research was to compare the bacterial destruction performance of two different application methods, three  $dO_3$  concentrations, and three treatment durations of ozonated water on peeled shrimp meat, and to select an optimal application for further iced shelf life and *Listeria* destruction studies.

## **4.2. Materials and Methods**

### **4.2.a. Sample procurement**

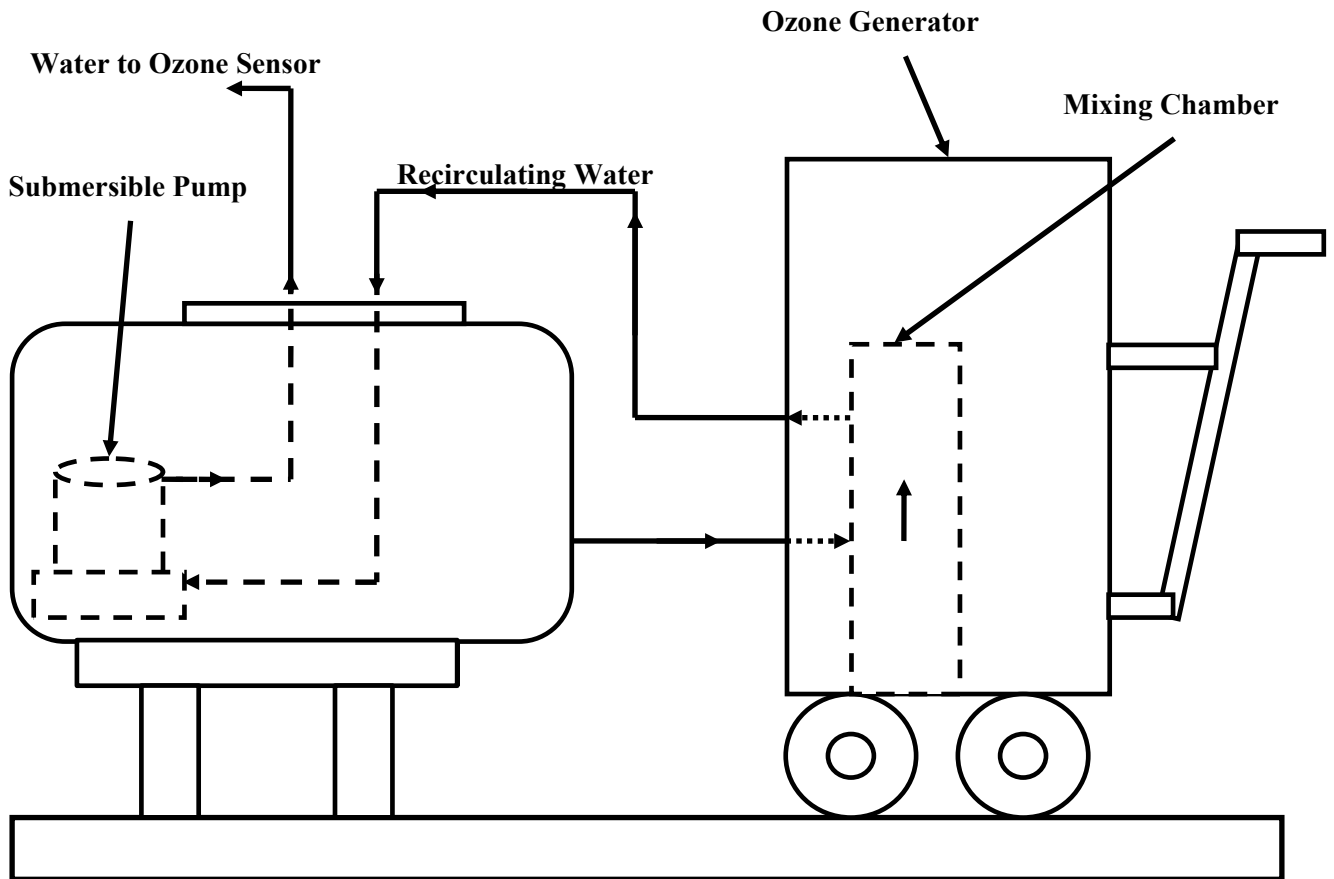
Mechanically peeled white shrimp (*Penaeus setiferus*) harvested from Gulf of Mexico waters were procured from a local shrimp processing plant. Medium shrimp, 50-60 count per pound, were removed from the processing line after peeling and before tri-polyphosphate addition and transported in ice to the Food Processing & Technology Pilot Plant at the LSU AgCenter in Baton Rouge, LA. The shrimp were stored in ice before sampling and processing.

### **4.2.b. Ozone generation**

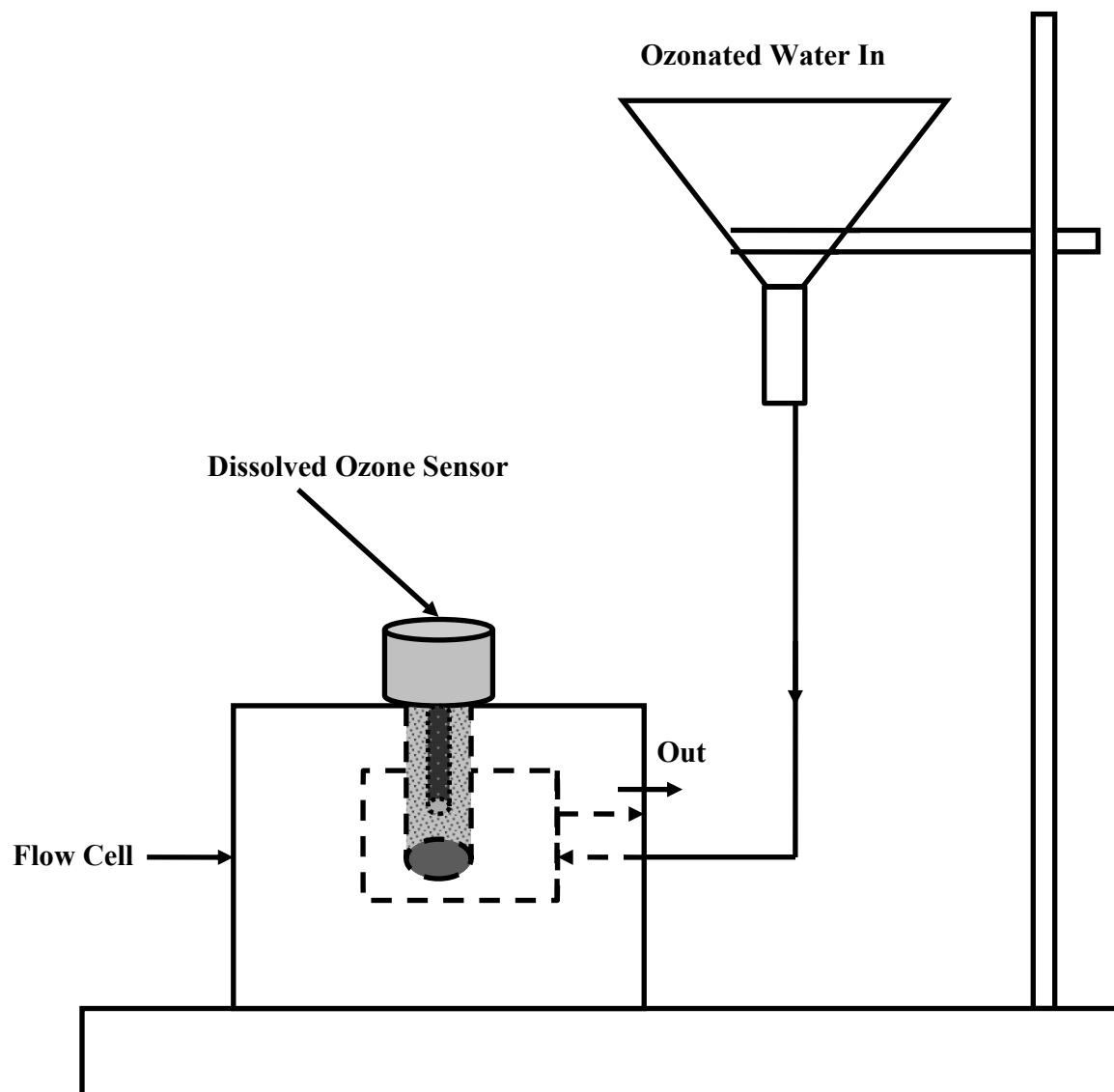
Ozonated water was generated using a DelOzone (San Luis Obispo, CA, 93401 USA)-AirLiquide (Houston, TX, 77056 USA) Infinity series (Model AGW 1500 G) corona discharge  $dO_3$  generator. A closed-loop recirculation system was constructed (Figure 4.2). This system recirculated water through the ozone generator and increased the  $dO_3$  concentrations to the three levels used in the study. Ozonated water was then transferred using a 1/2 hp submersible pump from the recirculation tank for use and measurement.

### **4.2.c. Ozone measurement**

Dissolved ozone concentration was measured using a Model A 15/64  $dO_3$  sensor (Analytical Technology, Inc. Oaks, PA, 19456 USA) inserted into an acrylic flow-cell and connected to a flow-through system (Figure 4.3).



**Figure 4.2: Recirculation Setup to Produce Ozonated Water**

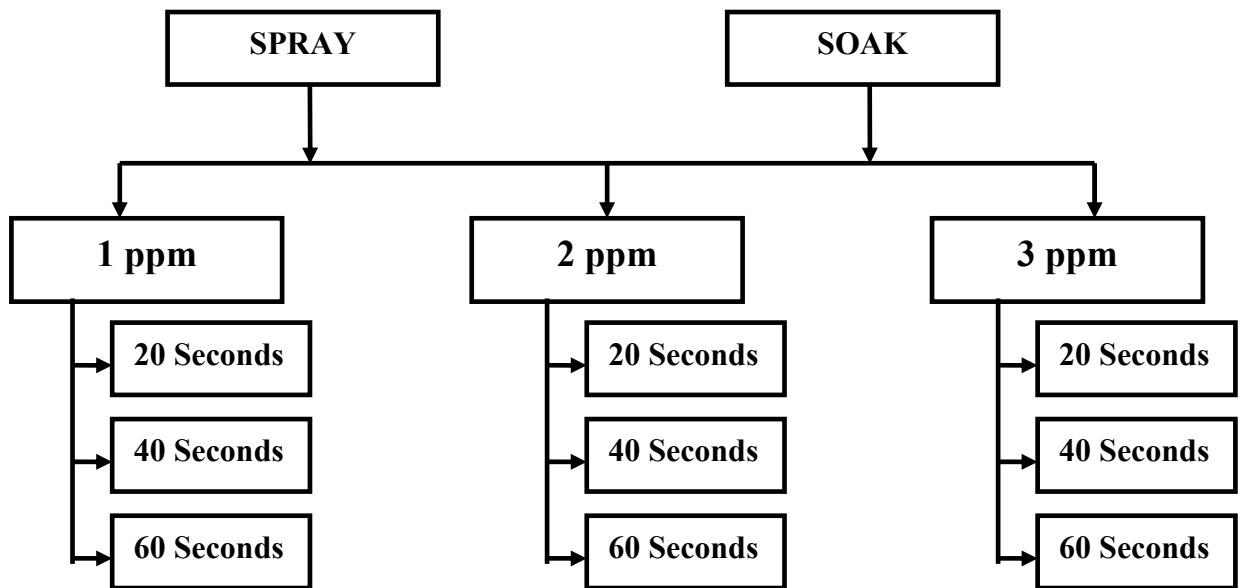


**Figure 4.3: Setup for Flow-thru Process Using a Flow cell and dO<sub>3</sub> Sensor**

Water enters the funnel and travels through the flow cell past the Clarke-Oxygen electrodes of the ozone sensor.  $dO_3$  concentration (ppm) are shown on the sensor digital display (Chawla et al., 2006). These ozone concentrations were verified by chemical methods, using Hach  $dO_3$ , AccuVac® Kits (Hach Company, Loveland, CO 80539) and the indigo blue titration method (Bader, 1982).

#### 4.2.d. Sample Treatment

A matrix of the different treatment combinations is shown in Figure 4.4. Two different application types, soaking and spraying, were compared. Three different  $dO_3$  concentrations 1 ppm, 2 ppm and 3 ppm were generated in  $10^\circ C$  and used for each application type. Shrimp samples were soaked or sprayed for 20, 40 and 60 s for each concentration.



**Figure 4.4: Matrix of Concentration and Contact Times for Application of  $dO_3$  using Soak and Spray Application**

To increase ozone production and control solubility, water temperature was maintained at 10°C. Each treatment (spray and soak for all combinations) used 19.3 l of water to 0.45 kg of shrimp, based on estimated usage at a local shrimp peeling facility. Three replicate processing trials were carried out for each treatment, and all treatment combinations for each trial were conducted on the same day.

Samples for each spray treatment were aseptically placed in a single layer on a sterile mesh.  $dO_3$  concentration of the spray water was measured immediately before spraying, and shrimp meat samples were sprayed from a fixed height of 26 cm to receive complete coverage for the specific time interval. Treated and control (non-ozonated water) samples were then aseptically transferred to sterile Whirl-pack bags and stored in ice for subsequent microbial and chemical analysis.

Samples for each soak treatment were dropped into a 38.5 l high density polyethylene HDPE tub containing the ozonated water and soaked for the specific time interval.  $dO_3$  in the soak water was measured immediately before filling the tub. Shrimp samples were then bag aseptically bagged and stored as the spray samples.

#### **4.2.e. Microbial Analysis**

Microbial analysis included Aerobic Plate Counts (APC) and *Pseudomonas fluorescens* counts. Each Whirl-pack bag containing the control or treated samples were placed in a stomacher (Seward STO 80, Cincinnati, OH 45222), serially diluted to 1/10 with phosphate buffer solution (PBS) and comminuted. The stomached sample was then serially diluted in PBS and



plated on APC Petrifilms (3M Corp., St. Paul, MN ) and then incubated at 37°C for 24 hours (Ginn, 1986). Dilutions were also surface-plated onto *Pseudomonas* F agar for *Pseudomonas fluorescens* enumeration and incubated at 35°C for 24 hours (King et al., 1954).

#### **4.2.f. Chemical Analysis**

Proximate analysis was carried out to determine the moisture, protein, fat and ash content of the shrimp samples, carbohydrate content was assumed to be negligible. Moisture and fat content was determined with a CEM microwave moisture/fat analyzer (CEM Corp., Matthews, NC 28106). The total nitrogen content was determined using a Primacs<sup>SN</sup> - Protein - Nitrogen Analyzer (Skalar Inc., Norcross, GA 30071), which uses the Dumas combustion method. Protein content was determined by multiplying the total nitrogen content by 6.25. Ash content was determined by combustion in a Muffle furnace at 450°C. TBARS test to determine lipid oxidation was done on the remaining, undiluted, stomached samples from the microbial analysis. TBARS test was used to detect malonaldehyde levels using the Thiobarbituric Acid Reacting Substances (Lemon, 1975) Fifteen grams of shrimp was digested with 30 ml of an extraction solution containing 7.5 % trichloroacetic acid, propyl gallate and ethylenediaminetetraacetic acid. The homogenate was separated by centrifuging the samples at 2000rpm for 15 minutes. 5 ml of the extract was then mixed with 5 ml of TBA reagent (0.02 M thiobarbituric acid in water) and heated in boiling water bath for 40 min. The solution was cooled and color development was measured at 530 nm using a spectrophotometer.

The standard curve was prepared using the TEP standard (1,1,3,3 tetraethoxypropane).

#### 4.2.g. Statistical Analysis

Triplicate samples were used for each analysis. One way ANOVA and paired comparisons of the treatment means with the control using students t test was carried out using JMP<sub>in</sub>® software (Copyright © 1989–2000, SAS Institute Inc., Cary, NC ). Significance was set at  $\alpha < 0.05$ .

#### 4.3. Results and Discussion

Proximate analysis showed little variability for shrimp meat composition moisture, protein, fat and ash.

**Table 4.1: Means and standard deviations for proximate composition of commercially peeled shrimp meat (pooled samples)**

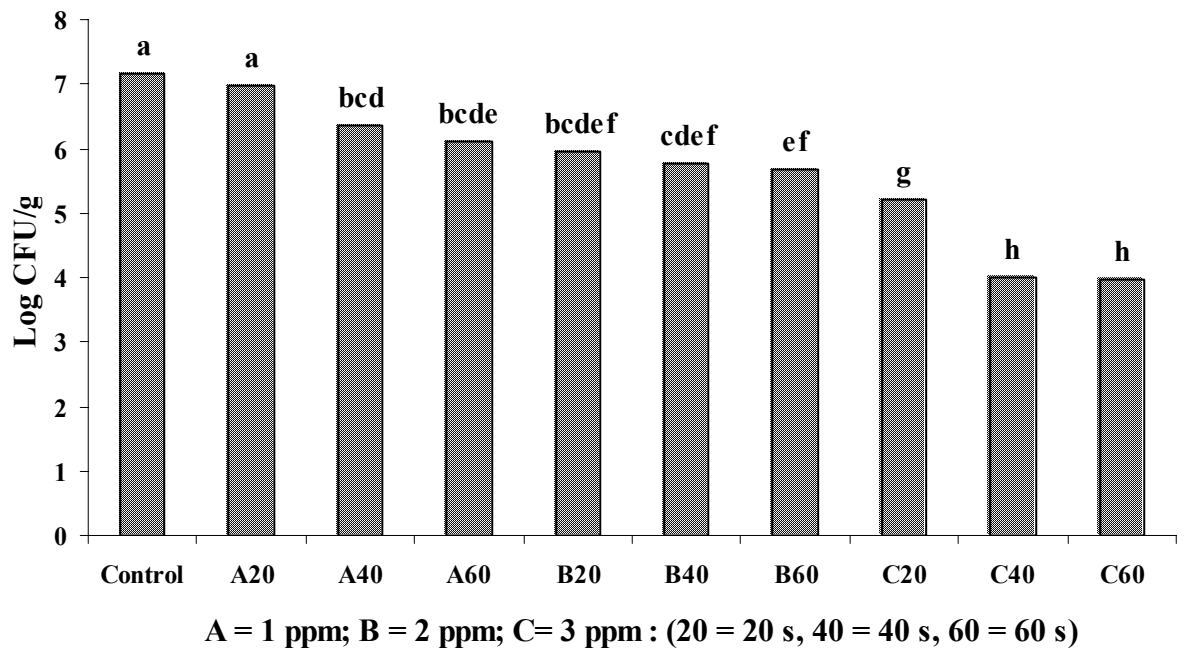
	Moisture %	Protein %	Fat %	Ash %
Mean	86	12.76	0.26	0.98
STD	0.75	0.09	.013	.04

Preliminary results showed that treating (spraying/soaking) shrimp with non ozonated water had no noticeable effect on bacterial reductions.

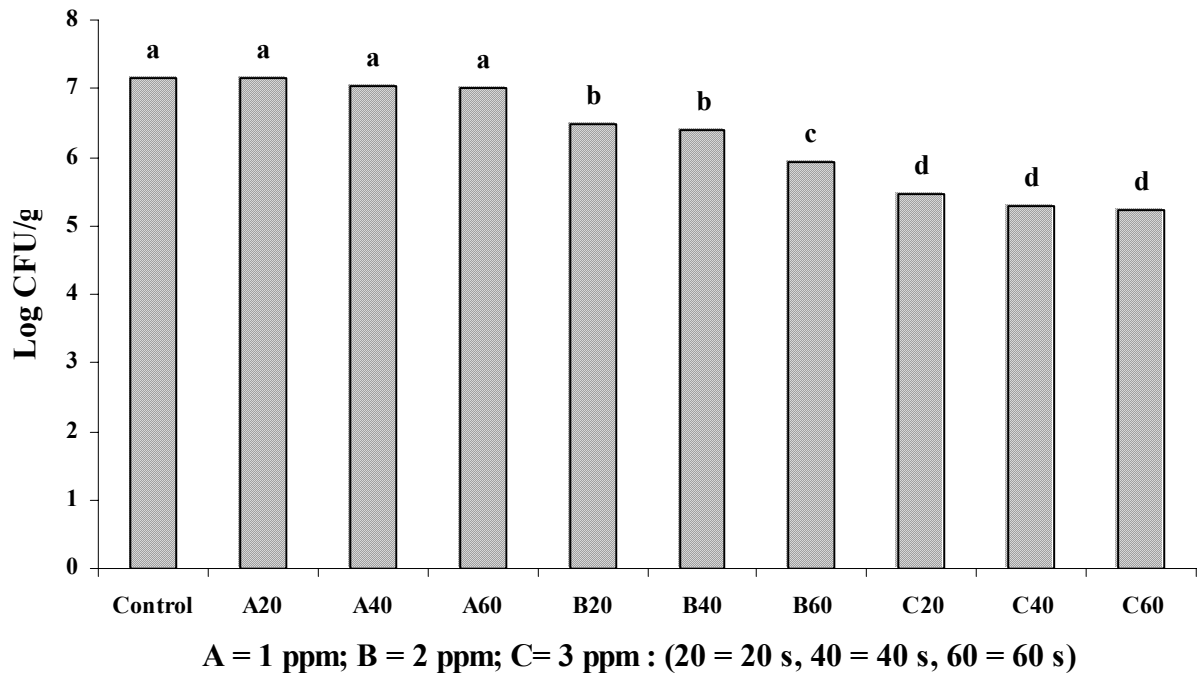
Destruction of spoilage bacteria was evaluated as an indicator of the efficacy of ozonated water treatment of peeled shrimp meat (Figure 4.5). For soak treatments, all combinations of dO<sub>3</sub> and application times investigated in this study resulted in significantly lower APC than control samples, except for the 1 ppm dO<sub>3</sub> for 20 s treatment. For the spray treatments, application of 1 ppm ozonated water for all three of the treatment times (20 s, 40 s, 60 s) did not result in significantly different APC than the control (Figure 4.6).

Spraying shrimp meat samples with 2 ppm and 3 ppm dO<sub>3</sub> did significantly reduce aerobic spoilage bacteria. Soaking peeled shrimp meat in ozonated water resulted in greater destruction of spoilage bacteria, with significantly greater APC reductions for most treatments, than equivalent ozonated water spray applications (Figure 4.7).

For each application type, treatment with higher dO<sub>3</sub> for the same amount of time resulted in greater reduction of spoilage bacteria (Figure 4.7). For all treatment groups except spraying with 2 ppm, there was little to no



**Figure 4.5: Aerobic Plate Counts Using Soak Application at Different Ozone Concentrations and Contact Times**

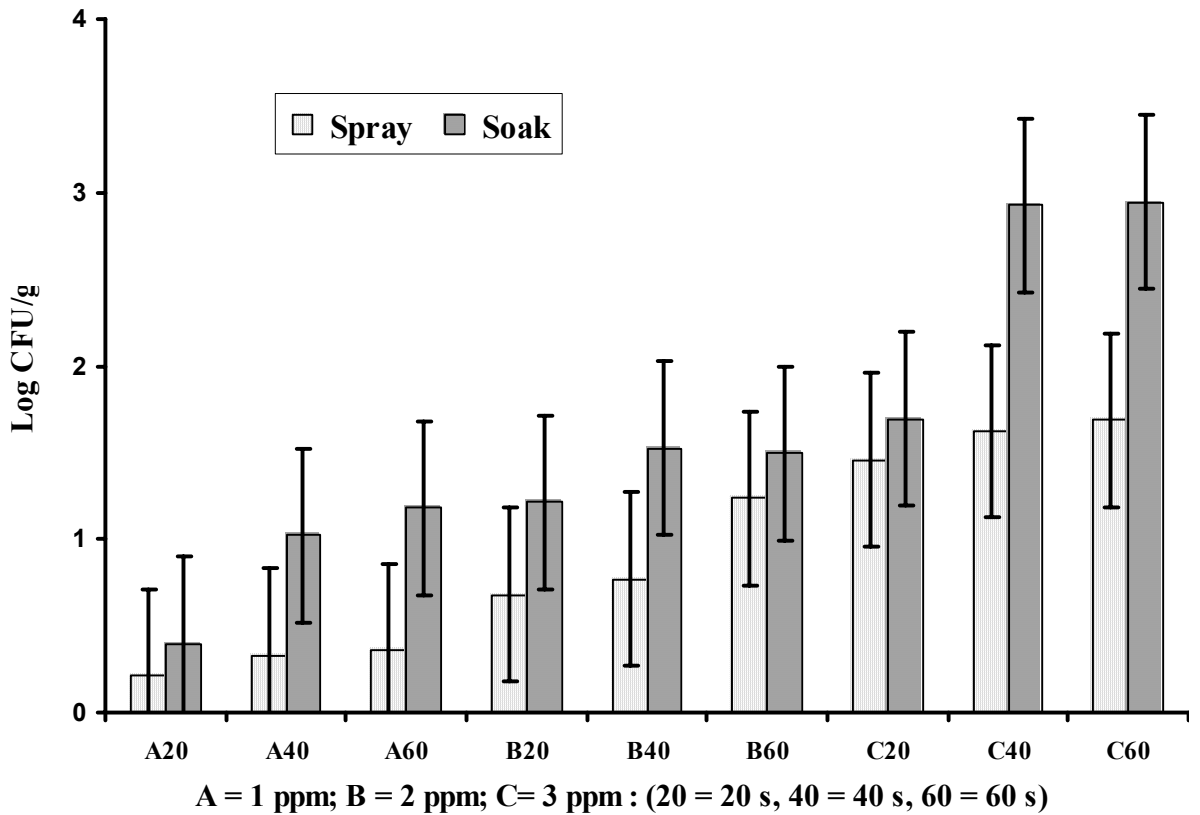


**Figure 4.6: Aerobic Plate Counts Using Spray Application at Different Ozone Concentrations and Contact Times**

increased reduction between 40 s and 60 s of application. For all groups except spraying at 1 ppm, 40 s and 60 s of treatment resulted in significantly greater APC reduction than 20 s. Additionally, for soaking, increasing the  $dO_3$  concentration by 1 ppm and treating for only 20 resulted in similar microbial reduction to soaking at the lower concentration for 40 s and 60 s (Figure 4.7).

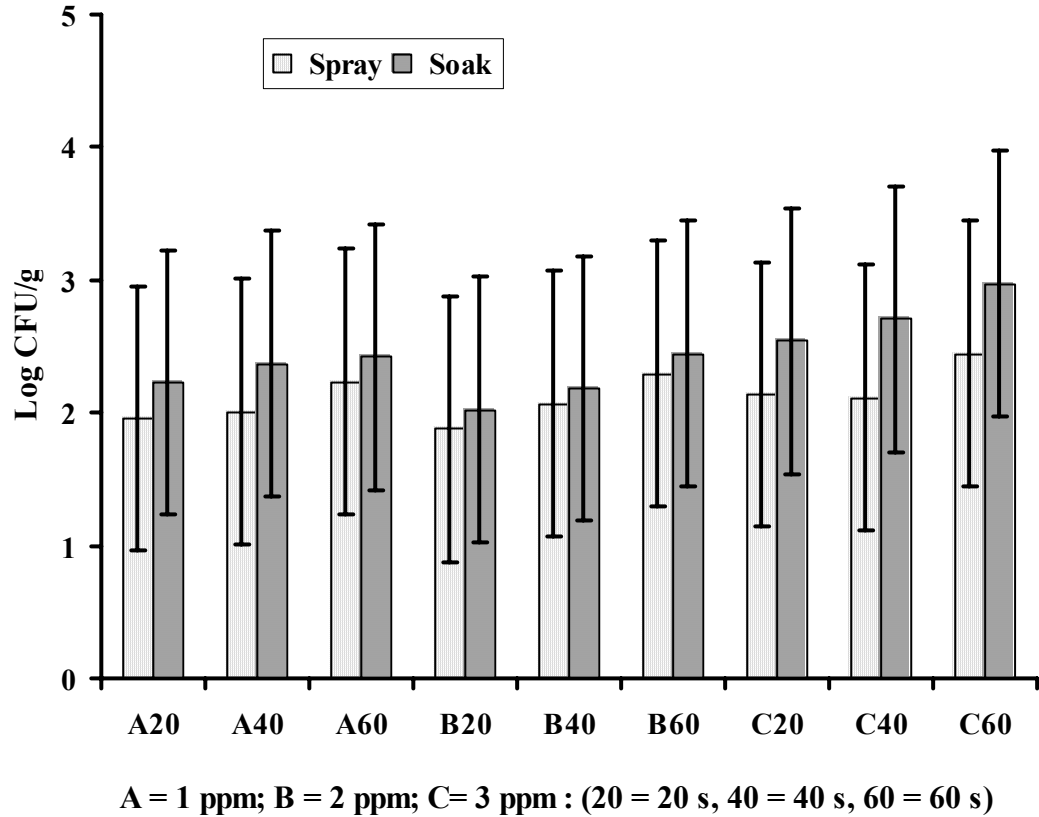
Soaking the peeled shrimp meat samples in 3 ppm ozonated water for 40 s and 60 s resulted in the greatest reduction in APC levels (Figure 4.7). This data also indicates that longer soak times above 60 s would not significantly increase destruction of spoilage bacteria. However, increased reduction of spoilage bacteria does result with increased concentration of  $dO_3$  in the treatment water. Increasing this  $dO_3$  level above 3 ppm may result in an increased destruction of bacteria on the peeled shrimp meat. Our experience in producing the  $dO_3$  concentrations used in this study suggest using 3 ppm as the

top limit, due to water conditions and off-gassing concerns expected to be encountered in the commercial shrimp facilities in the Gulf of Mexico region.



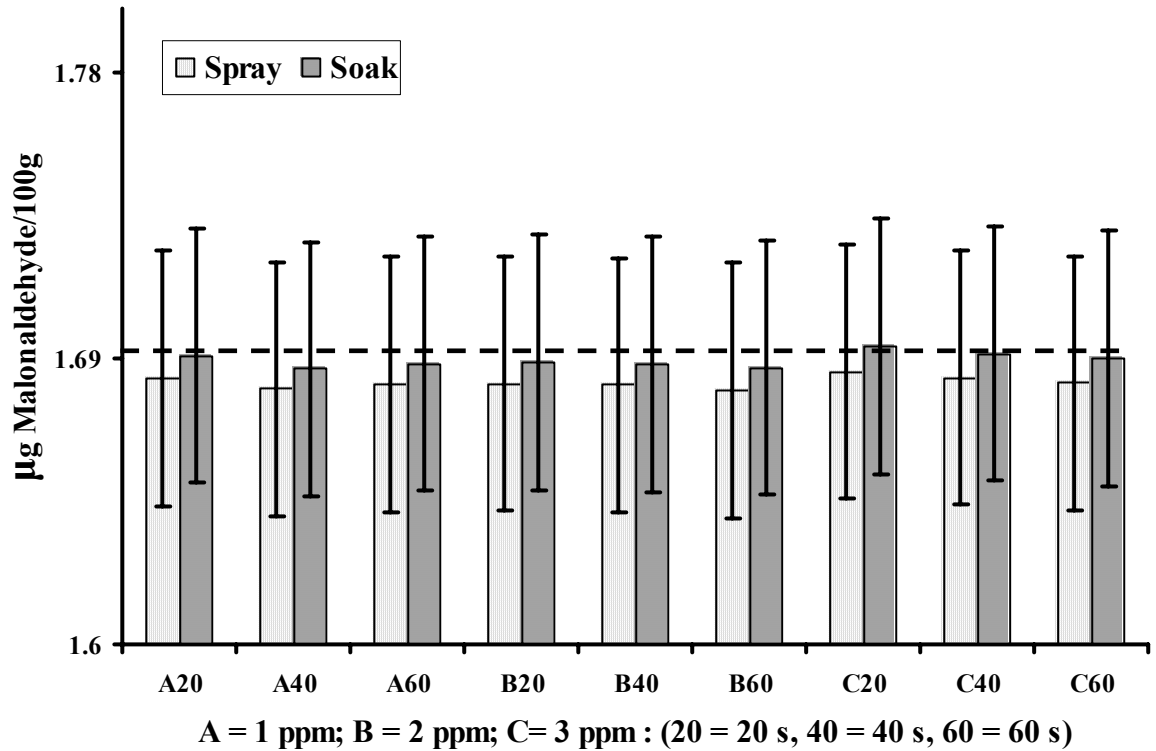
**Figure: 4.7 Comparison of Bacterial Reduction (from control) between Soak and Spray Application at Different Ozone Concentrations and Contact Times**

The highest levels of *P. fluorescens* destruction were also found for soaking in 3 ppm for 60 s treatment (Figure 4.8). Although initial *Pseudomonas*. levels were higher in the shrimp samples; the increased destruction at the highest dO<sub>3</sub> treatment was not as significant as the APC reduction. Gram negative bacteria like *Pseudomonas*. are more prone to injury than destruction by small doses of ozone (Kim, 1998).



**Figure 4.8: Comparison of *Pseudomonas fluorescens* Reduction (from control) between Soak and Spray Application at Different Ozone Concentrations and Contact Times**

Due to the strong oxidative strength of ozone, the shrimp samples were evaluated for lipid oxidation using the TBARS test. Proximate analysis of shrimp composition indicated < 0.3 % fat in shrimp (Table 4.1). A significant difference in the TBARS values was not observed between the control and the treated samples immediately after treatment for all treatment combinations investigated (Figure 4.9).



**Figure 4.9: TBARS Values for Peeled Shrimp Soaked and Sprayed with Ozonated Water at Different Ozone Concentrations and Contact Times**  
 \*(Dotted line indicates average micrograms of malonaldehyde/100g in control shrimp (1.69 µg/100g) with no treatment.)

#### 4.4. Conclusion

Ozonated water treatment presents an opportunity to improve product quality by reducing spoilage bacteria during mechanically peeled shrimp processing. Soaking peeled shrimp meat in ozonated water was found to be more effective than spraying shrimp with ozonated water, and higher ozone concentrations were more effective for reducing levels of spoilage bacteria levels on the shrimp. It is important to measure concentration of the treatment water near product application to be confident that the  $dO_3$  level produced in the water is actually being applied to the product surface. Water temperature

will significantly affect the  $dO_3$  concentration and decreasing water temperature to  $10^{\circ}C$  or below will facilitate generating and maintaining the desired  $dO_3$  level. The application of ozonated water did not increase lipid oxidation in the shrimp immediately after treatment.

Soaking peeled shrimp meat in water with 3 ppm  $dO_3$  for 60 s resulted in the highest reduction of spoilage bacteria of the treatments studied, and will be used to investigate the effects on product quality and shelf life during storage and *Listeria* destruction.



## **CHAPTER 5**

### **EFFECTS OF OZONATED WATER TREATMENT ON QUALITY, SHELF LIFE AND SAFETY IN PEELED SHRIMP MEAT**

#### **5.1. Introduction**

Shrimp has replaced canned tuna as the most popular seafood consumed in the U.S. This increased consumption has been driven by high volumes of low cost imports, as domestic production has remained steady. Louisiana leads the nation in shrimp production with over 63 % of the domestic landings of 210 million pounds harvested in 2004 (NMFS, 2006). Shrimp is the most economically valuable fishery in Louisiana, and mechanically peeled and frozen shrimp accounts for the major product form processed in the state and the Gulf of Mexico region (Schwab, personal communication 2005). However the viability of the domestic shrimp industry is under constant economic pressure from the high volumes of hand peeled and low cost imported shrimp (Anon, 2004).

Improved product quality is necessary for the domestic industry to compete with the imported product and support national shrimp industry marketing initiatives (DeSantis, 2003). The high volumes of shrimp landings in the Gulf of Mexico waters has resulted in the development of high volume mechanical peeling operators. Due to the variable pre and post harvest conditions of the wild shrimp fisheries, wild shrimp can develop increased levels of spoilage bacteria. Using ozonated water in the peeling operations presents an opportunity to reduce bacterial levels and improve the quality and

shelf life of peeled shrimp. This high quality shrimp will present the shrimp industry better chances to compete with the low cost imports.

Ozone is a USFDA and USDA approved antibacterial agent that can be applied to food products (FDA, 1982; USDA, 1997). Commercial ozone units can generate ozone using available water and power (DeOzone, 2004). Ozone has a short half life at ambient temperature and does not leave behind residues unlike chlorine. Ozone is effective against a wide range of bacteria, viruses, yeast, molds and protozoa (Anon. 2005; Guzel-Seydim, 2004). Ozone has seen use in the food processing industry as gaseous ozone and dissolved in water as ozonated water. Both have been used as a bactericide on a wide range of food products including meat, poultry, eggs, raw fruits and vegetables, seafood and fruit juices, as well as sanitation of product contact surfaces (Guzel-Seydim et al., 2003, Anon, 2005). Ozonated water has been shown to reduce levels of *Listeria monocytogenes* on food contact surfaces (Moore G, 2000). *L. monocytogenes* serotype (1/2a) and *L. monocytogenes* serotype (4b) have been isolated from seafood processing environments (Rorvik, 2000). Ozone has also been shown to be effective in reducing *Listera* levels on the surfaces of strawberries, cantaloupes, lettuce, beef carcasses, alfalfa sprouts and seafood (Jahncke, 2004; Reagan et al., 1996; Rodgers et al., 2004; Warriner et al., 2005).

Despite these advantages, the use of ozonated water technology has not been widely adopted in the seafood industry. Recent studies of ozonated water on salmon fillets and roe found increased oxidation during frozen storage of

these high lipid products (Carpo et al., 2004). In addition to incurred capital and operational costs, seafood processors have received limited understanding of  $\text{dO}_3$  solubility, including the effects of temperature and water quality. These inputs and off-gassing often result in the incorrect measurement and knowledge of actual ozone concentrations at the point of product application. A previously determined optimal time-concentration ozonated water treatment (Chawla et al., 2006) was used to investigate effects of ozonated water on raw product quality, shelf life and safety of peeled shrimp meat during iced storage.

## **5.2. Materials and Methods**

### **5.2.a. Sample procurement and treatment**

Medium white shrimp (*Litopenaeus setiferus*), and brown shrimp (*Farfantepenaeus aztecus*), 36 – 40 count stored in ice slush was landed and transported on ice to the Food Processing Technology Pilot Plant (FPTPP), Louisiana State University, Agricultural Center, LA, within 24 hours after harvest from Louisiana state waters. The untreated shrimp were then hand peeled and stored on ice until treatment or sampling. Ozonated water was generated at 10°C and concentrations measured using apparatus and procedures developed by Chawla et al. (2006). The peeled shrimp were divided into two equal groups and one group was treated using by soaking in 3ppm ozonated water for 60 seconds. Shrimp meat samples from both the untreated and treated control groups were stored in Ziploc® bags on ice for the duration of the shelf life study. Storage ice was changed periodically to maintain shrimp temperature. Shrimp meat samples were removed from both the groups at two

day intervals for immediate microbial and chemical analysis. Additional samples were removed at three day intervals and frozen in liquid carbon dioxide tunnel freezer (AirLiquide, Houston, TX, 77056 USA), and stored at –15°C until thawing for the consumer sensory study.

### **5.2.b. Shelf Life Study**

#### **5.2.b.i. Aerobic Plate Counts (APC)**

Triplicate samples of peeled shrimp meat were removed from ice storage every two days throughout the three week study to determine Aerobic Plate Counts (APC) using the standard Petri film method (AOAC, 1999). The Petri films were incubated at 37°C for 48 hours before enumeration and the results were expressed as CFU/g.

#### **5.2.b.ii. Proximate Analysis**

Proximate analysis was carried out to determine the moisture, protein, fat and ash content of the shrimp samples, carbohydrate content was assumed to be negligible. Moisture and fat content was determined with a CEM microwave moisture/fat analyzer (CEM Corp., Matthews, NC 28106). The total nitrogen content was determined using a Primacs<sup>SN</sup> - Protein - Nitrogen Analyzer (Skalar Inc., Norcross, GA 30071), which uses the Dumas combustion method. Protein content was determined by multiplying the total nitrogen content by 6.25. Ash content was determined by combustion in a Muffle furnace at 450°C.

### **5.2.b.iii. TBARS**

Triplicate samples of peeled shrimp meat were removed from iced storage at two day intervals and analyzed for malonaldehyde levels using the Thiobarbituric Acid Reacting Substances (TBARS) test to determine lipid oxidation (Lemon, 1975). Fifteen grams of shrimp was digested with 30 ml of an extraction solution containing 7.5 % trichloroacetic acid, propyl gallate and ethylenediaminetetraacetic acid. The homogenate was separated by centrifuging the samples at 2000 rpm for 15 minutes. 5 ml of the extract was then mixed with 5 ml of TBA reagent (0.02 M thiobarbituric acid in water) and heated in boiling water bath for 40 min. The solution was cooled and color development was measured at 530 nm using a spectrophotometer. The standard curve was prepared using the TEP standard (1,1,3,3 tetraethoxypropane). Moisture and fat content was determined with a CEM microwave moisture/fat analyzer (CEM Corp., Matthews, NC 28106).

### **5.2.c. Bioamines**

The following bioamines were analyzed: putrescine and cadaverine. All standard amine mixtures were prepared using 0.1 N HCL. Fluorinated anhydride derivatives of amines were created using pentafluoropropionic (PFP) anhydride as the derivitizing agent. The derivitization procedure was based on the methodology of Rogers et al. (2003). To a 1ml aliquot of hexanediamine internal standard working solution varying volumes of putrescine-cadaverine standard working solutions were added in a test tube along with 0.5 ml 1 N HCL. This mixture was dried by nitrogen flushing at 50 - 60°C. To the dried residue 1 ml ethyl acetate and

300 µl PFP anhydride was added and the mixture was heated at 50°C for 30 min. Within two hours after removal from water bath the resulting mixture was purified using solid phase extraction as described by Rogers et al. (2003). The final effluent collected from the solid phase extraction in 50% ethyl acetate-toluene solvent is stable for at least 3 months when stored refrigerated in dark. Two micro liter of this solution was injected into the GC.

### **5.2.c.i. GC Conditions**

A gas chromatograph - Finnigan/Tremetrics Model 9001 (Waltham, MA 02454), with <sup>63</sup>Ni electron capture detector was used with the following instrument modification for bioamine detection.

A DB-210 (30m\*0.23 mm id\*0.25 µm, 50% Trifluoropropylmethylpolysiloxane) column and a HP-225 (30m\*0.32 mm id\*0.25 µm, 50% CNPrPh ME Siloxane) column with the following temperature conditions were used for putrescine and cadaverine derivatives. Temperature (°C) injection port 270°C, detector 350°C, column uses helium as carrier gas with flow 3.0 ml/min; detector purge gas nitrogen 45 ml/min; detector makeup gas nitrogen 15 ml/min. Equipped with auto sampler CTC A200SE (Leap Technologies, Chapel Hill, NC). LabQuest Chromatography Data System was used to control instrumentation and integrate results (Finnigan Corp, Austin, TX)

Cold trapping was used to focus analytes on column initially with column oven at 80°C for one minute, increase temperature (20°C/min) to 190°C without hold, final increase temperature (8°C/min) to 240°C hold for 15 min., to get sharper resolution of peaks. Retention times of

pentafluoropropionic (PFP) derivatives on the DB 210 column were 8.36, 9.18, and 9.79 and on the HP 225 column were 10.04, 10.70, and 11.15 respectively for putrescine, cadaverine, and hexanediamine.

#### **5.2.c.ii. Calibration Curves**

Calibration curves were prepared by serially diluting a 1mg/ml solution of putrescine and cadaverine standard stock solution. The concentrations of bio amines used to prepare the standard curve were 0.5 µg/g, 1.0 µg/g, 2.0 µg/g, 5.0 µg/g, 10.0 µg/g and 0.25 µg/g, 0.5 µg/g, 1.0 µg/g, 2.5 µg/g, 5.0 µg/g for cadaverine and putrescine respectively. Each of these solutions were derivitized and the fluorinated derivatives were analyzed by GC using the electron capture detector as mentioned earlier. The peak areas were calculated and calibration curves were constructed based on triplicate runs of the standard solutions.

#### **5.2.c.iii. Shrimp Extraction**

Triplicate samples of peeled shrimp meat were removed from ice storage every three days and analyzed for putrescine and cadaverine concentrations according to the Association of Official Analytical Chemists (AOAC) Official Method 996.07 as modified by the FDA for shrimp using the improved extraction solvent (75% methanol + 25% 0.4N HCL in 0.5% KCL) (AOAC 1999; Rogers et al., 2003). Ten grams of shrimp was weighed and transferred to a blender bowl, to this shrimp 60 ml of 75% methanol + 25% 0.4N HCL in 0.5% KCL was added and the mixture was blended at high speed for 2 minutes. This slurry was then transferred to a 100 ml volumetric flask

rinsing the blender and the lid with the same extraction solvent and adding the rinses to flask. The mixture was heated to 60°C in a water bath for 15 min and then allowed to cool at room temperature. The volume was made up by diluting with the extraction solvent. This mixture was then chilled in an ice bath for > 45 min. The methanol extracts were then filtered through coarse filter paper. Five ml of this extract along with 1ml internal standard solution and 0.5 ml HCL was then evaporated to dryness by nitrogen flushing and heating at 50°C. After all solvents were evaporated 2-3 ml of 75% methanol was added and the mixture was dried again. To the dried residue of methanol extract 300µl of PFP anhydride and 1 ml of ethyl acetate was added and the mixture was heated to 50°C for 30 min in a water bath. The resultant mixture turned yellow and was purified using solid phase extraction within 2 hours, as described by Rogers et al. (2003).

#### **5.2.d. Consumer Sensory Study**

The consumer sensory study was conducted at, LSU campus, Baton Rouge, LA. On the morning of the consumer testing frozen samples were removed from frozen storage and thawed to 10°C in a cooler. Each of the 254 consumers were presented with 12 coded raw shrimp samples 30 g each to evaluate appearance and odor of the ozonated water treated and untreated samples over two days. Each consumer, was provided with a scale and descriptors, that was based on the National Marine Fisheries Service scale for grading raw frozen shrimp (NMFS, 1993), and graded the samples on a four point basis (4 = pass, 3 = borderline pass, 2 = borderline fail, 1 = fail).



### 5.2.e. *Listeria* Inoculation Study

#### Culture Growth Conditions

*Listeria monocytogenes* strain (serotype ½ a) and *Listeria monocytogenes* strain (serotype 4b) obtained from the Centers for Disease Control, Atlanta (CDC), GA., U.S.A., that was originally isolated from the blood of an infected individual, was used during this study (CDC, 2002). The *Listeria monocytogenes* cultures were grown for 18 hours in Brain Heart Infusion Broth (BHI) (Difco, Detroit, Mich., U.S.A.) at 37°C. The pure cultures were stored at -70°C and sub cultured twice in BHI Broth at 37°C for 24 h before being used. Shrimp, brine frozen at sea were locally procured and hand peeled and frozen until inoculation. The peeled shrimp samples were divided into two groups containing 1000g of shrimp each and irradiated with UV radiation for 15 minutes on both sides. The 18 h *L.monocytogenes* cultures were decimally diluted to 10<sup>6</sup> in phosphate-buffered saline (PBS). One milliliter of each *L. monocytogens* culture individually dispensed from the pipette was inoculated on each of the two groups of UV treated shrimp. The inoculums were allowed to air dry on the shrimp samples for 25 minutes under a laminar flow hood. Each of the two shrimp groups inoculated with the two strains of *Listera* was then split into halves containing 500g each. One half of each strain inoculated group was soaked in water for 60s (control) and the other half was soaked in 3 ppm ozonated water for 60s (treated). After treatment *L. monocytogenes* counts were determined by making 1:1 dilutions of shrimp with PBS, stomaching for 2 min, making serial dilutions and plating the dilutions on Oxford agar with a

selective supplement (20 mg/liter each of cycloheximide, colistin sulphate, acriflavine, cefoyetan, and fosfomycing; Oxoid, Hampshire, England). The plates were then incubated for 48 h at 37°C and the results were expressed as CFU/g.

### 5.2.f. Statistical analysis

Triplicate samples were used for each analysis. One way ANOVA and paired comparisons of means using students t test was carried out with JMP<sub>in</sub>® software (Copyright © 1989–2000, SAS Institute Inc., Cary, NC). Significance was set at  $\alpha < 0.05$ .

### 5.3. Results and Discussion

Proximate analysis showed little variability for shrimp meat composition moisture, protein, fat and ash.

**Table 5.1: Means and standard deviations, for proximate composition of commercially peeled shrimp meat (pooled samples)**

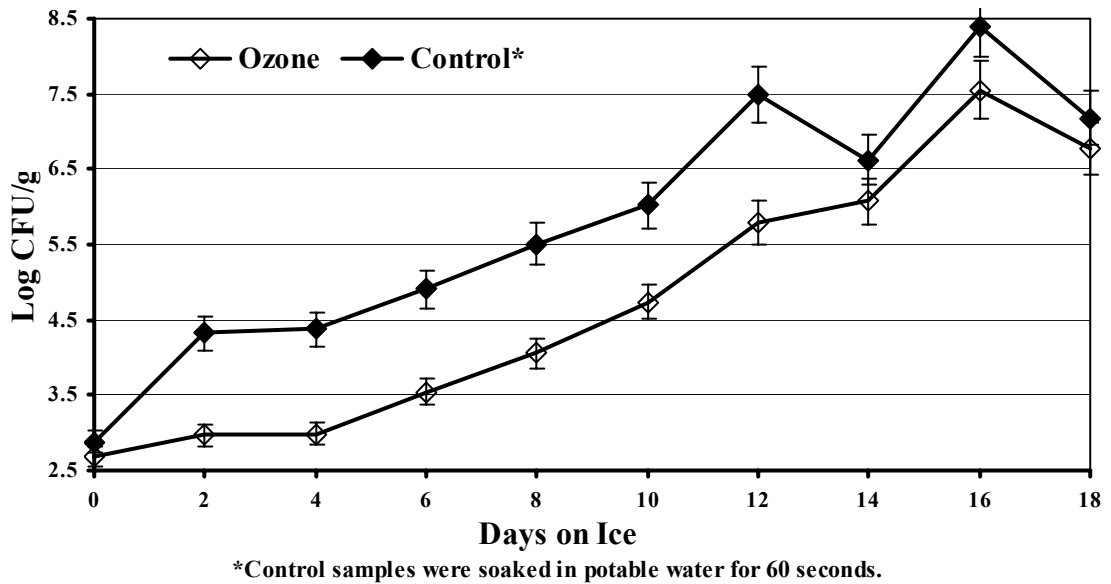
	Moisture %	Protein %	Fat %	Ash %
Mean	85	13.85	0.24	0.91
STD	0.51	0.29	.013	.04

Figure 5.1 shows the Aerobic Plate Counts of the treated and untreated shrimp samples stored on ice for a period of 18 days. Day 0 treated and untreated shrimp showed similar counts. This can be attributed to the fact that day 0 shrimp were of a high quality and correspond to < 48 hrs after harvesting, that were rapidly cooled with ice and maintained at that temperature under strict experimental conditions. An increase in bacterial

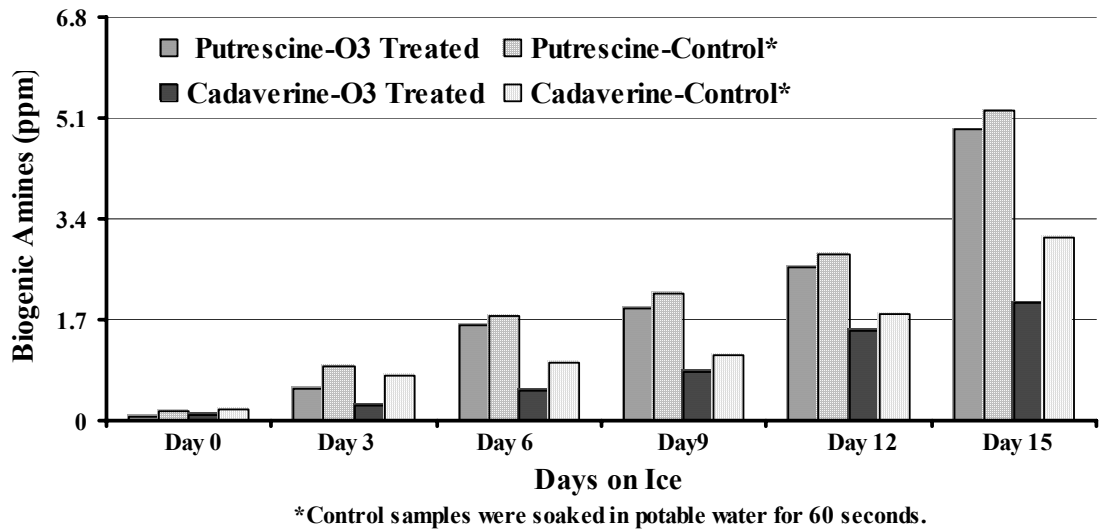
levels can be seen as the study progressed. Earlier work done in this laboratory with shrimp purchased from a commercial peeling plant showed a significant difference of 3 log CFU/g in bacterial loads between treated and untreated shrimp. Ozone treated shrimp showed significantly lower counts as compared to untreated shrimp on days 2, 4, 6, 8, 10 and 12. Treated shrimp took 14 days to reach a bacterial load of  $10^6$  CFU/g where as the untreated shrimp took 10 days to reach a similar bacterial load. As can be seen from Figure 5.1 the treated shrimp took a total of 16 days to reach bacterial loads greater than  $10^7$  CFU/g as compared to 12 days taken by untreated shrimp. The ozone treatment seems to have delayed the lag phase of growth of aerobic bacteria on shrimp.

These results indicated that low initial loads of bacteria seen in treated shrimp were a result of controlled handling of shrimp during this study however one can expect higher bacterial loads in shrimp after mechanical peeling and thus more opportunity for bacterial destruction with ozonated water treatment of commercial shrimp.

Biogenic amine formation in shrimp increased from < 1 ppm of putrescine and cadaverine in treated and untreated shrimp on day 0 to 5.2 ppm putrescine and 3 ppm cadaverine in untreated shrimp on day 15 Figure 5.2. Putrescine levels in shrimp increased at a faster rate as compared to the cadaverine levels in treated and untreated shrimp with the highest level of putrescine observed for day 15 untreated shrimp.



**Figure 5.1: Aerobic Plate Counts of Peeled Shrimp Soaked in 3ppm Ozonated Water for 60 seconds**

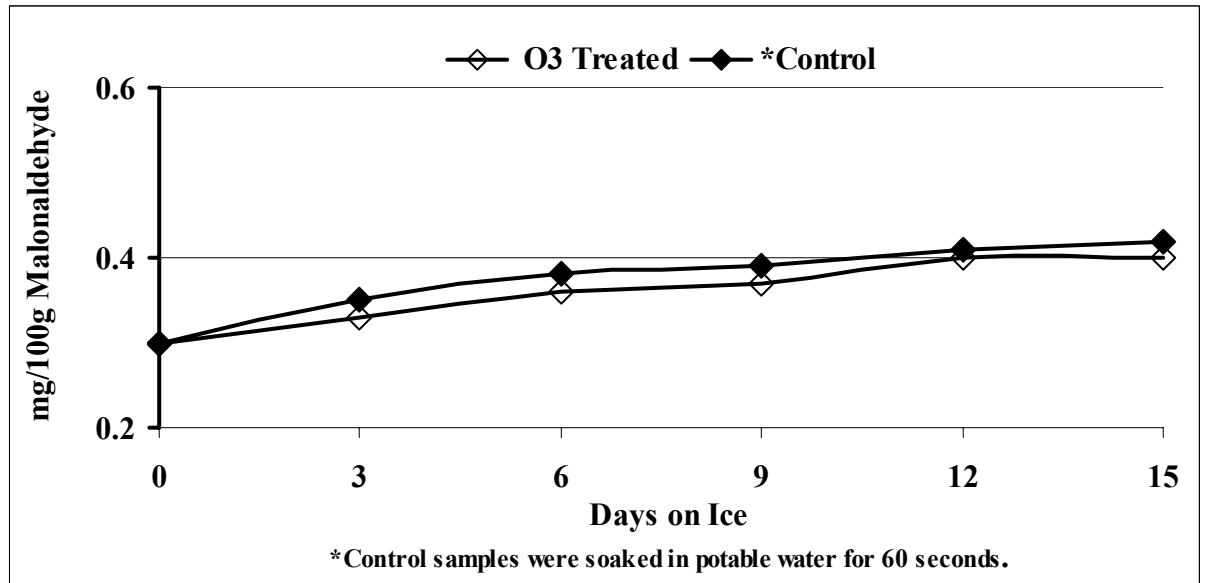


**Figure 5.2: Biogenic Amine Production in Peeled Shrimp Soaked in 3 ppm Ozonated Water for 60 seconds**

The putrescine levels in treated shrimp increased as the study advanced but at a rate slower than the untreated shrimp. This can be attributed to the differences in lag phase of the treated and untreated shrimp as indicated by the aerobic plate counts (Figure 5.1). Lack of significant differences in the treated and untreated shrimp may be attributed to the fact that different bacteria produce biogenic amines at different rates and show variable destruction rates and susceptibility to ozonated water treatment. The location of bacteria within the tissue may also play a role in destruction by ozone and hence the extent of bioamine produced.

Previous studies on the shelf life of shrimp stored at 0 C have shown that shrimp that failed sensory evaluation contained putrescine at > 4.8 ppm and cadaverine at > 1.3 ppm. For our study shrimp failing sensory evaluation fall between days 12 and 15, these shrimp samples also exhibit high bacterial counts as indicated by Figure 5.1.

No significant change in the TBARS value was observed between the untreated and the treated samples over the period of the study Figure 5.3. Due to the low levels of fat in the shrimp samples (<0.25 %) lipid oxidation as a result of the use of ozonated water for processing shrimp is not a concern.



**Figure 5.3: TBARS Values of Shrimp Soaked in 3ppm Ozonated Water for 60 seconds**

The ordinal rank data from the consumer sensory study was analyzed using PROC FREQ in SAS and computation of the Friedman statistic was done using GraphPad Prizm®. The Friedman non-parametric statistical analysis utilizing RBD can be used to analyze rank data. It is more powerful than Kramer test or Wilcoxon Rank Sum test as it eliminates unwanted sources of variability (O Mahony, 1986). The Friedman test for odor and appearance data indicates that the ozone treated shrimp and the untreated shrimp on different days of iced storage are significantly different in preference. Post tests included Dunn's Multiple Comparison Test done using the GraphPad Prizm software (Motulsky, 2003). The comparison of means of the treated and untreated shrimp showed no significant difference in appearance and odor on different days of storage on ice. The ordinal rank data from the four point scale

was extrapolated to two ordinal values of Pass (consisting of ranks 3 and 4 of the four point scale) and Fail (consisting of ranks 1 and 2 of the four point scale). The ozone treated shrimp did not exhibit odors attributed to the fail' class for the period of storage on ice Figures 5.5a. The untreated shrimp exhibited odors of the fail class on day 12 and day 15 Figure 5.5c. Untreated shrimp that exhibited odors of the fail class showed putrescine levels > 3 ppm and cadaverine levels of >2 ppm in day 12 and day 15 of storage on ice Figure 5.5. However ozone treated shrimp held on ice on day 15 showed > 3 ppm cadaverine but was classified as pass grade, indicating that consumers were more sensitive to rise in cadaverine levels as compared to rise in putrescine levels even though both bioamines are formed on at low temperature decomposition of shrimp (Rogers et al., 2004). The APC in untreated shrimp increased at a rate faster than the ozone treated shrimp Figure 5.1, however the consumers were not able to predict the growth of bacteria in shrimp on the basis of appearance Figures 5.6 b and 5.6d but they were able to do so on the basis of increase in spoilage odors Figure 5.5 c and 5.5d. The shrimp that were classified as failing sensory quality on the basis of decomposition odors showed APC of >  $10^7$  CFU/g. It can also be noted from Figures 5.5 and 5.6 that the consumers consisting of the student population of LSU were able to differentiate between the ozone treated and untreated shrimp on the basis of decomposition odors but not on the basis of appearance, indicating that appearance alone is not a suitable sensory criteria to indicate spoilage of shrimp as a result of bacterial growth.

Peeled shrimp soaked in 3ppm ozonated water for 60 sec and inoculated with *L. monocytogenes* serotype (1/2a) showed significant reduction of up to 4.6 logs CFU/g and inoculated with *L. monocytogenes* serotype (4b) showed significant reduction of up to 4.7 logs CFU/g as compared to control samples soaked in potable water for 60 seconds. (Figure 5.4). Ozonated water treatment is an effective means to eliminate the threat of *Listeria*. in shrimp processing environments. During the inoculation studies it was observed that *Listeria* adhered well to the shrimp shells more as compared to the peeled meat.

#### **5.4. Conclusion**

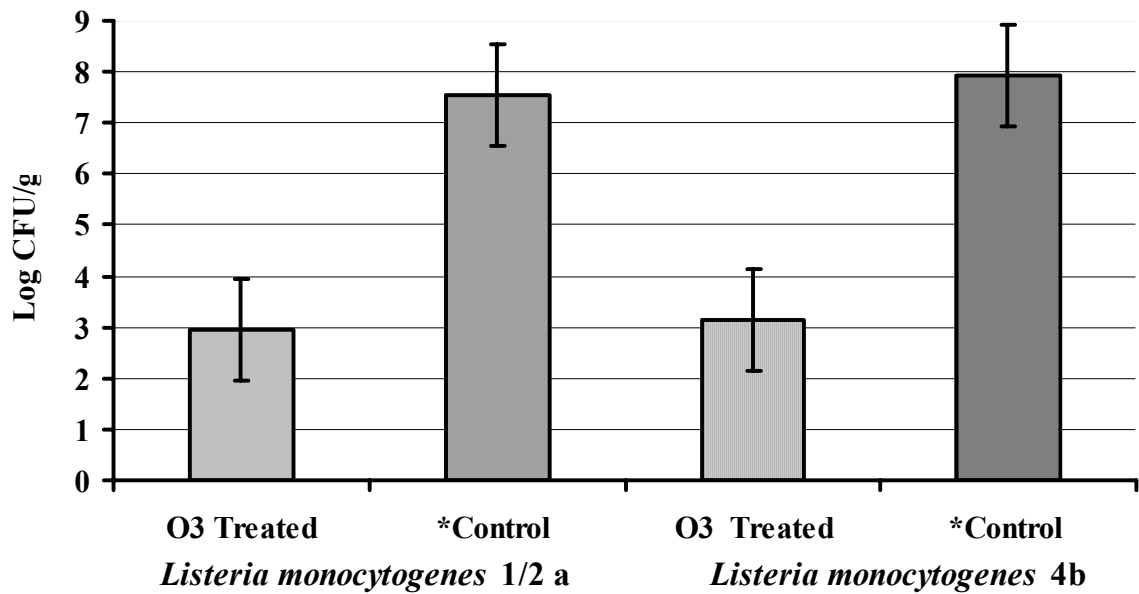
Ozonated water is an effective bactericidal agent and can improve the quality and safety of peeled shrimp meat. Further work needs to be done to evaluate the efficacy of ozonated water in improving quality and safety of mechanically peeled shrimp processed in a commercial environment. Shelf life of shrimp stored in ice based on bacterial loads was slightly extended by soaking in 3 ppm dO<sub>3</sub> for 60 seconds.

Bioamine production was not found to be reduced by the treatment, but consumer sensory scores did indicate higher acceptability of ozone treated shrimp samples at the end of the shelf life study. These conclusions are based on the results for treating well handled and controlled shrimp samples with, very low initial bacterial loads. Commercial processing operations will usually encounter shrimp with much higher bacterial levels, thus greater initial bacterial reduction can be expected due to ozonated water treatment.



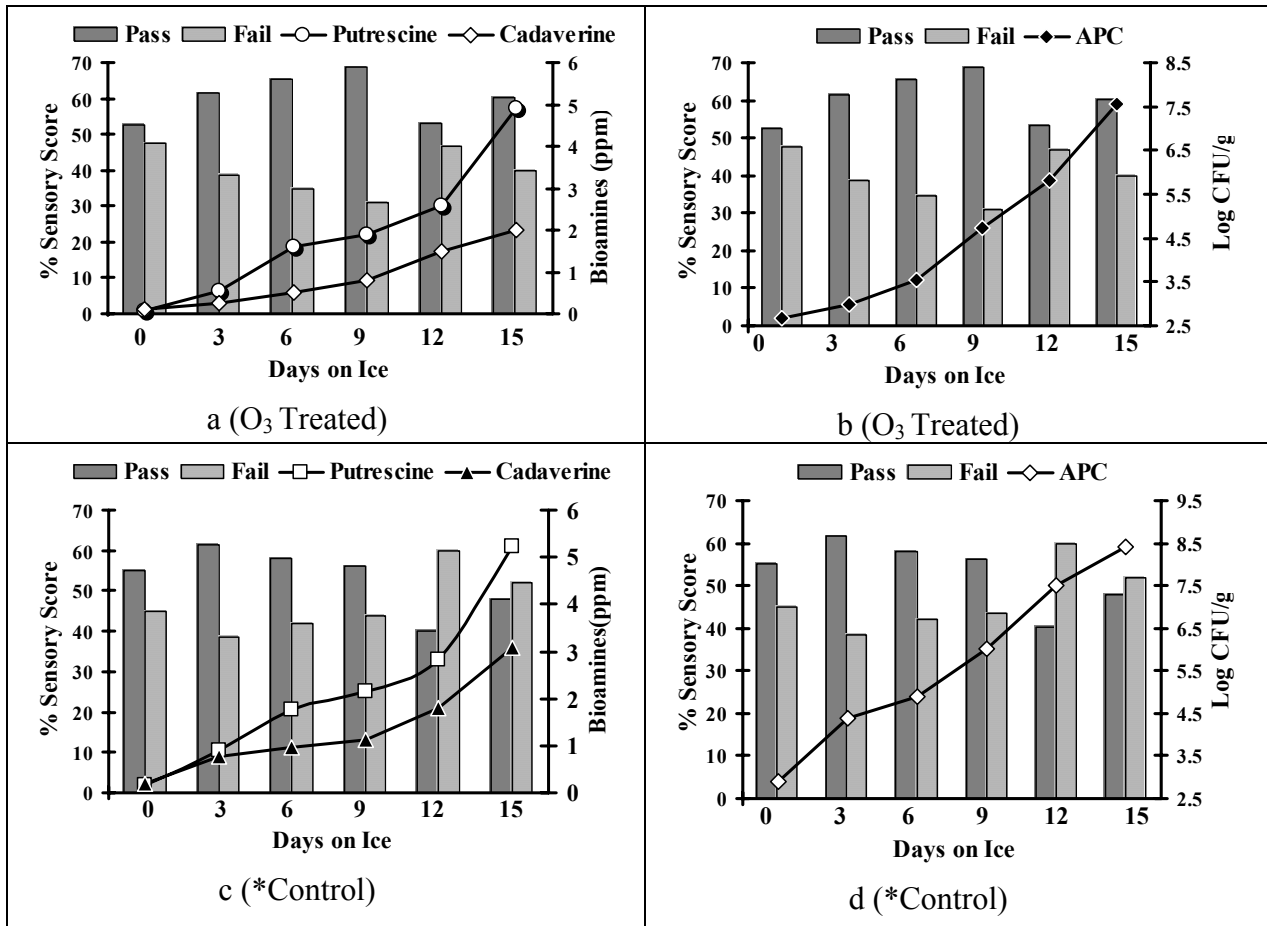
This may also result in reduction of spoilage bacteria, improved sensory scores and decreased bio amine content.

Additional work is needed to confirm these results with ozonated water systems designed for commercial shrimp peeling processes.



\*Control samples were soaked in potable water for 60 seconds

**Figure 5.4: Shrimp Innoculated with *Listeria monocytogenes* Strains Soaked in 3 ppm Ozoanted Water for 60 seconds**



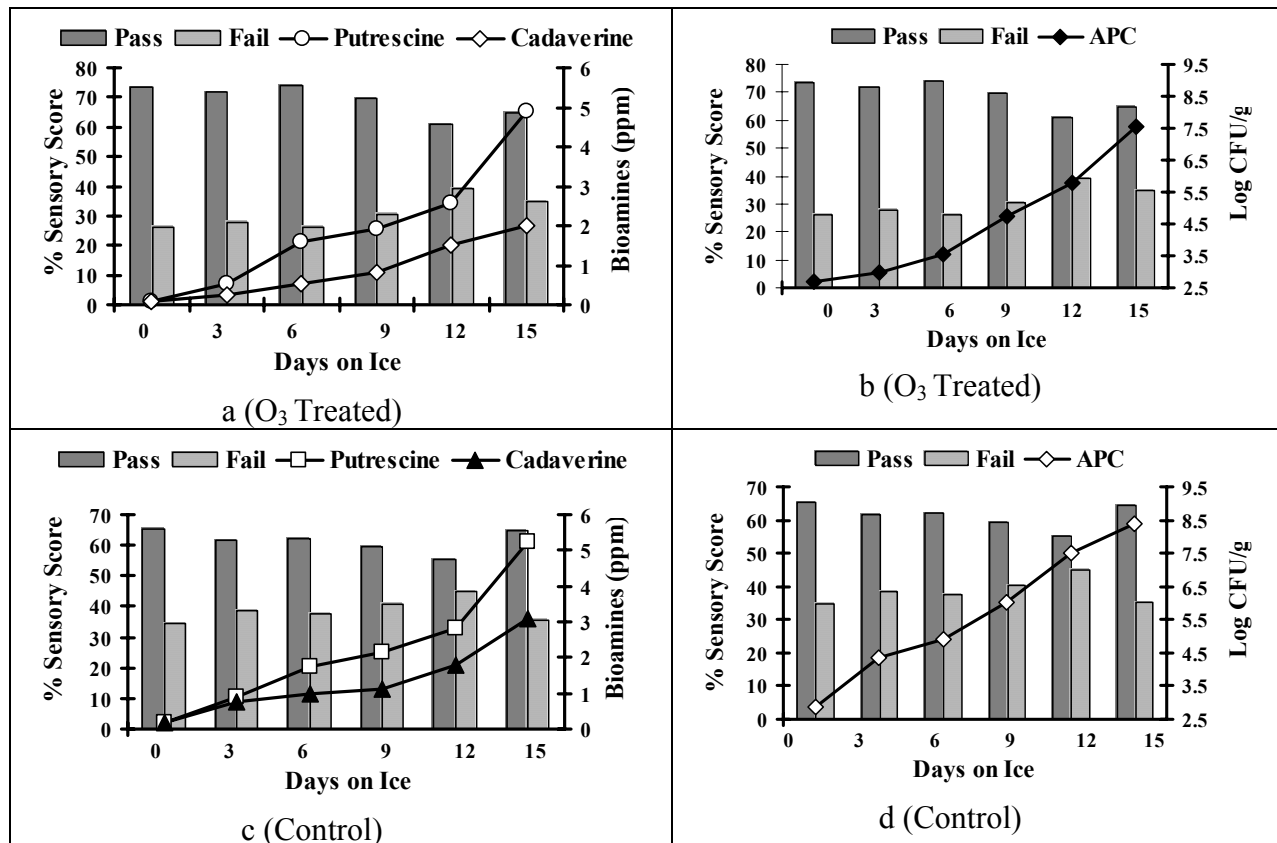
Pass = Grades (1 + 2)

Fail = Grades (3 + 4)

APC = Aerobic Plate Counts.

\*Control Samples were soaked in potable water for 60 seconds.

**Figure 5.5: Percentage Sensory Scores of Shrimp Odor Soaked in 3ppm Ozonated Water for 60 seconds**



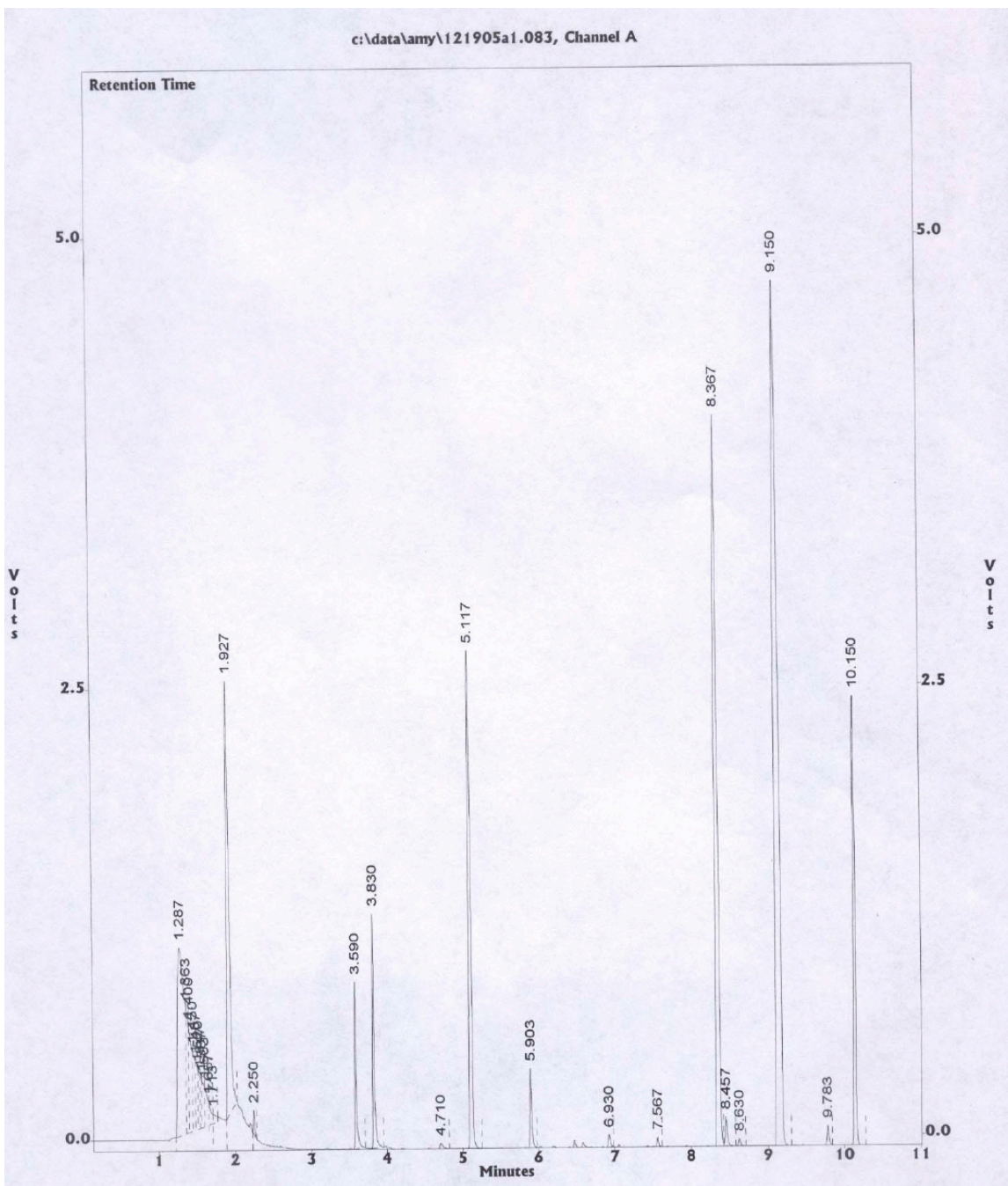
Pass = Grades (1 + 2)

Fail = Grades (3 + 4)

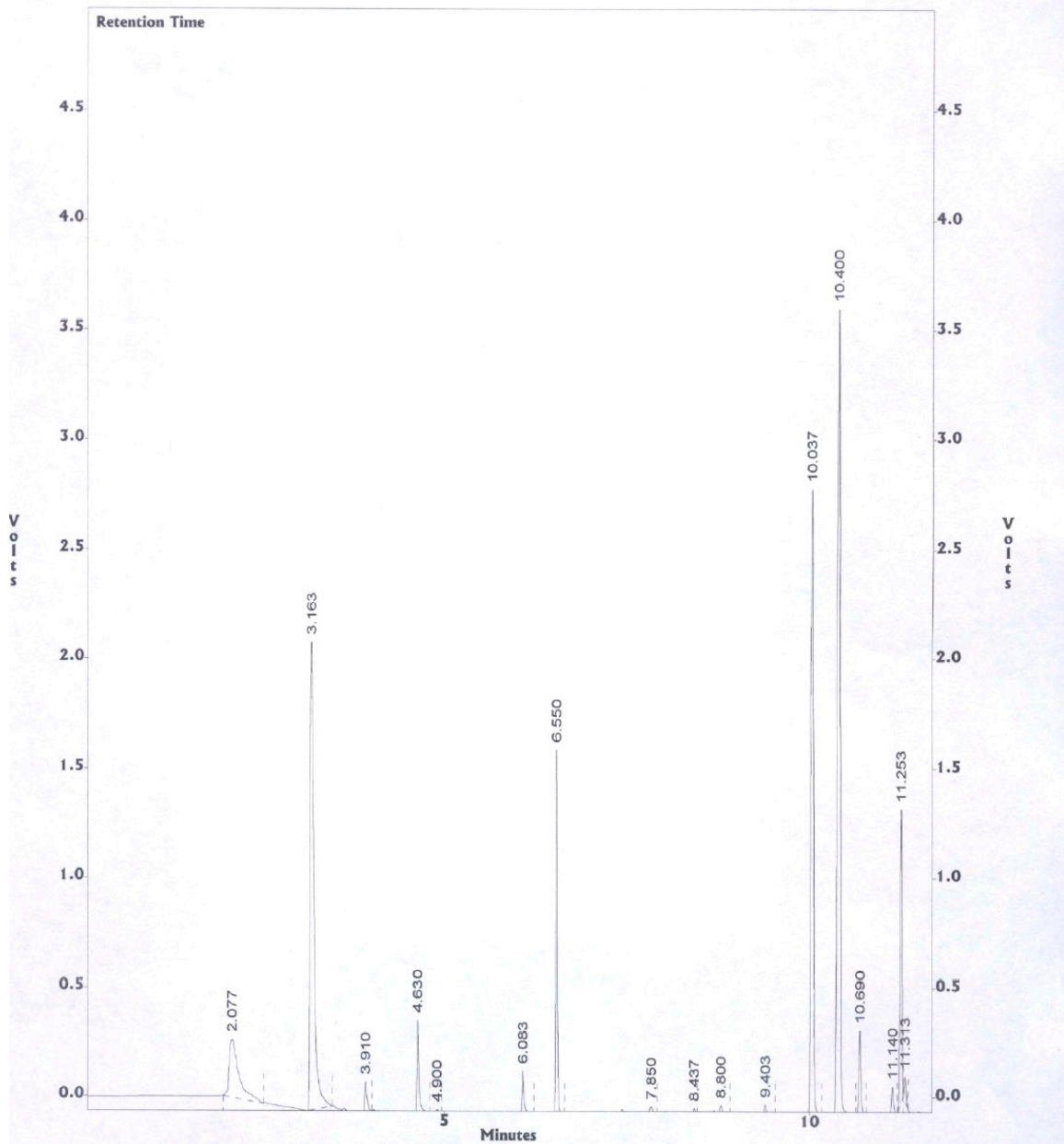
APC = Aerobic Plate Counts.

\*Control Samples were soaked in potable water for 60 seconds.

**Figure 5.6: Percentage Sensory Scores of Shrimp Appearance Soaked in 3ppm Ozonated Water for 60 seconds**



**Figure 5.7: Gas Chromatograph of PFP derivatives of untreated day 15 shrimp extract on DB-210; putrescine, 8.36 min RT; cadaverine, 9.15 RT; hexanediamine, 9.78 RT.**



**Figure 5.8: Gas Chromatograph of PFP derivatives of untreated day 15 shrimp extract on HP-225; putrescine, 10.03 min RT; cadaverine, 10.69 RT; hexanediamine, 11.14 RT.**

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## **APPENDIX : SENSORY QUESTIONNAIRES**

### **RESEARCH CONSENT FORM**

I, \_\_\_\_\_, agree to participate in the research entitled “Consumer Acceptance of Ozone Treated Shrimp,” which is being conducted by Dr. Jon Bell, Assist. Professor of the Department of Food Science at Louisiana State University, phone number (225) 578-5188.

I understand that participation is entirely voluntary and whether or not I participate will not affect how I am treated on my job. I can withdraw my consent at any time without penalty or loss of benefits to which I am otherwise entitled and have the results of the participation returned to me, removed from the experimental records, or destroyed. Two hundred consumers will participate in this research. For this particular research, about 15-minute participation will be required for each consumer.

The following points have been explained to me:

1. In any case, it is my responsibility to report prior participation to the investigators any allergies I may have.
2. The reason for the research is to gather information on consumer sensory acceptability of ozone treated shrimp samples. The benefit that I may expect from it is a satisfaction that I have contributed to solution and evaluation of problems relating to such examinations.
3. The procedures are as follows: Twelve coded samples will be placed in front of me, and I will evaluate them by normal standard methods and indicate my evaluation on score sheets. All procedures are standard methods as published by the American Society for Testing and Materials and the Sensory Evaluation Division of the Institute

of Food Technologists.

4. Participation entails minimal risk: As I do not have to ingest the samples but only evaluate them for their appearance and odor.
5. The results of this study will not be released in any individual identifiable form without my prior consent unless required by law.
6. The investigator will answer any further questions about the research, either now or during the course of the project.

The study has been discussed with me, and all of my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators listed above. In addition, I understand the research at Louisiana State University AgCenter that involves human participation is carried out under the oversight of the Institutional Review Board. Questions or problems regarding these activities should be addressed to Dr. David Morrison, Associate Vice Chancellor of LSU AgCenter at 578-8236. I agree with the terms above.

\_\_\_\_\_  
Signature of Investigator

\_\_\_\_\_  
Signature of Participant

Date: \_\_\_\_\_

Witness: \_\_\_\_\_

**Sensory Quality Indicators – Raw Shrimp**

	<b>Grade</b>	<b>Appearance and Texture</b>	<b>Odor</b>
<b>4</b>	High Pass	Translucent, Shiny, Firm, Resilient Moist, Shell Translucent	Fresh Ocean Air, Clean Seaweed, Pond water
<b>3</b>	Mid Pass to Borderline Pass	Sl. Opaque, Sl. Shiny, Sl. Firm, Sl. Resilient, Sl. Opaque shell Slight Mod. Opaque, Sl.. Dull, Surface Dry, Moderately Soft, Sl. Varied Color, Faded, Sl. Yellow Pink or Gray, Shell Sl. Pitted	Neutral Mod. Fishy, Cardboard y, Oxidized, Sl. Musty Sl. Sulfur

**Sensory Quality Indicators – Raw Shrimp**

2	Borderline Fail to Mid Fail	Opaque, Cooked Appearance, Dull, Sticky, Grainy, Soft, Sl. Mushy, Yellowish, Brownish, Sl. Gray, Pitted Shell Mod- Strong Opaque, Mod- Strong Yellow, Mod- Strong Gray, Mod. Mushy, Grainy, Sl. Pasty, Sl. Slimy	Sl. Sour, Sl. Cheesy, Sl. Rancid, Sl. Yeasty, Mod. Strong fishy, Sl. Indole, Sl. Taint, Sl. Sickly Sweet Sl. Ammonia, Sl. Musty/Moldy Mod. Sour, Sl. Fecal, Mod Rancid, Painty, Mod. Cheesy, Mod. Pungent, Mod Ammonia, Mod. Taint, Mod. Sickly Sweet, Mod. Musty/Moldy
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**Sensory Quality Indicators – Raw Shrimp**

<b>1</b>	Strong Fail	<p>Strongly Opaque, Cooked Appearance</p> <p>Strongly Gray, Strongly Mushy, Grainy, Pasty, Strongly Dry, Strongly Slimy.</p>	<p>Strongly Sour, Cheesy, Butyric, Mod-Strong Fecal, Strongly Putrid, Str. Ammonia, Str. Rancid, Painty, Str. Fermented, Str. Pungent, Str. Taint, Str. Sickly Sweet, Mod. Musty/Moldy</p>
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### Consumer Acceptance of Ozone Treated Shrimp

Sample Id.	Appearance	Odor
108	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
117	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
126	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
135	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
144	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
153	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
201	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
213	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
226	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
235	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
247	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1
252	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1	<input type="checkbox"/> 4 <input type="checkbox"/> 3 <input type="checkbox"/> 2 <input type="checkbox"/> 1

## VITA

Amrish Suresh Chawla, was born in the historical city of Meerut in the state of Uttar Pradesh, India, on July 6<sup>th</sup> 1980. He then moved to Mumbai, the business capital of India, for his primary education and later to the cultural city of Baroda for completing his secondary education.

He joined the B.Tech Dairy Technology program at the S.M.C. College of Dairy Science,, situated in the milk capital of India, in the city of Anand in 1998. This institute is recognized as a center of excellence by the apex Indian governmental body – Indian Council of Agricultural Research (ICAR). He graduated with a first class in 2002.

After graduation he joined Vasudhara Dairy, Billlimora, India as a Quality Control and Production Supervisor. Here, he worked on establishing a food microbiology laboratory and upgrading the key systems of food sanitation and safety in the plant. He was also part of the product development, ISO and HACCP certification teams.

In January 2004, Amrish started working on his master's degree at Louisiana State University at the Department of Food Science, under the guidance of Dr. Jon Bell. He is a candidate for that degree and will start working as a Research Associate with LSU upon graduation.