

EVALUATION OF CHLORINE DIOXIDE GAS AS A SANITIZER FOR FRESH FRUITS  
AND VEGETABLES

by

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(Under the Direction of Larry R. Beuchat)

ABSTRACT

Outbreaks of infections associated with consumption of fruits and vegetables and deterioration of produce quality caused by microbial growth have raised interest in using gas sanitizers. Lethality of gaseous chlorine dioxide ( $\text{ClO}_2$ ) was evaluated for its effectiveness in killing *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, yeasts, and molds inoculated or naturally present on blueberries, strawberries, raspberries, fresh-cut cabbage, carrots, and lettuce, and fresh uncut apples, tomatoes, onions, and peaches. Results show that pathogens on produce were reduced by treatment with up to 8.1 mg/L  $\text{ClO}_2$ . Treatment was least effective in sanitizing raspberries, fresh-cut lettuce, and onions. Yeasts and molds, in comparison to bacterial pathogens, were less susceptible to gaseous  $\text{ClO}_2$ . Evaluation to determine the effects of gaseous  $\text{ClO}_2$  treatment on sensory quality indicated that treated and untreated berries stored at 8°C for up to 10 days were not significantly different. Chlorine dioxide gas shows promise as a sanitizer on fresh produce.

INDEX WORDS: Chlorine dioxide, fruits, vegetables, *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, yeasts, molds, blueberries, strawberries, raspberries, fresh-cut, cabbage, carrot, lettuce, apple, tomato, onion, peach, sensory quality

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## DEDICATION

To my Lord God Almighty, who has given me life and shown me unconditional love. Thank you for blessing me with the opportunity to come and learn so that I may be a small instrument in your glorious work here on earth. May you continue to guide me and increase my faith in you, reminding me of all the ways you have provided even when I wasn't sure of my "next steps", and even now as I continue on with the journey to the future.

"By faith, Abraham, when called to go to a place he would later receive as his inheritance, obeyed and went, even though he did not know where he was going." "All these people were living by faith when they died. They did not receive the things promised; they only saw them and welcomed them from a distance." "God had planned something better for us so that only together with us would they be made perfect."

Hebrews 11:8, 13, 40

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW



## **INTRODUCTION**

Vegetables and to a lesser extent fruits have been associated with outbreaks of gastrointestinal infections in increasing frequency in the United States. This may be due in part to several factors, including changes in handling and production practices, increased consumption of fresh and lightly processed fruits and vegetables, and increased importation of produce from different regions or countries where standards for growing and handling produce vary and may be compromised (Beuchat, 1996). There are many potential sources of contamination of produce, beginning at the pre-harvest phase and ending in the consumer's kitchen. Among these sources are soil, feces, irrigation water, green or inadequately composed manure, air (dust), wild and domestic animals, human handling, harvesting equipment, transport containers, wash and rinse water, processing equipment (used for sorting, packing, or cutting), ice, transport vehicles, improper storage (temperature abuse, physical environment), improper packaging (including new packaging technologies such as modified or controlled atmosphere, or vacuum packaging), cross-contamination with other foods in storage or display areas, and improper handling after wholesale or retail purchase (Beuchat, 1996). It is important that different segments of the produce delivery system identify potential points of contamination and work on controlling microbial safety risks through the development and implementation of good agricultural practices (GAP), good manufacturing practices (GMP), and hazard analysis critical control point (HACCP) programs that best suit their particular segment of the produce industry.

## **FRESH PRODUCE ASSOCIATED WITH OUTBREAKS**

The presence of numerous genera of spoilage bacteria, yeasts, molds, and pathogenic microorganisms, including viruses, protozoan parasites, and bacteria on different types of fresh

produce has been recognized for many years. Some examples of fresh produce and unpasteurized produce products that have been associated with outbreaks of infections are strawberries (hepatitis A, *Campylobacter jejuni*, and *Cyclospora*); raspberries (*Cyclospora cayetanensis*, hepatitis A); cabbage/coleslaw (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Vibrio cholerae*, *Salmonella*); carrot (hepatitis A, *E. coli* 06:NM [ETEC], *Staphylococcus*); lettuce (*Salmonella* Enteritidis, *S. Heidelberg*, *S. Montevideo*, *S. Thompson*, *S. Braenderup*, *C. jejuni*, *E. coli* O157:H7, *Shigella sonnei*, *Staphylococcus*, Norwalk virus); unpasteurized or pasteurized apple cider (*E. coli* O157:H7, *Cryptosporidium parvum*), tomatoes (*Salmonella javiana*, *S. Montevideo*, *L. monocytogenes*, Norwalk virus), onions (*Shigella flexneri*, *Salmonella Agona*), and salad vegetables (hepatitis A, *S. flexneri*, *L. monocytogenes*, *Yersinia enterocolitica*, *E. coli* O157:H7, *Salmonella* Typhimurium, *Giardia lamblia*, *Cyclospora*, Norwalk virus) (Beuchat, 1996; Beuchat, 1998; Olsen *et al.*, 2000).

During 1993 – 1997, a total of 2,751 outbreaks of foodborne disease were recorded by the Centers for Disease Control and Prevention (CDC) (Olsen *et al.*, 2000). Fresh fruits and vegetables were associated with 12 outbreaks (2.5%) and 4,213 cases (24.1%) of infections in 1993; 17 outbreaks (2.6%) and 1,311 cases (8.1%) of infections in 1994; 9 outbreaks (1.4%) and 4,307 cases (24.2%) of infections in 1995; 13 outbreaks (2.7%), 1,807 cases (8.0%) of infections, and 1 death (25%) in 1996; and 15 outbreaks (3.0%), 719 cases (6.0%) of infections, and 1 death (50%) in 1997 (Olsen *et al.*, 2000). In general, the percentage of outbreaks associated with fresh fruits and vegetables was constant during these four years; however, the percentage of deaths attributed to fresh produce increased. This could be a result of a lack of accounting or reporting deaths caused by consumption of contaminated fresh fruits and vegetables in previous years or to an increase in virulence of pathogens that are present on produce or survive treatment with

sanitizers. Nevertheless, occurrence of foodborne illnesses is a problem that needs to be addressed; therefore, new and alternative measures need to be continuously developed and applied in order to further improve the security and safety of food for consumers.

## **PATHOGENIC MICROORGANISMS OF CONCERN**

Contamination of fresh produce with pathogens can occur in various ways. Pathogens such as *L. monocytogenes*, *Clostridium botulinum*, and *Bacillus cereus* are naturally present and ubiquitous in some soils, so it is not uncommon to find them on fresh produce. *Salmonella*, *E. coli* O157:H7, *C. jejuni*, *V. cholerae*, parasites, and viruses are more likely to be found in raw or improperly composted manure, irrigation water containing untreated sewage, or contaminated wash water which may come in contact with produce at different points in the preharvest and postharvest, e.g., during growing, harvesting, processing, and preparing produce for consumption (Beuchat and Ryu, 1997). Despite the broad spectrum of pathogens associated with fresh produce, it has been reported throughout the years that bacterial pathogens cause the largest percentage of outbreaks and cases of infections. In the survey conducted during 1993 – 1997, 75% of the total outbreaks and 86% of the cases of infection were bacterial pathogens (Olsen *et al.*, 2000). Outbreaks with unknown causes can be attributed in part to a lack of sensitive methodology to isolate pathogens, particularly many viruses, and parasites. The focus here will be on three pathogens, viz. *Salmonella enterica*, *L. monocytogenes* and *E. coli* O157:H7, most commonly involved.

### *Salmonella enterica*

Salmonellae are commonly found in feces of animals, birds, and some reptiles and amphibians; however, they are occasionally isolated from horticultural crops, soil, and water that has been used to wash raw fruits and vegetables (Wood *et al.*, 1991). Ercolani (1976) detected *Salmonella* in 68% of 120 samples of lettuce and 72% of 89 samples of fennel obtained from retail outlets in Italy. *Salmonella* was cultured from 4 of 50 vegetables examined in the United States (Rude *et al.*, 1984). In Spain, *Salmonella* was cultured from 7.5% of 345 samples of vegetables obtained from farms, a wholesale market, supermarkets, and small shops (Garcia-Villanova-Ruiz *et al.*, 1987). The organism was detected on artichoke, beet leaves, celery, cardoon, cabbage, cauliflower, lettuce, parsley, and spinach. Guo *et al.* (2002) reported *Salmonella* survives at high numbers in moist soil for at least 45 days. Watkins and Sleath (1981) demonstrated that *Salmonella* in sewage sludge applied to agricultural soils might persist for 5 weeks. On tomatoes, *Salmonella* Montevideo persisted for 49 days after tomato flowers were inoculated and was the most dominant serotype among five in the inoculum (Guo *et al.*, 2004). Tamminga *et al.* (1978) isolated salmonellae from 23 of 103 samples of vegetables grown in The Netherlands or imported to that country. Eggplant, cauliflower, peppers, endive, and lettuce were found to harbor the pathogen. Consumption of raw tomatoes was epidemiologically linked to 176 cases of *Salmonella* Javiana infection in Illinois, Michigan, Minnesota, and Wisconsin in 1990 (Wood *et al.*, 1991). The pathogen has been stated to be the leading cause of foodborne illness in the United States (Olsen *et al.*, 2000).

### ***Listeria monocytogenes***

*L. monocytogenes* is the most prevalent disease-causing microorganism resident in soil (Welshimer, 1960). Twenty-seven strains were isolated from soil and vegetation taken from 19 sites in The Netherlands (Welshimer and Donker-Voet, 1970). Plant material from which the organism was isolated included dead and decayed corn, soybean plants, and wild grasses. It was suggested that *L. monocytogenes* is a saprophyte that lives in a plant-soil environment and could therefore be contracted by humans and animals through many possible routes from many sources (Beuchat and Ryu, 1997). *Listeria* and also other potentially pathogenic bacteria have been reported to be isolated from sewage (Watkins and Sleath, 1981). *L. monocytogenes* was detected in 52 samples of sewage sludge, river water, and industrial effluents. In many samples, populations of *L. monocytogenes* were higher than those of salmonellae and the organism could survive longer. It has also been reported that populations of *L. monocytogenes* in soil remain essentially unchanged for 7 weeks after application of sludge containing *L. monocytogenes* and salmonellae. This provides for opportunities for fresh produce to be contaminated with *L. monocytogenes*, and therefore is one of pathogens of concern.

### ***Escherichia coli* O157:H7**

Enterohemorrhagic *E. coli* O157:H7 is recognized as a known cause of foodborne illness with its primary reservoir being found in cattle and other ruminant animals (Beuchat, 1996; Olsen *et al.*, 2000). However, outbreaks have been also been linked to fruits and vegetables, including cantaloupe, lettuce, alfalfa sprouts, radish sprouts, cantaloupe, and unpasteurized apple cider (Burnett and Beuchat, 2001; Kenney *et al.*, 2001). There are growing concerns pertaining to the ability of *E. coli* O157:H7 ability to grow on fresh-cut produce such as shredded lettuce,

sliced cucumber, and other produce packaged under modified atmosphere packaging at storage, since there has been an increased consumption of minimally processed produce products (Abdul-Raouf *et al.*, 1993). *E. coli* O157:H7 has been reported to grow rapidly in cantaloupe and watermelon cubes stored at 25°C and also in apple cider at 8°C, confirming that exposure of the pathogen to mildly acid conditions and less than optimal nutrient conditions is not sufficient to control the pathogen (del Rosario, and Beuchat, 1995; Zhao *et al.*, 1993). There are concerns about how to prevent contamination or remove *E. coli* O157:H7 and other pathogens from raw fruits and vegetables. One method currently used in the food industry is the applications of cleaners, disinfectants, and sanitizers.

## **CLEANERS, DISINFECTANTS, AND SANITIZERS**

There are many types of chemical agents available for cleaning, reducing, and/or killing microorganisms on hard non-porous inert surfaces and on uneven food surfaces. Cleaners are composed of various chemicals, generally formulated to enhance the cleaning ability of water (McLaughlin and Zisman 2002). Disinfectants are products, which kill disease-causing microorganisms, usually vegetative (non-spore forming) bacteria, yeasts, molds, and viruses, and are commonly applied to inert surfaces. The effectiveness of a disinfectant may depend on the exposure time, temperature, concentration, and composition of the targeted surface to be disinfected (Lawrence, 1968). Sanitizers, on the other hand, are agents (whether thermal, chemical, or even radiation) that reduce and control microbial contaminants on equipment that comes in contact with produce or on actual surfaces of produce at safe levels as determined by public health requirements. Although a sanitizer significantly reduces populations of microorganisms, it does not necessarily eliminate all forms. Definitions of food-contact

sanitizers typically stipulate that a 1-min contact time should reduce the microbial population by 5 logs and treatment will not leave a residue which may be harmful to subsequent users of the article or product, as well as the elimination of any contamination which might be esthetically objectionable (McCulloch, 1945; Lawrence, 1968).

### ***TYPES OF CHEMICAL SANITIZERS***

The recommended means for consumers to reduce contamination on raw fruits and vegetables is washing with tap water (0.025 – 2.0 µg/ml chlorine) (Troller, 1993). Although washing produce in tap water may have some effectiveness in removing soil and other debris, it should not be relied upon to completely remove microorganisms (Beuchat *et al.*, 1998).

Different sanitization methods and several types of sanitizers have been used to reduce populations of pathogens on produce or prevent growth of pathogenic and spoilage microorganisms that may cause foodborne illnesses and/or loss of food quality. Liquid chlorine, chlorine dioxide, electrolyzed water, iodine-containing compounds, peroxyacetic acids, hydrogen peroxide, and ozone are some of the chemical sanitizers currently approved for use in the food industry.

#### ***Chlorinated Water (HOCl)***

The most commonly used commercial sanitizer for fresh produce is chlorinated water (50 – 200 µg/ml) (Rodgers *et al.*, 2004; Beuchat *et al.*, 1998). This is because the antimicrobial spectrum of chlorine-based sanitizer is broader than that of many other approved sanitizers. Bacteria, viruses, molds, yeasts, spores, algae, and protozoa are inhibited to at least in some degree, with bacterial spores more resistant than vegetative cells, gram-positive bacteria tending

to be more resistant than gram-negative species, with fungal spores appearing to be slightly more resistant than vegetative bacterial cells to hypochlorites, and with many types of viruses being significantly more resistant than bacteria (Troller, 1993). Antimicrobial activity depends on the amount of free available chlorine (as hypochlorous acid, HOCl) in water that comes in contact with microbial cells (Beuchat and Ryu, 1997). Several modes of action of chlorine have been proposed (1) disruption of protein synthesis; (2) oxidative decarboxylation of amino acids to nitrites and aldehydes; (3) reactions with nucleic acids, purines, and pyrimidines; (4) unbalanced metabolism after the destruction of key enzymes; (5) induction of deoxyribonucleic acid (DNA) lesions with an accompanying loss of DNA-transforming ability; (6) inhibition of oxygen uptake and oxidative phosphorylation, coupled with leakage of some macromolecules; (7) formation of toxic N-chloro derivatives of cytosine; and (8) creation of chromosomal aberrations (Marriott, 1999).

The effectiveness of liquid chlorine in removing pathogens on fresh produce has been assessed. Fresh fruits and vegetables exposed to 100 – 2,000 µg/ml chlorine can result in 2 to 4 log reductions in microbial populations, with the washing effect from water responsible for an initial 1 log reduction (Rodgers *et al.*, 2004). Total counts of aerobic microorganisms and fecal coliforms were markedly reduced on leafy salad greens upon treatment with increased concentrations of chlorine up to 50 µg/ml (Mazollier, 1988). A further increase in concentration up to 200 µg/ml did not have an additional substantial effect. Washing cantaloupes contaminated with *Salmonella* Stanley with water, 1000 µg/ml chlorine, and 5% hydrogen peroxide revealed that the highest log reductions (3.4 log<sub>10</sub> CFU/cm<sup>2</sup>) were achieved with chlorine (Ukuku and Sapers, 2001). A standard procedure for washing lettuce leaves in tap water was reported to remove 92.2% of the microflora (ca. 10<sup>7</sup> CFU/g); however, inclusion of 10



µg/ml available free chlorine (pH ca. 9) reduced the count by 97.8% (Adams, 1989). Other examples of the biocidal effect of free available chlorine reported are: 0.5 µg/ml at 25°C (pH 7.0) resulted in 100% inactivation of *Clostridium botulinum* toxin type A within a 30-sec exposure time (Brazis *et al.*, 1959); 0.5 µg/ml at 5°C (pH 6.0) resulted in 99.99% removal of Simian rotavirus in 15 sec (Berman *et al.*, 1984); 100 µg/ml at 20°C (pH 9.0) resulted in 99.99% inactivation of *Yersinia enterocolytica* in 5 min (Orth *et al.*, 1989)

Although chlorinated water is widely used to wash and sanitize fresh fruits and vegetables, there are some questions regarding its effectiveness in some types of fresh produce as well as its potentially negative impact on the human health. The maximum log<sub>10</sub> reductions of *L. monocytogenes* on shredded lettuce and cabbage treated with 200 µg/ml chlorine for 10 min were 1.3 – 1.7 log<sub>10</sub> CFU/g and 0.9 – 1.2 log<sub>10</sub> CFU/g, respectively (Badaway *et al.*, 1985). Numbers decrease only marginally with increased exposure time from 1 to 10 min, which agrees with observations made by Brackett (1987) that the removal of *L. monocytogenes* from Brussels sprouts by chlorine treatment occurs primarily during the first 30 sec of exposure. Nguyen-the and Carlin (1994) concluded that the elimination of *L. monocytogenes* from the surface of vegetables by chlorine is unpredictable and limited.

Chlorine also has been repeatedly reported to react with trace amounts of organic material on fresh produce to form various carcinogenic organochlorine compounds (Richardson *et al.*, 1998). Chlorine compounds are also rapidly inactivated by organic material, which is inherent to raw produce. Some of the organic materials that inactivate chlorine include tyrosine, tryptophan, cystine, egg albumin, peptone, body fluids, tissues, microbes, and vegetable matter. It appears, however, that sugars and starches do not affect the germicidal activity of chlorine (Dychadala, 1968). The effectiveness of chlorine decreases with increased pH. For example, it

has been reported that 25 µg/ml available chlorine killed 99% of *Bacillus metiens* spores within 2.5 min at pH 6, 5 min at pH 8, and 465 min at pH 12.86 (Rudolph *et al.*, 1941). Chlorine is very corrosive to stainless steel and other metals, necessitating limiting contact time with food handling equipment, and deteriorates during storage when exposed to light or to a temperature of above 60°C. Its efficacy is dependent on the morphology of the surface of produce, because microbial cells are inaccessible to chlorine if located in creases, crevices, pockets, and natural openings in the skin (Beuchat, 2000). In addition, pathogens of concern to water quality, such as *Giardia lamblia* and especially *Cryptosporidium parvum*, are known to be resistant to chlorine at concentrations typically applied for water treatment (Carpenter *et al.*, 1999; Gyurek *et al.*, 1998; Peeters *et al.*, 1989)

### ***Chlorine Dioxide***

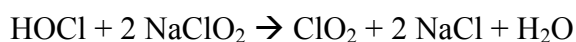
Chlorine dioxide (ClO<sub>2</sub>) is occasionally employed for purification of water. It has a unique ability to break down phenolic compounds and remove phenolic tastes and odors from water (Dychdala *et al.*, 1968). Since it is less reactive than chlorine toward organic compounds and produces fewer toxic, mutagenic by-products, it is a good alternative sanitizer to chlorinated water (Richardson *et al.*, 1998). Aqueous ClO<sub>2</sub> can be employed, according to the FDA (1998), to sanitize equipment (maximum of 200 µg/ml), whole fresh fruits, whole vegetables, shelled beans, and peas with cuticles intact (not exceeding 5 µg/ml), as well as peeled potatoes with a maximum wash concentration of 1 µg/ml. Although a concentration of 5 µg/ml of ClO<sub>2</sub> would appear to be too low to be effective, it is actually equivalent to 20 µg/ml chlorine. Chlorine dioxide is able to control bacteria in chilled water used for poultry processing, can extend the shelf life of broiler carcasses, and reduces the incidence of *Salmonella* on poultry (Lillard, 1979;

Thiessen *et al.*, 1984; and Villarreal *et al.*, 1990). Research done on fresh fruits and vegetables has also shown ClO<sub>2</sub> to be an effective sanitizer. This is demonstrated by a > 99% mortality to conidia and sporangiospores of several fungal pathogens on apples and other fruits resulting from a 1-min treatment of 3 or 5 µg/ml liquid ClO<sub>2</sub> (Roberts and Reymond, 1994). These observations indicate that ClO<sub>2</sub> has desirable properties as a sanitizing agent for postharvest decay management when application of postharvest fungicides are not desired or allowed (Beuchat, 2000). Another favorable feature is the lack of reaction of ClO<sub>2</sub> with ammonia to form chloramines, and more importantly, its oxidizing capability is 2.5 times greater compared to chlorine. The bactericidal efficacy of ClO<sub>2</sub> was found not to be affected at pH 6 to 10, while chlorine is not as effective in its killing capacity at alkaline pH's (Ridenour and Ingols, 1947). Chlorine dioxide is less corrosive than other chlorine sanitizers because of the low concentration necessary to be effective (Marriott, 1999), and is not inactivated by organic material as most chlorine-containing sanitizers are, which is helpful when treating produce which consists largely of organic material. Its oxidation-reduction potential, however, is close to that of chlorine (Dychdala, 1968) and it has about the same spectrum of antimicrobial activity as hypochlorite (Troller, 1993).

In most cases, because of its instability, ClO<sub>2</sub> is generated at the site where it is intended to be used (Marriott, 1999). This can be accomplished by several ways:



(3) Reacting hypochlorous acid with sodium chlorite:



The mode of action for ClO<sub>2</sub> has not been completely determined, although research by Bernade et al. (1965) revealed through the use of <sup>14</sup>C-labeled amino acids that ClO<sub>2</sub> disrupts protein synthesis in *E. coli*. Olivieri et al. (1984) reported that ClO<sub>2</sub> is highly reactive against amino acids in viral proteins, thus altering proteins to result in changes in outer membrane permeability. Berg et al. (1986) reported that the permeability of the outer membrane in *E. coli* was sensitive to ClO<sub>2</sub> and was also influenced by growth conditions, since starved cells caused the membrane-associated cations, calcium, and magnesium to be more accessible to ethylenediaminetetraacetic acid (EDTA). The role of EDTA is to chelate these ions in bacterial membranes and increase the permeability of incoming molecules (Leive, 1965). Respiratory damage induced by ClO<sub>2</sub> in *E. coli* was also shown to occur; however, this was a sublethal event in the inactivation of *E. coli*, since even at the lowest dose (0.5 µg/ml), where respiration was severely impaired, there was no measurable inactivation (Berg et al., 1986). Mechanisms for killing *Bacillus subtilis* spores have been studied, whereby it is reported that DNA damage is not the cause of spore inactivation (Young and Setlow, 2003). Rather, inactivation is by the damage of inner membrane in spores, resulting in a major permeability change in the plasma membrane of germinated spores. However, removal of the intact spore coat must first be achieved either by use of chemicals, e.g., NaOH combined with a detergent extraction procedure (Nicholson and Setlow, 1990; Driks, 1999), or by mutation of *cotE* that codes for a protein important in outer coat assembly (Driks, 1999), since this is the major factor contributing to spore resistance to both hypochlorite and ClO<sub>2</sub> (Young and Setlow, 2003).

Gaseous ClO<sub>2</sub>, similar to its aqueous counterpart, has good biocidal activity. Application of 4.0 mg/L of ClO<sub>2</sub> gas for 10 min has been reported to cause a 5.5-log reduction of *L. monocytogenes* on the skin of apples (Du et al., 2002). Except for ClO<sub>2</sub> gas, as observed by Du

*et al.* (2002) and allyl isothiocyanate vapor (Lin *et al.*, 2000), chemical treatments did not achieve a 5-log reduction of bacteria on apples (Du *et al.*, 2002). Surface-injured green peppers treated with 1.24 mg/L ClO<sub>2</sub> gas for 30 min at 22°C and 90 – 95% relative humidity (RH) resulted in a 6.45-log<sub>10</sub> CFU/5 g reduction compared to reductions of 1.5 – 1.67 log<sub>10</sub> CFU/5 g resulting from washing injured surfaces with water (Han *et al.*, 2000). Gaseous ClO<sub>2</sub> also has potential as a sanitizer for reducing yeast and mold populations in food processing plants and on fruits and vegetables. Inoculation of epoxy-coated stainless steel strips identical to those used in juice tanks with *Eurotium*, *Penicillium*, *Candida*, and *Saccharomyces cerevisiae* at populations > 4 log<sub>10</sub> CFU/2.5 x 7 cm<sup>2</sup> area, followed by ClO<sub>2</sub> gas treatment, resulted in reductions in populations to below detectable limits and controlled subsequent growth (Han *et al.*, 1999).

There is disparity among the data reported regarding ClO<sub>2</sub> inactivation of *Cryptosporidium parvum* oocysts. One researcher stated that 120 mg-min/L (*Ct* value [product of disinfectant concentration and contact time]) ClO<sub>2</sub> at 22°C was capable of inactivating 2.1 log<sub>10</sub> CFU of *Cryptosporidium* oocysts (Finch and Li, 1999), while Chauret *et al.* (2001) reported that *Ct* values of 75, 550, and 1,000 mg-min/ml ClO<sub>2</sub> were required to achieve approximately 2.0 log<sub>10</sub> CFU inactivation of oocysts from three different sources using a most-probable-number (MPN) cell culture infectivity assay. In this same study, a *Ct* value of 1,000 mg-min/L ClO<sub>2</sub> caused only a 0.5-log<sub>10</sub> CFU reduction with *in vitro* excystation, indicating that the variance in resistance of *C. parvum* oocysts to ClO<sub>2</sub> depends partly on the analytical method used as well as the source of oocysts (Chauret *et al.*, 2001). Lethality of ClO<sub>2</sub> against protozoan agents is clearly difficult to confirm, not to mention determining the mechanism of lethality of to parasites.

The disadvantages of ClO<sub>2</sub> are that it's unstable and explosive when concentrated and decomposes at temperatures greater than 80°C when exposed to light (Beuchat, 2000).

### *Electrolyzed water*

In recent years, acidic electrolyzed water (AEW) or acidic electrolyzed oxidizing (EO) water and neutral electrolyzed water (NEW) have been evaluated as sanitizers. These solutions are generated by electrolysis of a dilute NaCl solution passing through the anode of a membrane electrolyzer (Deza *et al.*, 2003). They are considered a non-thermal alternative for sanitizing produce, which does not require the handling and storing of potentially dangerous chemicals other than low concentrations of NaCl (0.05 to 0.1%) and thus have less harmful impact on the environment (Kroyer, 1995).

### *Acid Electrolyzed Water (AEW)*

AEW has been shown to have a strong bactericidal effects on most pathogens due to its low pH (2-4), high oxidation-reduction potential (ORP > 1000 mV), and ability to oxidize due to components such as hypochlorous acid (Kim *et al.*, 2000; Len *et al.*, 2000). Exposing mixtures of *E. coli*, *Salmonella* Enteritidis, and *L. monocytogenes* to AEW for 5 min reduced populations by 7 logs CFU/ml (Venkitanarayanan *et al.*, 1999). AEW has also been demonstrated to have a strong fungicidal effect. Bonde *et al.* (1999) observed that treatment of wheat seed for 20 min with AEW eliminated molds such as *Aspergillus*, *Cladosporium*, and *Penicillium* spp. Buck *et al.* (2002) reported that test fungi suspended in distilled water combined with AEW (1:9 water:AEW) for various time periods resulted in a significant reduction or prevention of the germination of 22 fungal species. There are, however, disadvantages to AEW in that it can be neutralized with nonselective reducing agents (Oomori *et al.*, 2000). The effectiveness of AEW may also be reduced in the presence of surfactants such as Triton X-100 and Tween (1 and 10%) (Buck, 2002).

### Neutral Electrolyzed Water (NEW)

NEW is generated like AEW, but part of the product formed at the anode is redirected into the cathode chamber, thus increasing the content of  $\text{ClO}^-$  ions (Deza *et al.*, 2003). The advantages of a neutral pH in NEW are that it does not contribute as aggressively as AEW in the corrosion of processing equipment or irritation of hands, and is more stable, since chlorine loss is significantly reduced at pH 6 – 9 (Rojas and Guevara, 2000; Len *et al.*, 2002). NEW (pH 6 – 8 and 20 mg/L active chlorine) has been reported to cause reductions of up to 2 – 6  $\log_{10}$  CFU/g on the total microbial counts in fresh-cut vegetables without a significant effect on tissue pH, surface color, and general appearance (Izumi, 1999). Deza *et al.* (2003) observed that washing tomatoes inoculated with non-pathogenic *E. coli*, *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* with deionized water (control) reduced the number of viable cells by approximately 2  $\log_{10}$  CFU/cm<sup>2</sup> within 30 or 60 s. Upon treatment with NEW (containing 89  $\mu\text{g/ml}$  chlorine), populations on tomatoes were reduced by an average of 4.18  $\log_{10}$  CFU/cm<sup>2</sup> within 30 s and 4.74  $\log_{10}$  CFU/cm<sup>2</sup> within 60 s. Sensory evaluation was also conducted on uninoculated tomatoes washed with NEW or with tap water and no significant differences ( $p \leq 0.05$ ) in appearance, aroma, and taste were found for treated and control tomatoes.

### ***Iodine Compounds***

The major iodine compounds used for sanitizing food processing equipment are iodophors, alcohol-iodine solutions, and aqueous iodine solutions (Marriott, 1999). Principal applications are also in hand sanitizing solutions, where iodophors effectively kill vegetative cells of bacteria without causing skin irritations that are associated with chlorine rinses and result

in fewer build-ups of resistant populations that sometimes occur with treatments containing quaternary ammonium compounds (Troller, 1993).

Iodophors are combinations of iodine with carriers such as polyvinylpyrrolidone or a surface-active agent such as alkyphenoxypolyglycerol ether. Like chlorine-containing germicides, iodophors are active against both gram-positive and gram-negative bacteria, viruses, yeasts, and molds. Some of the food-related bacteria that are inactivated by iodophors (6 – 13  $\mu\text{g/ml}$ , pH 6.7 – 7) at 20 – 25°C are *Vibrio parahaemolyticus*, *E. coli*, *Streptococcus lactis*, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, and *Salmonella* Derby (Odling, 1981). Not only do iodophors play a role in removing microorganisms, they also reduce bacterial populations in biofilms. A study by Joseph *et al.* (2001) revealed that *Salmonella* Weltevreden in biofilm on plastic surface was reduced by 3  $\log_{10}$  CFU/cm<sup>2</sup> when treated with 20  $\mu\text{g/ml}$  chlorine in 25 min, while treatment with 20  $\mu\text{g/ml}$  of I<sub>2</sub> for 25 min resulted in 4- $\log_{10}$  CFU/cm<sup>2</sup> reductions. *Salmonella* in biofilm grown on cement had 2- $\log_{10}$  CFU/cm<sup>2</sup> reductions when treated with 50  $\mu\text{g/ml}$  Cl<sub>2</sub> for 25 min and 6- $\log_{10}$  CFU/cm<sup>2</sup> reductions when treated at a concentration of 50  $\mu\text{g/ml}$  I<sub>2</sub> for 25 min; and *Salmonella* biofilm grown on stainless steel was reduced by 2  $\log_{10}$  CFU/cm<sup>2</sup> when treated with 50  $\mu\text{g/ml}$  of Cl<sub>2</sub> for 25 min and 5.5  $\log_{10}$  CFU/cm<sup>2</sup> with 50  $\mu\text{g/ml}$  of I<sub>2</sub> for 25 min. All planktonic cells (approximately 6  $\log_{10}$  CFU/ml) of *S. Weltevreden* and *Salmonella* FCM 40 were killed after exposure to 10  $\mu\text{g/ml}$  Cl<sub>2</sub> for 10 min and 10  $\mu\text{g/ml}$  I<sub>2</sub> for 5 min, indicating that treatment with I<sub>2</sub> is more effective than with Cl<sub>2</sub> at the same concentration in killing both Salmonellae (Joseph *et al.*, 2001). In a study to determine the minimum inhibitory concentrations (MIC) of various sanitizer compounds against *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Gluconobacter oxydans*, and *Saccharomyces cerevisiae* associated with spoilage of orange juice, it was found that compounds effective at the lowest



concentrations were iodophors, ClO<sub>2</sub>, and quaternary ammonium compounds. Concentrations of 0.0012 – 0.0098% (w/v; aqueous) iodophors were needed to achieve an inactivation rate of 6 log<sub>10</sub> CFU/ml/min for the four microorganisms, while treatment with hypochlorite, citric acid, and peracetic acid required concentrations of 0.027 – 0.034%, 6.3 to > 10%, and 0.011 – 0.026%, respectively, to inactivate 6 log<sub>10</sub> CFU/ml/min (Winniczuk and Parish, 1997). This shows that while these spoilage microorganisms (including yeasts) are not necessarily pathogens, their survival or susceptibility is still indicative that iodophors are effective in removing or killing undesirable organisms that can be found on citrus surfaces.

The lethality of iodophors can be attributed to the antibacterial action of diatomic iodine, which disrupts bonds that hold cell proteins together and inhibits protein synthesis (Anon, 1996). Other advantages of iodophors are that they are somewhat more stable than chlorine compounds in the presence of organic matter and they tend to retain their activity at relatively low pH (2.5 – 3.5), which makes them especially effective in dairy plants. However, at pH > 8.0, some decrease in sanitizing effectiveness can be expected (Troller, 1993). Iodophors are more costly than chlorine-based sanitizers; however, they normally are used at much lower concentrations (12.5 – 25 µg/ml). Some disadvantages of iodophors, other than cost, are corrosiveness (especially on galvanized iron), release of free iodine at temperatures > 43.3°C, a high chemical oxygen demand in plant wastewaters, reduced activity at temperatures >120°C (due to iodine vaporization) or at and near 5°C, and color reaction, especially with starch-containing products due to the formation of purple complexes with starches (Troller, 1993). Iodophors are less effective than chlorine in killing bacterial spores and bacteriophage, and have no antiviral activity (Payan *et al.*, 2001). Craven *et al.* (1981) reported that *Burkholderia cepacia* was able to survive in povidone-iodine solutions containing 1% iodine and persisted in these solutions as

long as 68 weeks. A study by Pyle *et al.* (1994) showed that *Pseudomonas aeruginosa* surviving treatment with iodine exhibited decreased susceptibility to iodine and chlorine. This phenotype persisted for at least four transfers in the absence of selective pressure, and susceptibility was affected by carbon source and cyclic AMP (Chapman, 2003). Although, these studies do not involve food plants, they do illustrate that some sort of resistance can develop in cells exposed to povidone-iodine solutions. Application of iodophors at specific concentrations and to specific products must therefore be evaluated (Troller, 1993).

### ***Peroxyacetic Acid***

Peroxyacetic acid or peracetic acid (PAA),  $C_2H_4O_3$ , formed by reacting hydrogen peroxide and acetic acid, was approved by FDA (1986) as a food-grade sanitizer at concentrations not exceeding 100  $\mu\text{g/ml}$ , probably partly due to its explosive character in concentrated solutions. Since PAA is an organic acid, it is often used in conjunction with cleaning, because the acid can neutralize excess alkalinity from the cleaners, prevent formation of alkaline deposits, and of course, sanitize (Marriott, 1999). The advantages of PAA are that it is non-corrosive, unlike chlorine, but has a higher oxidation potential (1.81 electron-volts) than chlorine sanitizers (1.36 – 1.57 eV) but less than ozone (2.07 eV); it is unaffected by changes in temperature, produces little to no toxic or mutagenic by-products after reaction with organic material, and remains effective in the presence of organic matter, unlike ozone (Rodgers *et al.*, 2004).

Studies have reported that PAA is effective against a broad spectrum of microorganisms whereby its disinfection efficacy can be ranked on a general basis as: bacteria>viruses>bacterial spores>protozoan cysts (Liberi and Notarnicola, 1999; Rudd and Hopkinson, 1989). Often, PAA

is used as an oxidizer and applied as a disinfectant, bactericide, fungicide, or germicide, and also used in bleaching textiles, paper, oils, waxes, and starch (Anonymous, 2004). However its major use in the food industry is to control deposits, odor, and biofilm on food-contact surfaces, and microbial population/growth in wastewater and on food-contact surfaces (Anonymous, 2000). The primary mode of action is oxidation, whereby it oxidizes the outer cell membrane of vegetative bacterial cells, endospores, yeasts, and mold spores by transfer of electrons to the microorganism. It has also been suggested that PAA disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of cell walls (Baldry and Fraser, 1988; Leaper, 1984). Intracellular PAA may also oxidize essential enzymes, impairing biochemical pathways, active transport across membranes, and intracellular solute levels (Fraser *et al.*, 1984). Several studies have described the effectiveness of PAA: contamination from feces was reduced by 97% with peracetic acid (1.5 – 2 µg/ml, contact time 20 min), attaining the *E. coli* limit recommended by Italian law ( $\leq 5000$  MPN/100 ml) for the return of treated wastewaters to surface waters (Stampi *et al.*, 2001); approximately 5 µg/ml residual PAA reduced populations of total coliforms and fecal coliform in secondary effluent by 4 to 5 logs after a 20-min contact time (Morris, 1993); and 15 and 30 mg/L PAA concentrations in demineralized water resulted in log reductions of bacteriophages MS2 and  $\phi$  x 174 by 4 logs over a 5-min of contact time (Baldry and French, 1989a). Laboratory and full-scale trials in Brazil and Italy showed that PAA was a better disinfectant than sodium hypochlorite for wastewater disinfection in tropical and warm-temperature climates (Baldry *et al.*, 1995)

Despite the effectiveness of PAA in killing bacterial, there are concerns that pathogenic bacteria may develop tolerance to peroxidative stress. In a study conducted by Zook *et al.* (2001), *E. coli* O157:H7 933 exhibited increased tolerance to peroxidative stress when acutely

exposed to a sublethal concentration of the experimental sanitizer containing 27.5% H<sub>2</sub>O<sub>2</sub>, 8% acetic acid, and 5.8% PAA. There has been limited work done to determine the effectiveness of higher concentrations of PAA against Norwalk-like viruses (Hilgren and Salverda, 2000). In a study comparing various chemical sanitizers, Rodgers *et al.* (2004) observed that PAA (80 µg/ml) was the least effective sanitizer because it took the longest time for inactivation of *E. coli* O157:H7 and *L. monocytogenes* on whole and sliced apples, whole and sliced lettuce, strawberries, and cantaloupe. In addition, the lowest log reductions (~4.4 log CFU/g on produce) were achieved by PAA treatment compared to reductions of ~5.6 log CFU/g resulting from treatment with 5 µg/ml ClO<sub>2</sub> or 3 µg/ml ozone, or ~4.9 log CFU/g from treatment with chlorinated trisodium phosphate (CTP) containing 200 µg/ml chlorine and 3 µg/ml of chlorine dioxide. Another drawback is the cost of PAA. A 12% PAA solution is four to five times more expensive than sodium hypochlorite in the U.S., making it less in demand compared to chlorine-based sanitizers (Kitis, 2004).

### ***Ozone***

Ozone (O<sub>3</sub>) naturally present in the earth's upper atmosphere acts as an oxidant and disinfectant, which may control microbial and chemical hazards. Currently, FDA permits O<sub>3</sub> for use in treating drinking water and recycled water in poultry plants at concentrations that do not exceed 0.1 µg/ml (FDA, 1995). Ozonated water has been shown to be an effective sanitizer for fresh produce, with microbial populations on lettuce reportedly reduced up to 4.6 log<sub>10</sub> CFU/g following a 5-min exposure to 1.3 mM ozone (Kim *et al.*, 1999a). Immersion of black peppercorns contaminated with *Salmonella*, *S. aureus*, *B. cereus*, *Penicillium* spp., or *Aspergillus* spp. in water sparged with gaseous ozone (6.7 µg/ml) for 10 min resulted in reductions of

microbial counts by 3 – 4 logs (Zhao and Cranston, 1995). Stainless steel coupons inoculated with *E. coli*, *Serratia liquefaciens*, *Staphylococcus aureus*, *L. innocua*, and *Rhodotorula rubra* upon treatment with ozonated water at 2 and 5 µg/ml for 1 h, were reduced 1 – 7 logs, significantly greater ( $p \leq 0.05$ ) than those observed for untreated controls (~0.5 log reduction), showing that ozone is effective in reducing microbial populations (Moore *et al.*, 2000).

Ozonated water also has activity against a wide range of microorganisms, including *S. Typhimurium*, *Y. enterocolitica*, *S. aureus*, *L. monocytogenes*, enteric viruses, aflatoxigenic aspergilli, and oocytes of parasites, such as *Cryptosporidium* and *Giardia*; it destroys pesticides and chemical residues such as chlorinated by-products, since it rapidly dissipates into oxygen; and it converts non-biodegradable organic materials into biodegradable forms (Restaino *et al.*, 1995; Beuchat *et al.*, 1999; Graham, 1997; and Hwang *et al.*, 2001). In addition to functioning as a bactericidal agent, O<sub>3</sub> has some effectiveness in preventing mold formation in food storage rooms that are inclined to be damped (Philips, 1968). Grapes exposed for 20 min to ozone (8 µg/ml) had considerably reduced counts of bacteria, molds, and yeasts (Sarig *et al.*, 1996)

The efficacy of ozone is largely due to its strong oxidizing power, whereby it rapidly reacts with microorganisms, resulting in high lethality (Beuchat, 2000; Kim *et al.*, 1999b). Ozone is lethal to a wider spectrum of microorganisms than is chlorine and other common sanitizers through the oxidation of the cell membrane (Giese and Christenser, 1954; Kim *et al.*, 1999a). Ozone initially targets the bacterial membrane glycoproteins, glycolipids, or certain amino acids, such as tryptophan, and also acts on the sulfhydryl groups of certain enzymes, resulting in disruption of normal cellular activity (Greene *et al.*, 1993). Ozone may also inactivate microorganisms by causing genetic damage to pyrimidine bases, with thymine being more sensitive than cytosine and uracil (Scott, 1975). Other advantages of using O<sub>3</sub> are that,

unlike chlorine, its efficiency is not affected by pH. Ozone may extend shelf life of oranges, strawberries, raspberries, grapes, apples, and pears (Beuchat, 2000).

The disadvantages of using ozone as a sanitizer are that it is difficult to generate and it is unstable. Rajkowski and Rice (2004) and Moore *et al.* (2000) showed that ozonated water, when used to wash seeds, cut produce, or processing equipment containing biofilms or high organic load, results in chemical reactions with the released seed/produce nutrients or organic material in such a way that the efficacy is compromised. This was observed when the ozone concentration is reduced to a level at which it can no longer function as a sanitizer and microbial counts are only reduced. Confirmation of these observations has been made in studies by Naito and Shiga (1989), Sharma *et al.* (2002), and Wade *et al.* (2003). Proper cleaning is suggested to remove soil and other debris before application of ozone to enhance its effectiveness as a disinfectant. Food quality deterioration may result from application of ozone. In some cases, oxidative spoilage may be caused by treatment with ozone, resulting in discoloration of some fruits and vegetables, such as peaches, carrots, and broccoli florets (Badiani *et al.*, 1996; Liew and Prange, 1994; and Lewis *et al.*, 1996). These alterations in sensory attributes, however, do not occur in all foods; it depends on the chemical composition of food, ozone dose, and treatment conditions (Kim *et al.*, 1999b).

### ***Hydrogen Peroxide***

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is an oxidizing agent more commonly used as antiseptic because it is able to change the environment in such a way that conditions are unsuitable for growth of specific organisms (Price, 1968). There have been many instances in which H<sub>2</sub>O<sub>2</sub> has been used to control and prevent growth of pathogens on fresh produce. A study conducted by

Sapers *et al.* (1999) showed that immersion of apples in 5% H<sub>2</sub>O<sub>2</sub> solution reduced populations by 2 – 3 log<sub>10</sub> CFU/g versus 1 – 2 log<sub>10</sub> CFU/g achieved by the conventional washing and sanitizing agents such as chlorinated water. A 5% H<sub>2</sub>O<sub>2</sub> wash, applied to intact cantaloupes prior to cutting was shown to be effective in extending the shelf life of fresh-cut fruits (Sapers *et al.*, 2001b) and reducing the population of *Salmonella* Stanley (> 2 log<sub>10</sub> CFU/cm<sup>2</sup>) on the surface of inoculated cantaloupes (Ukuku and Sapers, 2001). Treatment of lettuce with 2% H<sub>2</sub>O<sub>2</sub> at 50°C caused population reductions of ≤ 4 logs of *E. coli* O157:H7 and *S. Enteritidis* and a 3-log reduction for *L. monocytogenes*, with only slight browning during storage (Lin *et al.*, 2002). Lettuce given the same treatment and stored at 5°C for as long as 15 days was rated superior to or at least as high in quality as untreated controls by a consumer panel (McWatters *et al.*, 2002).

Although, H<sub>2</sub>O<sub>2</sub> is classified by the U.S. Food and Drug Administration as Generally Recognized as Safe (GRAS) for specified food applications (FDA, 1986) and may be a good alternative sanitizer, current FDA regulations do not permit the use of H<sub>2</sub>O<sub>2</sub> as a washing agent for produce except when used at low concentrations in combination with acetic acid to form peroxyacetic acid and where fruits and vegetables are not considered raw agricultural commodities (FDA, 1999). Recent action by the U.S. Environmental Protection Agency, however, now exempts use of ≤ 1% H<sub>2</sub>O<sub>2</sub>, applied to all postharvest agricultural food commodities, from the requirement of a tolerance (FDA, 2002). Inactivation of human pathogens and molds present on produce has been achieved in several studies using ≤ 1% H<sub>2</sub>O<sub>2</sub> as a sanitizer, sometimes in conjunction with acid or other disinfectants (Sapers and Sites, 2003). In a study of mushroom-washing technology, the microbial population in water used to wash dirty mushrooms was reduced by 2 logs by treatment with 0.5% H<sub>2</sub>O<sub>2</sub> (Sapers and Simmons, 1998). Treatment of eggplant and sweet red pepper with 0.5% Sanosil-25 (a proprietary

disinfectant containing 0.24% H<sub>2</sub>O<sub>2</sub>) suppressed *Botrytis cinerea* and *Alternaria alternaria* and reduced postharvest decay during storage (Fallik *et al.*, 1994). Reductions in populations of *E. coli* O157:H7 on apples that were treated with 1% H<sub>2</sub>O<sub>2</sub> were similar at 20°C (2.8 log reduction) and 40°C (3.0 log reduction) and also comparable to reductions obtained by treatment with 5% H<sub>2</sub>O<sub>2</sub> at 60°C (3.51 log reduction) for 2 min, indicating that the lethality of 1% H<sub>2</sub>O<sub>2</sub> is similar to that of 5% H<sub>2</sub>O<sub>2</sub>. Population reductions (2.6 log) obtained with 200 µg/ml of Cl<sub>2</sub> were slightly lower than those achieved by treatment of inoculated apples with 1% H<sub>2</sub>O<sub>2</sub> at 20°C (Sapers and Sites, 2003), indicating that 1% H<sub>2</sub>O<sub>2</sub> may be a possible alternative to chlorinated water.

There are disadvantages in using H<sub>2</sub>O<sub>2</sub> as a sanitizer, e.g., its lack of stability and effectiveness in solution over time. Sapers *et al.* (2001a) reported that a 1% H<sub>2</sub>O<sub>2</sub> pre-wash solution, though effective in reducing the number of lesions indicative of bacterial spoilage on mushrooms, eventually resulted in lost strength during prolonged use. This is due to reactions with mushrooms and suspended soil. Thus, the 1% H<sub>2</sub>O<sub>2</sub> pre-wash solution had to be replaced periodically. In some cases, the effectiveness of sanitizers varies between different produce. At concentrations of 1% and 5%, H<sub>2</sub>O<sub>2</sub> has been reported by various studies to be effective in sanitizing lettuce, apples, and mushrooms; however, the efficacy of 1% H<sub>2</sub>O<sub>2</sub> in killing non-pathogenic *E. coli* on cantaloupes was relatively poor, a lower concentration of H<sub>2</sub>O<sub>2</sub> (1% vs. 5%) achieving  $\leq 1$  log reduction of the population.

In general, there are many different types of cleaners, disinfectants and sanitizers available to remove, kill, or help prevent growth of pathogens on fresh produce and surfaces it may come in contact. Each chemical agent has specific attributes that need to be taken into consideration when choosing the appropriate sanitizer for a particular situation. Studies have shown that gaseous ClO<sub>2</sub> has promise as an alternative sanitizer for fruits and vegetables as



research laboratories and the produce industry continue to deliver safe, high-quality products to the consumers. Research presented here was conducted to determine the efficacy of gaseous  $\text{ClO}_2$  in killing or inactivating three pathogens, *Salmonella enterica*, *E. coli* O157:H7, and *L. monocytogenes*, as well as yeasts and molds, on fresh whole (uncut) and fresh-cut fruits and vegetables.

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## CHAPTER 2

# EFFICACY OF GASEOUS CHLORINE DIOXIDE AS A SANITIZER FOR KILLING *SALMONELLA*, YEASTS, AND MOLDS ON BLUEBERRIES, STRAWBERRIES, AND RASPBERRIES<sup>1</sup>

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<sup>1</sup>Sy, Kaye V. and Larry R. Beuchat 2004. To be submitted to Journal of Food Protection

## ABSTRACT

Breakage of tissues and the creation of residual moisture on the surface of small fragile fruits intended for the fresh produce market as a result of washing with water or treatment with aqueous sanitizers have raised interest in evaluating gaseous chemicals for their efficacy as produce sanitizers. The objective of the research reported here was to examine gaseous chlorine dioxide ( $\text{ClO}_2$ ) for its effectiveness in killing or removing *Salmonella*, yeasts, and molds on blueberries, strawberries, and red raspberries. An inoculum (100  $\mu\text{l}$ , ca.  $5.5 \log_{10}$  CFU) containing five serotypes of *Salmonella enterica* was deposited on the skin, calyx tissue, or stem scar tissue of blueberries, skin or stem scar of strawberries, and skin of red raspberries, dried for 2 h at  $22^\circ\text{C}$ , then held for 20 h at  $4^\circ\text{C}$  and 2 h at  $22^\circ\text{C}$  before treatment. Sachets containing reactant chemicals were formulated to release gaseous  $\text{ClO}_2$  at concentrations of 4.1, 6.0 – 6.2, and 8.0 – 8.1 mg/L of air within 30, 60, and 120 min, respectively, at  $23^\circ\text{C}$ . Treatment with 8.0 mg of  $\text{ClO}_2/\text{L}$  significantly ( $\alpha = 0.05$ ) reduced the population of *Salmonella* on blueberries by 2.2 – 4.1  $\log_{10}$  CFU/g. Lethality was higher to cells in inoculum placed on the skin, compared to the stem scar tissue. Populations of *Salmonella* on strawberries treated with 8.1 mg of  $\text{ClO}_2/\text{L}$  were reduced by 3.7 – 4.3  $\log_{10}$  CFU/g; significant reductions of 1.4  $\log_{10}$  CFU/g of raspberries were achieved. Treatment with 4.1 – 8.1 mg of  $\text{ClO}_2/\text{L}$  caused reductions in populations of yeast and mold on blueberries, strawberries, and raspberries of 1.2 – 2.8, 0.8 – 4.1, and 2.1 – 3.2  $\log_{10}$  CFU/g, respectively. Lethality of  $\text{ClO}_2$  to *Salmonella*, yeasts, and molds was higher when fruits were treated at 75 – 90% relative humidity than at lower relative humidity. Results indicate that gaseous  $\text{ClO}_2$  has promise as a sanitizer for small fruits.

## INTRODUCTION

Over the past several years, there has been an increased per capita consumption of fresh fruits, due in part to a desire for health-promoting diets. Increased consumption, among other factors, has undoubtedly contributed to a higher number of cases of foodborne illness associated with fruits and vegetables (Beuchat, 2002). Small fruits are among the produce that have been linked to outbreaks of enteric infections. Strawberries have been implicated in outbreaks of hepatitis A (Niu *et al.*, 1992) and raspberries have been associated with outbreaks of *Cyclospora cayetanensis* infection (Herwaldt, 2000). *Salmonella* can survive on produce throughout their shelf life (Beuchat, 2002; Institute of Food Technologists, 2001; Du *et al.*, 2002; Asplund and Nurmi, 1991).

It is recognized that raw fruits and vegetables are occasionally contaminated with foodborne pathogens; however, there is a general lack of efficacy of sanitizers in killing or removing these pathogens (Beuchat, 1998). This can be partially attributed to difficulties in delivering aqueous chemical sanitizers to areas on the surface of produce in which pathogens may be lodged (Burnett and Beuchat, 2001). Treatment with aqueous chemical solutions can also leave residual moisture on fruits and vegetables, which can promote the growth of molds. Infection of produce with molds can in turn increase the pH of tissues and enhance the growth of *Salmonella* (Wade and Beuchat, 2003), *Escherichia coli* O157:H7 (Riordan *et al.*, 2000), and *Clostridium botulinum* (Draughon *et al.*, 1988)

Sanitizers such as gaseous chlorine dioxide (ClO<sub>2</sub>) have been explored as alternatives to aqueous chemicals for sanitizing fruits and vegetables eaten raw. Gaseous ClO<sub>2</sub> has some advantages over chlorinated water in that it can break down phenolic compounds and remove phenolic tastes and odors from the water, does not react with ammonia, and has 2.5 times the

oxidation capacity of chlorine; its bactericidal efficacy is not markedly affected by pH and it has greater sporicidal activity (Dychdala, 1968; Reina, 1995; Cords and Dychdala, 1993). Loss in permeability control with nonspecific oxidative damage of the outer membrane and inhibition of respiration are among the events associated with lethality of ClO<sub>2</sub> to vegetative bacterial cells (Berg *et al.*, 1986).

Several studies have shown gaseous ClO<sub>2</sub> to be effective in killing enteric pathogens on fruits and vegetables. Treatment of uninjured green peppers with 3 mg/L gaseous ClO<sub>2</sub> resulted in a reduction of *Listeria monocytogenes* by more than 6 log<sub>10</sub> CFU/5 g (Han *et al.*, 2001b). Treatment of peppers with 0.6 mg/L gaseous ClO<sub>2</sub> for 30 min at 22°C and 90 – 95% relative humidity caused a 7.3-log<sub>10</sub> CFU/5 g reduction of *E. coli* O157:H7 on the uninjured surface (Han *et al.*, 2000b). Treatment of cut apples with gaseous ClO<sub>2</sub> at a concentration of 3.3 mg/L for 20 min resulted in a reduction of *E. coli* O157:H7 of 5.9 log<sub>10</sub> CFU/5 g (Du *et al.*, 2003). Sapers *et al.* (2003) reported that the number of *E. coli* inoculated onto apples was reduced by 4.5 log<sub>10</sub> CFU/g, with minimal quality loss, by treatment with gaseous ClO<sub>2</sub> at 0.3 mg/L.

Gaseous ClO<sub>2</sub> (0.5 – 2.0 ppmv/g of material) effectively controlled the spread of molds in libraries (Weaver-Meyers *et al.*, 1998). Although the fungicidal effect was demonstrated on books, gaseous ClO<sub>2</sub> also has potential as a sanitizer for reducing yeast and mold populations in food processing plants and on fruits and vegetables. Inoculation of epoxy-coated stainless steel strips identical to those used in juice tanks with *Eurotium*, *Penicillium*, *Candida*, and *Saccharomyces cerevisiae* at populations > 4 log<sub>10</sub> CFU/2.5 x 7 cm area, followed by gaseous ClO<sub>2</sub> treatment, resulted in reductions to populations below detectable limits (Han *et al.*, 1999). Other gaseous chemicals have shown promise as sanitizer for fruits and vegetables. Treatment of fruits (Sholberg and Gaunce, 1995) and mung bean seed (Delaquis *et al.*, 1999) with gaseous

acetic acid and apples (Sapers *et al.*, 2003) and prunes (Simmons *et al.*, 1997) with vapor-phase hydrogen peroxide has been reported to reduce microbial populations and extend shelf life.

The efficacy of gaseous ClO<sub>2</sub> gas in killing or removing *Salmonella*, yeasts, and molds on small fruits has not been reported. The objective of this study was to evaluate gaseous ClO<sub>2</sub> for its effectiveness in killing *Salmonella* inoculated onto the surface of blueberries, strawberries, and red raspberries. Inactivation of yeasts and molds naturally occurring on the fruits was also determined.

## MATERIALS AND METHODS

**Bacteria used and maintenance of cultures.** Serotypes of *Salmonella enterica* isolated from alfalfa sprouts (serotype Agona), feces of patients in tomato-associated outbreaks of salmonellosis (serotypes Baildon and serotype Montevideo), orange juice (serotype Gaminara), and a cantaloupe-associated outbreak (serotype Michigan) were used. All serotypes were grown in tryptic soy broth (TSB) (BBL/Difco, Sparks, Md.) supplemented with nalidixic acid (Sigma, St. Louis, Mo.) (TSBN) at a concentration of 50 µg/ml at 37°C for 24 h. Cultures were combined with glycerol (85:15, v:v, culture:glycerol) and stored at -30°C until used.

**Preparation of inoculum.** Frozen cell suspensions of the five *S. enterica* serotypes were thawed and streaked onto tryptic soy agar (TSA) (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (1 g/L) (TSANP). The TSANP plates were incubated at 37°C for 24 h before picking colonies to be transferred into 10 ml of TSBN. Tubes were incubated at 37°C for 24 h. A minimum of two consecutive 24 h transfers were made via loop inoculum (ca. 10 µl) into 10 ml of TSBN. Cells were harvested by centrifugation at 2,000 x g for 15 min (Centra CL2 centrifuge International Equipment Co, Needham Heights, Mass.). The supernatant

was decanted and cells were resuspended in 5 ml of sterile 5% (v:v) horse serum (Sigma). Suspensions of each serotype were combined to give 20 ml of a five-serotype mixture of *S. enterica* containing approximately equal populations ( $9 \log_{10}$  CFU/ml) of each serotype. Populations were determined by serially diluting suspensions in sterile 0.1% peptone and surface plating duplicate 0.1 ml samples on TSANP plates were incubated at 37°C for 24 h before colonies were counted.

**Produce tested.** Blueberries (*Vaccinium corymbosum* L.), strawberries (*Fragaria ananassa* Duchesne), and red raspberries (*Rubus idaeus* L.) were purchased at a local market in Griffin, Ga. and stored at 4°C for a maximum of 2 days before using in experiments. Prior to inoculation with *Salmonella*, the fruits were adjusted to  $22 \pm 1^\circ\text{C}$  over a 1- to 2-h period. Samples consisting of twelve blueberries, five strawberries, or six raspberries, all free of visible wounds, cuts, and bruises, were placed in single layers on plastic trays (14 cm long x 14 cm wide x 2.5 cm high) in preparation for inoculation.

**Inoculation of berries.** Blueberries ( $20 \pm 1$  g), strawberries ( $100 \pm 10$  g), and raspberries ( $20 \pm 1$  g) at  $22 \pm 1^\circ\text{C}$  in plastic weigh trays were spot-inoculated with 100  $\mu\text{l}$  of a five-serotype mixture of *S. enterica* using a micropipettor. Inoculum was deposited on the skin, calyx tissue, or stem scar tissue of blueberries; separate samples were used for each inoculum site. Inoculum was applied on either the skin or stem scar area of separate samples of strawberries. Only the external skin surface of raspberries was inoculated.

To prevent inoculum from running off the sides of fruits and to facilitate drying, small approximately equal volumes of inoculum were applied to several berries in the same sample or up to five locations at each site on the same berry. All fruits were inoculated in a biosafety hood and dried for 2 h at  $22 \pm 2^\circ\text{C}$ , followed by storing in plastic containers at 62% relative humidity

for 20 h at 4°C. Prior to treatment with gaseous ClO<sub>2</sub>, fruits were placed in a biosafety hood for 2 h at 22 ± 2°C.

**Relative humidity.** Nine samples of blueberries (three samples with the skin inoculated, three with the calyx tissue inoculated, and three with the stem scar tissue inoculated), six samples of strawberries (three with the skin inoculated and three with the stem scar area inoculated), or three samples of raspberries with the skin inoculated were placed in a Fisherbrand transparent plexiglass desiccator cabinet (45.7 cm long x 30.5 cm wide x 30.5 cm high) (Figure 2.1). Samples were placed on the bottom three shelves of the four-shelf cabinet. High (67 – 95%) relative humidity was achieved by placing 20 ml of hot water (initially at 97 – 99°C) in a shallow plastic dish (8.6 x 8.6 x 2.2 cm) on the bottom shelf during treatment of berries with ClO<sub>2</sub>. A RadioShack Brushless 12VDC cooling fan (6.9 x 6.9 x 2.5 cm) (RadioShack, Fort Worth, Tex.) was strategically placed on each of the four shelves to circulate the air. A Fisher Scientific Thermo-Hygro recorder (model no. 11-661-13) was used to monitor relative humidity and temperature inside the treatment cabinet.

**Gaseous ClO<sub>2</sub> treatment.** Fruits inoculated with ca. 6.0 – 6.5 log<sub>10</sub> CFU of *Salmonella*/g were placed in the cabinet and treated with either air (control) or gaseous ClO<sub>2</sub> for 0, 30, 60, and 120 min. Sachets consisting of two compartments containing reactant chemicals (granular porous solid impregnated with sodium chlorite and an activator, granular porous solid impregnated with acid, ferric chloride) were supplied by ICA TriNova, Inc., Marietta, Ga. Breakage of the septum between the two compartments, followed by mixing the chemicals, initiated the production of ClO<sub>2</sub> gas (Figure 2.2). The mixture of chemicals in three sachets was formulated to release gaseous ClO<sub>2</sub> into the cabinet (31.1 L) at concentrations of 0 mg/L (control), 4.1 mg/L, 6.0 – 6.2 mg/L, and 8.0 – 8.1 mg/L in 30, 60, and 120 min, respectively, at

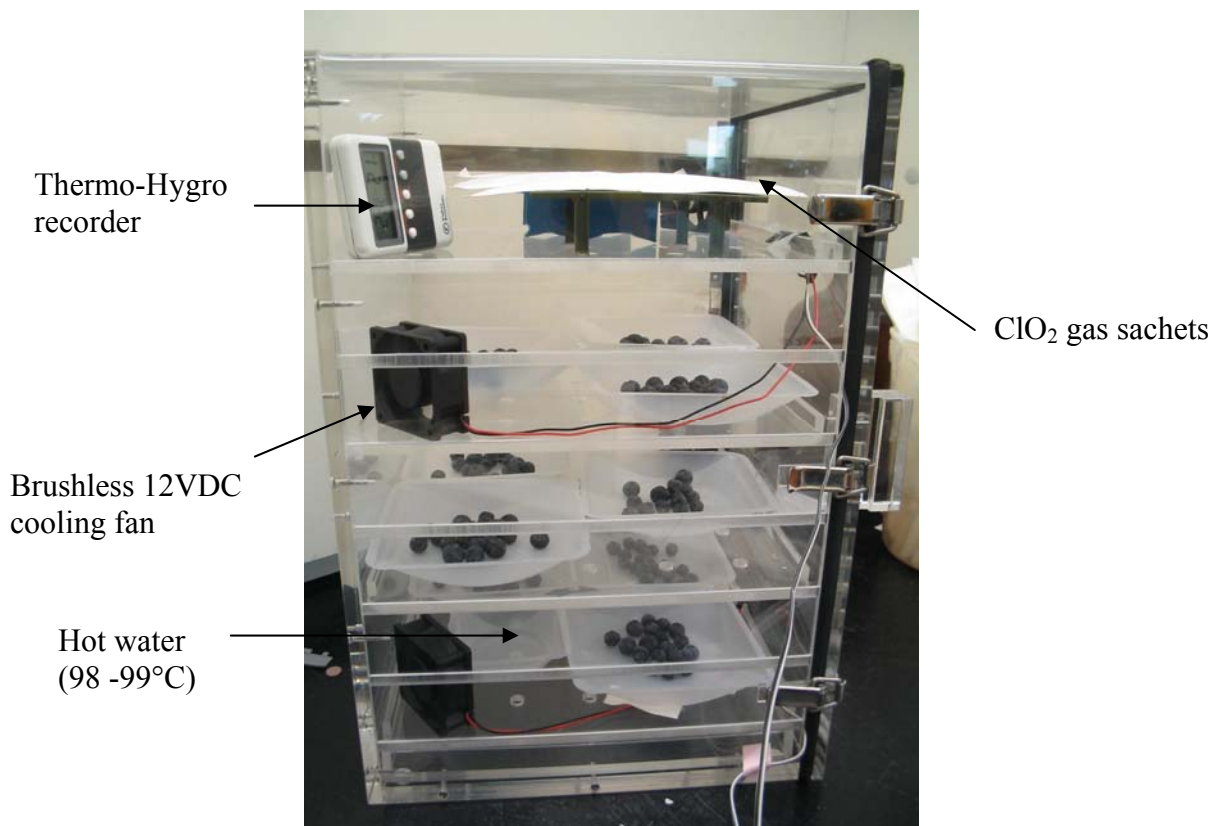


Figure 2.1. *Nine samples of blueberries treated with ClO<sub>2</sub> gas in a plexiglass desiccator cabinet.*

23 ± 1°C. The gaseous ClO<sub>2</sub> concentrations released into the treatment chamber can also be defined as ppmv, since a gas phase concentration of 1 mg/L is equivalent to 362 ppmv. An alternative way to present concentrations of 4.1 mg/L, 6.0 – 6.2 mg/L, and 8.0 – 8.1 mg/L gaseous ClO<sub>2</sub> released into the treatment chamber are 1,484 ppmv, 2,172 – 2,244 ppmv, and 2,896 – 2,932 ppmv in 30, 60, and 120 min, respectively. Concentrations of ClO<sub>2</sub> released during a 2-h period are shown in Figures 2.3 and 2.4. Concentrations were determined by titrating the amount of iodine formed by the reaction of ClO<sub>2</sub> with potassium iodide using sodium thiosulfate as a titrant. The detailed procedure and a description of ClO<sub>2</sub> chemistry are published elsewhere (Aieta *et al.*, 1984; Anon, 1975).



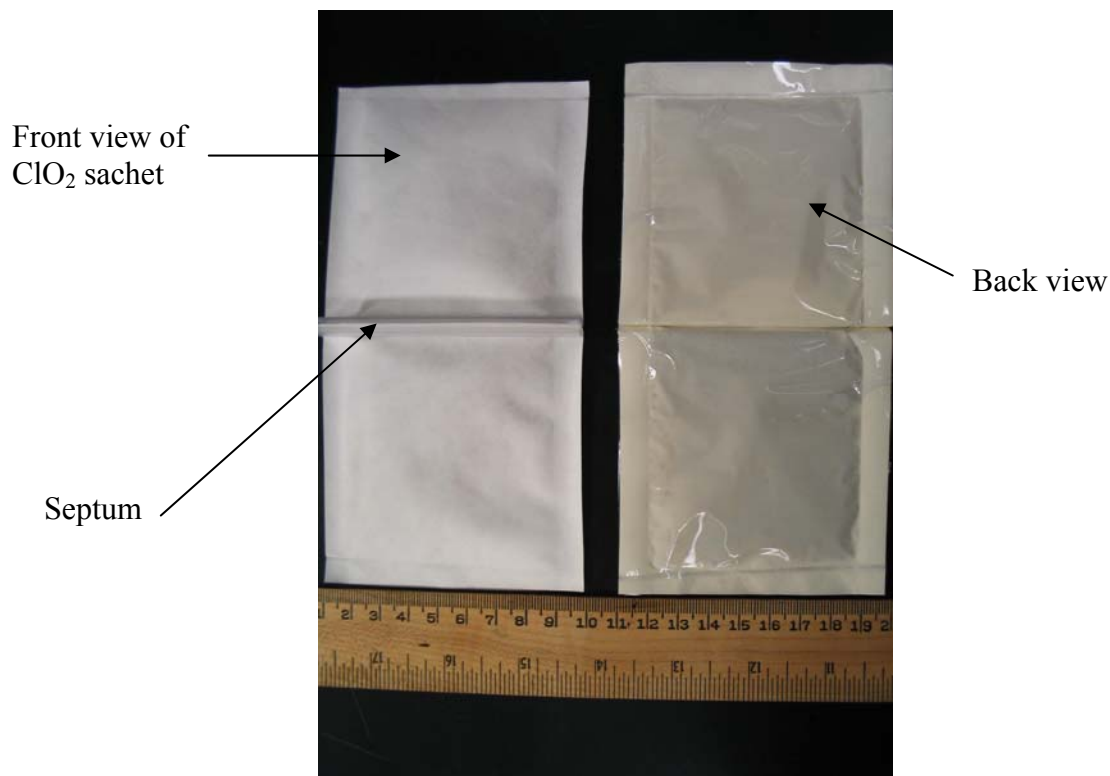


Figure 2.2. *Breakage of septum of two compartments and mixture of reacting chemicals in sachets release ClO<sub>2</sub> gas.*

Immediately following placement of the fruit samples on the bottom three shelves in the treatment cabinet, hot water (20 ml) in a plastic dish was placed on the bottom shelf, while three sachets containing the reactant chemicals were simultaneously placed on an elevated mesh platform placed on the top shelf to deliver maximum levels of relative humidity and desired concentrations of gaseous ClO<sub>2</sub>. The control samples were handled in an identical manner, except ClO<sub>2</sub> sachets were not placed in the cabinet. Closing and securing the door to which a rubber gasket was affixed sealed the cabinet. This created a ClO<sub>2</sub> gas-sanitizing environment to treat inoculated berries.

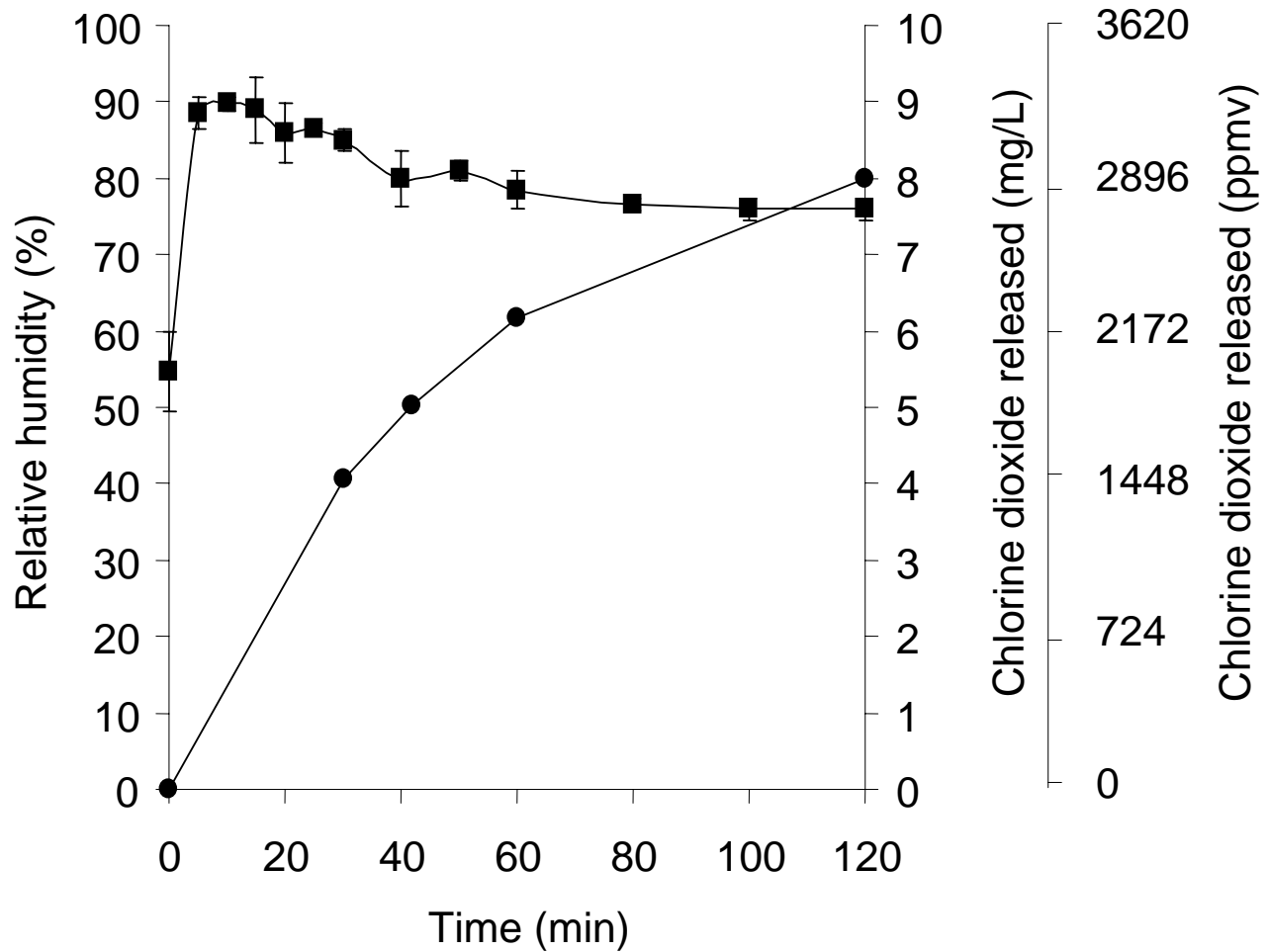


Figure 2.3. Atmospheric relative humidity ( ■ ) and chlorine dioxide ( ● ) released into the atmosphere in the cabinet used to treat blueberries.

**Efficiency of recovery of *Salmonella*.** Preliminary studies were conducted to determine the most efficient method to recover *Salmonella*, yeasts, and molds from blueberries, strawberries, and raspberries. The skin of fruits was inoculated with 100  $\mu$ l of *Salmonella* suspension at populations of 7.81  $\log_{10}$  CFU/g of blueberries and raspberries and 7.11  $\log_{10}$  CFU/g of strawberries. Two methods (washing and stomaching) were evaluated for their efficiency in removing *Salmonella* and naturally occurring yeasts and molds.

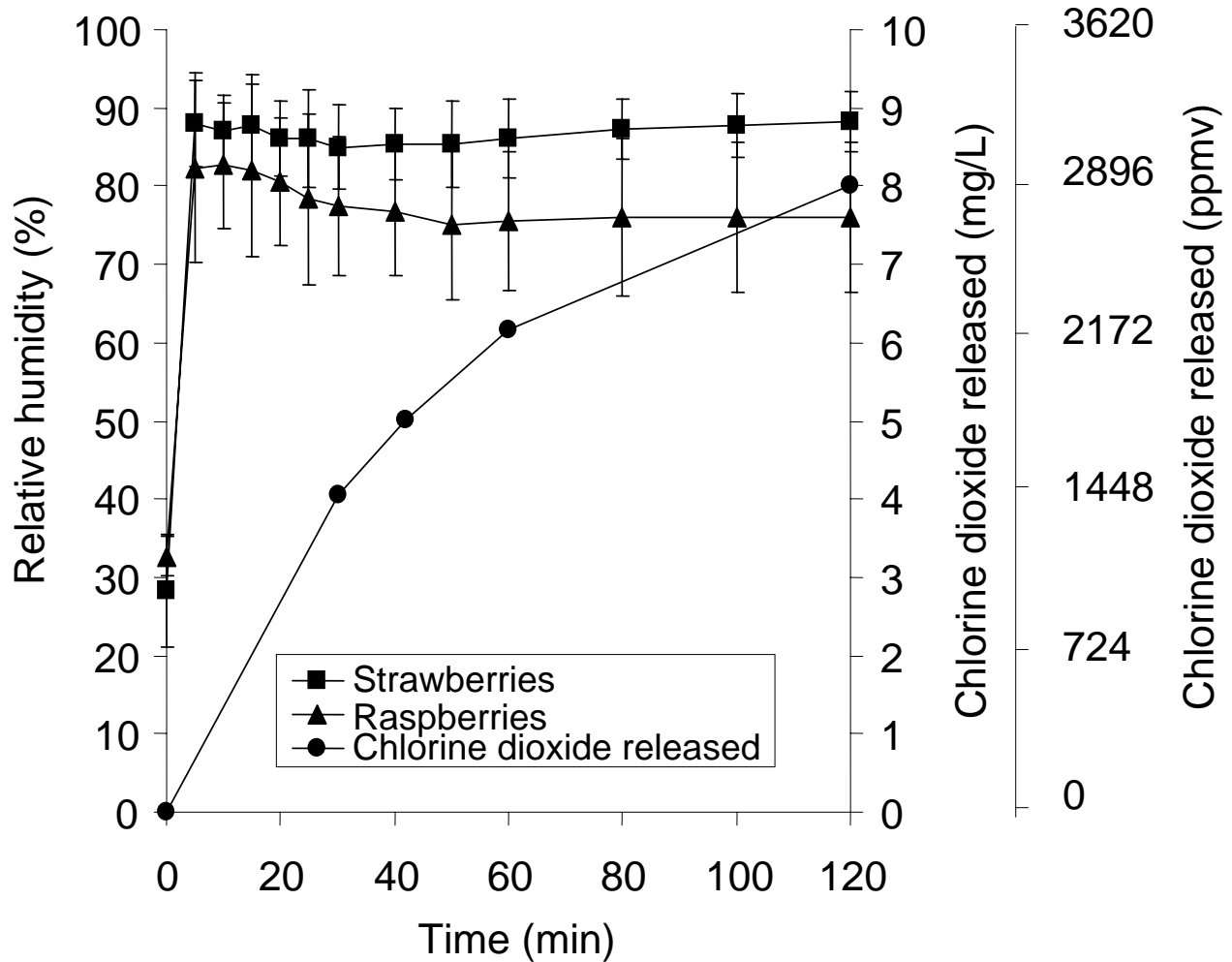


Figure 2.4. Atmospheric relative humidity and amount of chlorine dioxide released into the atmosphere in the cabinet used to treat strawberries and raspberries.

Strawberries on which *Salmonella* inoculum had dried for 2 h at  $22 \pm 2^\circ\text{C}$  were placed in a stomacher 400 bag (Seward Medical, London, U.K.) and 100 ml of Dey-Engley (DE) broth (BBL/Difco) was added. The strawberries were gently hand-rubbed for 1 min to wash the external surface of each fruit. Samples of blueberries and raspberries inoculated with *Salmonella* on which 100  $\mu\text{l}$  of *Salmonella* suspension had dried for 2 h at  $22 \pm 2^\circ\text{C}$  were placed in a stomacher 80 bag and 40 ml of DE broth was added to each bag. Blueberries and raspberries

were washed with DE broth by placing samples on a platform shaker (New Brunswick Scientific, Innova 2000, Brunswick, N.J.) and shaking at 150 rpm for 1 min.

Pummeling samples in a Stomacher blender (Seward Medical, Ltd., London, U.K.) was also evaluated as a procedure to remove *Salmonella*, yeasts, and molds from berries. Samples were prepared as described for the wash method, except that, instead of washing berries, strawberries were pummeled at normal speed for 1 min in a Stomacher 400 blender and blueberries and raspberries were pummeled in for 1 min in a Stomacher 80 blender.

**Microbiological analyses.** Undiluted DE wash broth and homogenates (0.25 ml in quadruplicate and 0.1 ml in duplicate), as well as samples (0.1 ml in duplicate) serially diluted in sterile 0.1% peptone, from berries examined in preliminary studies were surface plated on TSANP and xylose lysine desoxycholate (XLD) agar (pH 7.4) supplemented with nalidixic acid (50 µg/ml) and 1 g/L of sodium pyruvate (XLDNP agar) to enumerate *Salmonella*. Dichloran rose bengal chloramphenicol (DRBC) agar (pH 5.6) (BBL/Difco) was used to enumerate yeasts and molds. The TSANP and XLDNP agar plates were incubated at 37°C for 24 h before presumptive-positive *Salmonella* colonies were counted. Five to ten presumptive-positive colonies were randomly selected for confirmation using a *Salmonella* latex test (Oxoid, Basingstoke, U.K.), lysine iron agar (LIA) (BBL/Difco), and triple sugar iron (TSI) agar (BBL/Difco). The DRBC agar plates were incubated at 25°C for 5 days before yeast and mold colonies were counted. Following removal of samples for plating on TSANP, XLDNP agar, and DRBC agar, the pH of DE wash broth and the berry/DE broth homogenates was measured.

Inoculated, untreated berry samples not exposed to air (control) or gaseous ClO<sub>2</sub> treatments in the cabinet, as well as samples held for up to 120 min in air (control) or gaseous ClO<sub>2</sub> in the cabinet, were analyzed for populations of *Salmonella*, yeasts, and molds. Untreated

and treated blueberry and raspberry samples were placed in separate Stomacher 80 bags and DE broth (40 ml) was added to each bag. Strawberry samples were placed in Stomacher 400 bags and 100 ml of DE broth was added. Blueberries and raspberries were washed with the DE broth by placing samples on a platform shaker and shaking at 150 rpm for 1 min. Strawberries were gently hand-rubbed in DE broth for 1 min. The undiluted DE wash broth (0.25 ml in quadruplicate and 0.1 ml in duplicate), as well as samples (0.1 ml in duplicate) of wash broth serially diluted in sterile 0.1% peptone from berry samples exposed to air or gaseous ClO<sub>2</sub> in the treatment cabinet, were surface plated on TSANP and DRBC agar to enumerate *Salmonella* and yeasts/molds, respectively. The TSANP plates were incubated at 37°C for 24 h before presumptive-positive *Salmonella* colonies were counted and subjected to confirmation tests. The DRBC agar plates were incubated at 25°C for 5 days before yeast and mold colonies were counted.

Following removal of samples from wash broth for plating on TSANP and DRBC agar, 40 ml of 2X lactose broth (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (1 g/L) (LBNP) was added to each bag containing DE wash broth and blueberries or raspberries that had been treated with gaseous ClO<sub>2</sub>; 100 ml of 2X LBNP was added to each sample of strawberries in DE wash broth. Mixtures of fruit samples with DE broth and LBNP were incubated at 37°C for 24 h. If fruit samples treated with ClO<sub>2</sub> did not yield one or more colonies of *Salmonella* on TSANP, the pre-enriched mixture was examined for the presence of *Salmonella*. A loop (ca. 10 µl) of each DE broth/berry/LBNP mixture was streaked on XLDNP agar and the plates were incubated at 37°C for 24 h before examining for the presence of presumptive *Salmonella* colonies. In addition, 0.1 ml samples of the pre-enriched mixture were inoculated into 10 ml of Rappaport-Vassiliadas (RV) enrichment broth (pH 5.1) (BBL/Difco).

The RV broth was incubated at 42°C for 24 h and streaked on XLDNP agar. If samples of pre-enriched mixture did not yield presumptive-positive *Salmonella* colonies on the XLDNP agar plates. XLDNP agar on which enriched samples were streaked was incubated at 37°C for 24 h before examining for presumptive *Salmonella* colonies, followed by confirmation.

**Sensory evaluation.** An untrained panel of 31 individuals from the Center of Food Safety and Department of Food Science and Technology subjectively evaluated treated (4.1 mg/L ClO<sub>2</sub>) and untreated, uninoculated blueberries, strawberries, and raspberries. Panelists were familiarized with sensory qualities of berries through discussions and a 20-min examination of visual and aroma characteristics of fresh, untreated blueberries, strawberries, and raspberries. Both treated and control berries were placed in covered plastic bins (50.8 cm long x 34.3 cm wide x 12.7 cm high) and stored for 0, 3, 7, and 10 days at 8°C, before evaluating for sensory quality. Panelists were asked to carefully examine the appearance, color, aroma, and overall quality of the berries. Sensory attributes were rated by assigning scores of 1 – 9 on a 9-point hedonic scale, with 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. Samples consisting of 20 g of blueberries, 100 g of strawberries, or 20 g of raspberries were placed on white plastic weigh boats and assigned with random three-digit codes. These samples were then presented to the panel in random order on a white bench. All evaluations were conducted within 90 min after treating the samples with ClO<sub>2</sub> gas (day 0) or within 1 h of removing the samples from storage at 8°C.

**Statistical analyses.** Preliminary experiments consisted of three samples of each fruit subjected to each recovery method. All other experiments were replicated three times and each replicate experiment consisted of three samples exposed to the same treatment conditions. Undiluted washes or homogenates were plated in quadruplicate (0.25 ml samples) or duplicate

(0.1 ml samples) and serially diluted samples were plated in duplicate (0.1 ml samples). Mean values were analyzed to determine significant differences ( $\alpha = 0.05$ ) in populations of *Salmonella* or yeasts/molds on fruits subjected to various recovery methods and ClO<sub>2</sub> treatment concentrations. Mean values were also analyzed to determine significant differences ( $\alpha = 0.05$ ) in sensory attributes of berries as affected by treatment. Data were subjected to SAS (Statistical Analysis Systems Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests.

## RESULTS AND DISCUSSION

Dip, spray, and spot application are among the options for inoculating the surface of fruits and vegetables with pathogens for the purpose of conducting sanitizer efficacy studies (Beuchat *et al.*, 2001). Spot inoculation was chosen because it mimics contamination that might occur in the field, during harvesting, or in the packinghouse as a result of contact of fruits with handlers, soil, animals, fecal material, or contaminated produce. A known number of cells can be applied to produce using spot inoculation, enabling reductions in the number of viable cells caused by sanitization treatment to be more accurately calculated (Lang *et al.*, 2004).

**Comparison of recovery methods.** Preliminary studies comparing washing and stomaching as methods to recover *Salmonella* revealed that the number of *Salmonella* recovered on TSANP from stomached blueberries was significantly lower ( $\alpha = 0.05$ ) than the number recovered by washing the berries (Table 2.1). This observation is contrary to that of others (Knudsen *et al.*, 2001) in which surface rinsing methods recovered slightly lower populations of *Salmonella* on strawberries than those recovered by stomaching. Populations recovered from stomached blueberries were significantly lower ( $\alpha = 0.05$ ) compared to populations recovered

TABLE 2.1. Recovery of *Salmonella* and yeasts/molds from strawberries, blueberries, and raspberries as affected by method of retrieval of cells

Fruit	Retrieval Method	Population (log <sub>10</sub> CFU/g) <sup>a</sup>					
		TSANP		XLDNP agar		DRBC agar	
		Recovered	Reduction <sup>b</sup>	Recovered	Reduction <sup>b</sup>	Recovered	
Blueberry	Inoculum <sup>c</sup>	7.81		7.23			
	Wash	A 7.49	0.32	A 6.36	0.87	A 3.00	
	Stomach	B 6.49	1.32	B 5.31	1.92	A 0.00	
Strawberry	Inoculum	7.11		6.53			
	Wash	A 6.23	0.88	A 5.72	0.51	A 5.18	
	Stomach	A 6.24	0.87	B 5.22	1.01	B 4.79	
Raspberry	Inoculum	7.81		7.23			
	Wash	A 7.09	0.72	A 6.35	0.88	A 5.02	
	Stomach	A 6.97	0.84	B 6.07	1.16	A 5.28	

<sup>a</sup>Populations of *Salmonella* recovered on TSANP and XLDNP agar, and yeasts and molds on DRBC agar, respectively wash washing or stomaching blueberries, strawberries, and raspberries for 1 min. Within each fruit and recovering medium, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>b</sup>Reduction (log<sub>10</sub> CFU/g) compared to the number in the inoculum recovered on TSANP or XLDNP agar.

<sup>c</sup>Population (log<sub>10</sub> CFU/g) spot-inoculated on fruit.



from washed blueberries when samples were plated on XLDNP agar. The number of *Salmonella* recovered from stomached strawberries and raspberries were significantly lower ( $\alpha = 0.05$ ) compared to populations recovered from washed strawberries and raspberries when samples were plated on XLDNP agar. Populations of yeasts and molds recovered from stomached and washed berries are also shown on Table 2.1. Stomached strawberries but not blueberries and raspberries, showed significantly lower ( $\alpha = 0.05$ ) populations of yeasts and molds compared to washed berries.

Stomaching results in the release of acidic juice from berry tissues, which decreased the pH of the DE broth from 7.60 to 4.05, 4.09, and 4.04 in blueberry, strawberry, and raspberry homogenates, respectively, and may have caused injury or death of some of the *Salmonella*. Although some salmonellae can survive in acidic environments (pH > 4.0), they cannot survive in foods with lower pH or at higher pH in foods containing certain types of acids (D'Aoust, 2000). Since the external pH of strawberries, blueberries, and raspberries ranges from 2.9 – 3.5 (Splittstoesser, 1987), release of acids caused by stomaching may have caused stress and sublethal injury of *Salmonella*. Debilitation of cells would further contribute to reduced numbers of *Salmonella* recovered from homogenized fruits. Washing, rather than homogenizing, was used as the processing method to remove *Salmonella*, yeasts, and molds from the surface of berries treated with ClO<sub>2</sub>.

A nutrient-rich medium selective for nalidixic acid-adapted cells was used to enumerate *Salmonella* on inoculated fruits. Preliminary studies with uninoculated samples showed that the vast majority of background microflora did not grow on TSANP or XLDNP agar. The performance of TSANP was equal to or better than XLDNP agar for supporting colony development by *Salmonella* (Table 2.1). To maximize the recovery of ClO<sub>2</sub>-stressed *Salmonella*,

direct plating on TSANP was preferred over plating on XLDNP agar, which contains ingredients that may impose a secondary stress. Han *et al.* (2002) observed that surface-plating on conventional selective media resulted in poor recovery of *E. coli* O157:H7 and *L. monocytogenes* on green peppers treated with ClO<sub>2</sub>. Although there was an increase in the number of both pathogens recovered from peppers subjected to resuscitation procedures, counts were still lower than those obtained on non-selective media.

A high relative humidity (75 – 90%) was maintained in the chamber atmosphere to enhance the lethality of ClO<sub>2</sub> to *Salmonella*, yeasts, and molds (Figures 2.3 and 2.4). The lethality of gaseous ClO<sub>2</sub> to *E. coli* O157:H7 on peppers (Han *et al.*, 2001a) is known to increase as the atmospheric relative humidity is increased. Treatment of juice storage tanks with 8 mg/L ClO<sub>2</sub> has also been shown to be more effective in killing spoilage microorganisms as the relative humidity is increased from 56 to 94% (Han *et al.*, 1999).

**Treatment of blueberries.** Blueberries inoculated on the skin, calyx, or stem scar tissue with concentrations of 4.1 mg/L, 6.2 mg/L, and 8.0 mg/L within 30, 60, and 120 min, respectively, at 76 – 90% relative humidity and 22 – 25°C. Log reductions (CFU/g) of *Salmonella* resulting from these treatments are shown in Table 2.2. Compared to blueberries not treated ClO<sub>2</sub> (control), all treatments with gaseous ClO<sub>2</sub> caused significant reductions ( $\alpha = 0.05$ ) in the number of viable cells, regardless of the site on which inoculum was applied. The highest log reductions on blueberries inoculated at all three locations resulted from treatment with 8.0 mg/L ClO<sub>2</sub>, although reductions achieved using 4.1, 6.2, or 8.0 mg/L ClO<sub>2</sub> were similar. There was no significant difference between the sanitizing effect of gaseous ClO<sub>2</sub> gas on the population of *Salmonella* inoculated onto the calyx and stem scar tissue. However, there was a significantly higher log reduction ( $\alpha = 0.05$ ) on blueberries inoculated on the skin and treated

TABLE 2.2. *Recovery of Salmonella from blueberries treated with gaseous ClO<sub>2</sub>*

Inoculation Site	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>a</sup>				
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>		
Skin	0	0	A	4.94	Y		
	30	4.1	B	2.49	Y	2.45	2/2
	60	6.2	B	1.39	Y	3.55	4/5
	120	8.0	B	0.86	X	4.08	3/5
Calyx	0	0	A	5.13	Y		
	30	4.1	B	3.04	XY	2.09	
	60	6.2	B	3.45	XY	1.68	
	120	8.0	B	2.90	X	2.23	2/3
Stem scar	0	0	A	5.86	X		
	30	4.1	B	3.21	X	2.65	1/1
	60	6.2	B	3.57	X	2.29	
	120	8.0	B	2.81	X	3.05	1/2

<sup>a</sup>Populations of *Salmonella* recovered on TSANP after treatment of blueberries for 0, 30, 60, and 120 min at 23°C. The population of *Salmonella* inoculated onto blueberries was 6.51 log<sub>10</sub> CFU/g. The detection limit was 1 CFU/ml of DE wash (2 CFU/g of blueberries).

<sup>b</sup>Within inoculation site, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within the same ClO<sub>2</sub> concentration, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Within inoculation site, reduction (log<sub>10</sub> CFU/g) compared to the population recovered from blueberries receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, washed blueberry samples positive for *Salmonella*, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

with gaseous ClO<sub>2</sub> at concentrations of 4.1 mg/L or 6.2 mg/L compared to reductions on blueberries on which the inoculum had been applied to the stem scar tissue and treated with respective concentrations of ClO<sub>2</sub>. This is attributed to a larger percentage of cells on the skin being exposed to ClO<sub>2</sub> and therefore more vulnerable to its lethality compared to cells protected by stem scar tissues. Results are in agreement with observations on the higher log reductions in populations of *L. monocytogenes* on the skin (5.5 log<sub>10</sub> reduction) versus stem cavity (3.6 log<sub>10</sub> reduction) or calyx (3.2 log<sub>10</sub> reduction) of apples treated with gaseous ClO<sub>2</sub> at 4.0 mg/L for 10 min at 21°C and 90% relative humidity (Du *et al.*, 2002). Reductions were 1 – 3 logs higher than those achieved in our study using 4.1 mg/L ClO<sub>2</sub> to treat blueberries. Differences in sensitivity of various enteric pathogens as well as differences in surface structures and morphology of apples and blueberries may result in differences in the level of protection of cells against exposure to ClO<sub>2</sub>, which in turn may have affected its biocidal efficacy in these studies. Different systems were used to produce gaseous ClO<sub>2</sub> in the two studies. The sachets used in our study released 4.1 mg/L within 30 min, whereas the ClO<sub>2</sub> gas-treatment system used by Du *et al.* (2002) released 4.0 mg/L within seconds, enabling a shorter exposure time (10 min) to achieve reductions in pathogen populations greater than those we observed after treatment for 30 min.

The yeast and mold populations on blueberries were significantly reduced ( $\alpha = 0.05$ ), compared to reductions on control samples, by treating with gaseous ClO<sub>2</sub>, regardless of the site of inoculation with *Salmonella* (Table 2.3). There were no significant differences ( $\alpha = 0.05$ ) in log reductions in yeast and mold populations on blueberries inoculated with *Salmonella* at a specific site and treated with 4.1, 6.2, or 8.0 mg/L ClO<sub>2</sub>. There were also no significant differences in log reductions in yeast and mold populations on blueberries inoculated with *Salmonella* at three different sites and treated with the same concentration of ClO<sub>2</sub>. The natural

TABLE 2.3. Recovery of yeasts and molds from blueberries treated with ClO<sub>2</sub>

Inoculation <sup>a</sup> Site	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>b</sup>		
			Recovered <sup>c</sup>	Reduction <sup>d</sup>	
Skin	0	0	A 3.41	X	
	30	4.1	B 0.58	X	2.83
	60	6.2	B 0.67	X	2.74
	120	8.0	B 1.11	X	2.30
Calyx	0	0	A 3.40	X	
	30	4.1	B 2.21	X	1.19
	60	6.2	B 1.30	X	2.10
	120	8.0	B 0.90	X	2.50
Stem scar	0	0	A 3.39	X	
	30	4.1	B 0.88	X	2.50
	60	6.2	B 1.02	X	2.37
	120	8.0	B 1.34	X	2.05

<sup>a</sup>Site of which *Salmonella* was applied.

<sup>b</sup>Populations of yeasts and molds recovered on DRBC agar after treatment of blueberries for 0, 30, 60, and 120 min and 23°C. The detection limit was 1 CFU/ml of DE wash (2 CFU/g of blueberries).

<sup>c</sup>Within inoculation site, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within the same ClO<sub>2</sub> concentration, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>d</sup>Within inoculation site, reduction (log<sub>10</sub> CFU/g) compared to the population recovered from blueberries receiving no ClO<sub>2</sub> treatment (0 mg/L).

distribution and survival of yeasts and molds on the blueberries was apparently unaffected by handling or by the procedure used to inoculate with *Salmonella*.

The skin of blueberries showed a dry, slightly chalky appearance after treatment with 6.2 mg/L ClO<sub>2</sub> for 60 min at 79% relative humidity. Blueberries treated with 8.0 mg/L ClO<sub>2</sub> for 120 min at 76% relative humidity showed some pink discoloration around the calyx area. This is a result of bleaching and other biochemical changes caused by ClO<sub>2</sub> gas.

**Treatment of strawberries.** Strawberries inoculated with *Salmonella* on the skin or stem scar area were treated with gaseous ClO<sub>2</sub> at release concentrations of 4.1 mg/L, 6.0 mg/L, and 8.1 mg/L within 30, 60, and 120 min, respectively, at 85 – 88% relative humidity and 22 – 25°C. Populations of the pathogen recovered are shown in Table 2.4. Treatment with 4.1 mg/L ClO<sub>2</sub> caused significant reductions ( $\alpha = 0.05$ ) in populations of *Salmonella* of 1.90 and 2.00 log<sub>10</sub> CFU/g of strawberries inoculated on the stem scar and skin, respectively. Larger, but not significantly different reductions were caused by treatment with 6.0 mg/L and 8.1 mg/L ClO<sub>2</sub> compared to treatment with 4.1 mg/L. Unlike blueberries, the effectiveness of a given concentration of ClO<sub>2</sub> in killing *Salmonella* on strawberries was unaffected by the site of inoculation. For each treatment concentration, the number of *Salmonella* recovered from strawberries inoculated on the skin or stem scar was not significantly different. This may be due in part to similarities in porosity of skin and stem scar surfaces. This is in contrast to the protective effect afforded by the stem scar tissues in blueberries. Rogers *et al.* (2004) examined the effectiveness of aqueous ClO<sub>2</sub> in killing *E. coli* O157:H7 and *L. monocytogenes* on raw produce. Treatment of strawberries with 3 or 5 mg/L ClO<sub>2</sub> for 20 – 30 sec caused a ca. 5-log reduction in population. Initial populations of *E. coli* O157:H7 and *L. monocytogenes* of 6.1 and 5.8 log<sub>10</sub> CFU/g, respectively, were reduced to < 1 log<sub>10</sub> CFU/g within a 5-min treatment with 3 mg/L ClO<sub>2</sub>. These larger reductions, compared to those observed for *Salmonella* in our study, may have resulted from differences in sensitivity of pathogens to ClO<sub>2</sub> and to other experimental

TABLE 2.4. *Recovery of Salmonella from strawberries treated with gaseous ClO<sub>2</sub>*

Inoculation Site	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>a</sup>			
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>	
Skin	0	0	A 4.30	X		
	30	4.1	B 2.30	X	2.00	
	60	6.0	B 1.11	X	3.19	0/1
	120	8.1	B 0.63	X	3.67	0/6
Stem scar	0	0	A 4.51	X		
	30	4.1	B 2.61	X	1.90	0/1
	60	6.0	B 2.02	X	2.49	1/4
	120	8.1	B 0.18	X	4.33	1/4

<sup>a</sup>Populations of *Salmonella* recovered on TSANP after treatment of strawberries for 0, 30, 60, and 120 min at 23°C. The population of *Salmonella* inoculated onto strawberries was 6.05 log<sub>10</sub> CFU/g. The detection limit was 1 CFU/ml of DE wash (1 CFU/g of strawberries).

<sup>b</sup>Within inoculation site, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within the same ClO<sub>2</sub> concentration, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Within inoculation site, reduction (log<sub>10</sub> CFU/g) compared to the population recovered from strawberries receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, washed strawberry samples positive for *Salmonella*, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

parameters but are more likely a reflection of different methods of inoculation and removal of viable cells from strawberries in the aqueous treatment solution, thus excluding them from populations detected on berries.

The yeast and mold populations on strawberries were significantly reduced when treated with gaseous ClO<sub>2</sub> (Table 2.5). Populations recovered from strawberries treated with 4.1 mg/L,

TABLE 2.5. Recovery of yeasts and molds from strawberries treated with gaseous ClO<sub>2</sub>

Inoculation <sup>a</sup> Site	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>b</sup>	
			Recovered <sup>c</sup>	Reduction <sup>d</sup>
Skin	0	0	A 4.76	X
	30	4.1	B 3.93	X 0.83
	60	6.0	B 3.53	X 1.23
	120	8.1	B 0.71	X 4.05
Stem scar	0	0	A 4.87	X
	30	4.1	B 2.65	Y 2.22
	60	6.0	B 2.45	X 2.42
	120	8.1	B 0.79	X 4.08

<sup>a</sup>Site of which *Salmonella* was applied.

<sup>b</sup>Populations of yeasts and molds recovered on DRBC agar after treatment of strawberries for 0, 30, 60, and 120 min and 23°C. The detection limit was 1 CFU/ml of DE wash (1 CFU/g of strawberries).

<sup>c</sup>Within inoculation site, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within the same ClO<sub>2</sub> concentration, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>d</sup>Within inoculation site, reduction (log<sub>10</sub> CFU/g) compared to the population recovered from strawberries receiving no ClO<sub>2</sub> treatment (0 mg/L).

6.0 mg/L, and 8.1 mg/L gaseous ClO<sub>2</sub> were not significantly different but treatment with 8.1 mg/L caused higher reductions (4.08 and 4.05 log<sub>10</sub> CFU/g on the stem scar and skin, respectively), compared to treatment with 6.0 mg/L. The greater effectiveness of gaseous ClO<sub>2</sub> in killing yeasts and molds on strawberries, compared to blueberries, may have been caused by the slightly higher relative humidity achieved and maintained in the cabinet during treatment of strawberries (Figure 2.4), attesting to the synergistic role high relative humidity plays in



enhancing the lethality of gaseous ClO<sub>2</sub>. On the other hand, strawberries had a higher initial population of yeasts and molds (4.76 – 4.87 log<sub>10</sub> CFU/g), compared to populations on blueberries (3.39 – 3.41 log<sub>10</sub> CFU/g) or raspberries (4.09 log<sub>10</sub> CFU/g). Different genera or species of yeasts and molds on the three types of berries as well as the type, location, and number of mycelia, spores, and conidia would also be expected to vary in sensitivity to ClO<sub>2</sub>, and this would be reflected in differences in reductions in counts resulting from treatment.

**Treatment of raspberries.** Raspberries inoculated with *Salmonella* only on one location, the skin, were treated with gaseous ClO<sub>2</sub> at release concentrations of 4.1 mg/L, 6.0 mg/L, and 8.1 mg/L within 30, 60, and 120 min, respectively, at 75 – 83% relative humidity and 22 – 25°C. Reductions in populations of *Salmonella* are shown on Table 2.6. Treatment with gaseous ClO<sub>2</sub> at concentrations of 4.1 mg/L, 6.0 mg/L or 8.1 mg/L significantly reduced the number of *Salmonella*. There was no significant differences between the log reductions caused by treating raspberries with the three concentrations of ClO<sub>2</sub>. The lower reductions in populations of *Salmonella* on raspberries, compared to reductions on blueberries and strawberries treated with the same concentrations of ClO<sub>2</sub>, are attributed in part to the lower relative humidity during treatment of raspberries (Figure 2.4) compared to blueberries and strawberries (Figures 2.3 and 2.4, respectively) with ClO<sub>2</sub>. Failure to achieve a higher relative humidity may have been influenced by the high respiration rate (114 – 245 mg CO<sub>2</sub>/kg/h at 20°C) of raspberries compared to respiration rate of strawberries (102 – 196 mg CO<sub>2</sub>/kg/h at 20°C) and blueberries (52 – 87 mg CO<sub>2</sub>/kg/h at 20°C) (Ryall and Pentzer, 1982a). The evolution of higher amounts of CO<sub>2</sub> associated with the higher respiration rate of raspberries may protect the surface from contact with gaseous ClO<sub>2</sub>, thereby potentially reducing the lethality of ClO<sub>2</sub> to *Salmonella*. Reduced access of ClO<sub>2</sub> to areas between the drupelets of raspberries where part of

TABLE 2.6. Recovery of *Salmonella*, yeasts, and molds from raspberries treated with gaseous  $\text{ClO}_2$

Microorganism	Treatment time (min)	Amount of $\text{ClO}_2$ released (mg/L)	Population ( $\log_{10}$ CFU/g) <sup>a</sup>			
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>	
<i>Salmonella</i>	0	0	A	4.83		
	30	4.1	B	4.42	0.41	
	60	6.0	B	3.92	0.91	1/3
	120	8.1	B	3.47	1.36	0/3
Yeasts and molds	0	0	A	4.09		
	30	4.1	B	2.04	2.05	
	60	6.0	B	0.87	3.22	
	120	8.1	B	1.71	2.38	

<sup>a</sup>Populations of *Salmonella*, yeasts, and molds recovered on TSANP and DRBC agar after treatment of raspberries for 0, 30, 60, and 120 min at 23°C. The population of *Salmonella* inoculated onto raspberries was 6.78  $\log_{10}$  CFU/g. The detection limit was 1 CFU/ml of DE wash (2 CFU/g of raspberries).

<sup>b</sup>Mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction ( $\log_{10}$  CFU/g) compared to the population recovered from raspberries receiving no  $\text{ClO}_2$  treatment (0 mg/L).

<sup>d</sup>Number of treated, washed raspberry samples positive for *Salmonella*, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

the inoculum may have lodged would also protect *Salmonella* from exposure to  $\text{ClO}_2$ . Tissue juice released from broken trichomes may provide a site of harborage for the pathogen. Some or all of these factors may have impacted the efficacy of gaseous  $\text{ClO}_2$  in killing *Salmonella* on raspberries to a greater extent than on blueberries or strawberries. Treatment of uninjured green pepper surfaces with gaseous  $\text{ClO}_2$  at a concentration of 0.60 mg/L for 30 min at 20°C under 90

– 95% relative humidity has been reported to result in a reduction in population of *E. coli* O157:H7 of 7.27 log<sub>10</sub> CFU/5 g (Han *et al.*, 2000b). Treatment of injured surfaces (crevices created uniformly by cutting of sterile blade) with 1.2 mg/L ClO<sub>2</sub> resulted in a 6.45-log<sub>10</sub> CFU/5 g of peppers. Their study also demonstrated, through counts of CFUs and confocal laser scanning microscopy, that *E. coli* O157:H7 preferentially attached to injured surfaces on peppers, which protected it from contact with ClO<sub>2</sub>, resulting in significantly lower reductions in viable cells. Another study showed that *E. coli* O157:H7 preferentially attached to coarse and porous intact surfaces and injured surfaces of peppers (Han *et al.*, 2000a). Similar protective phenomena may have occurred on raspberries and, to a lesser extent, on blueberries and strawberries examined in our study.

Populations of yeasts and molds on raspberries treated with gaseous ClO<sub>2</sub> were significantly reduced compared to populations on control raspberries (Table 2.6). However, as with reductions in populations of *Salmonella*, differences in populations of yeasts and molds recovered from raspberries treated with 4.1 mg/L, 6.0 mg/L, or 8.1 mg/L were not significantly different ( $\alpha = 0.05$ ).

**Sensory evaluation of berries.** The efficacy of 4.1 mg/L ClO<sub>2</sub> in removing or killing pathogens met recommendations given by an EPA Scientific Advisory Panel, wherein sanitizers tested against at least five strains of outbreak-related *Salmonella* would result in a 2- log<sub>10</sub> reduction in pathogen populations as a reasonable performance standard (Federal Register, 1997). Berries were therefore treated with 4.1 mg/L ClO<sub>2</sub> to determine if sensory attributes can be maintained while simultaneously achieving an average of 2 log<sub>10</sub> CFU/g reductions. Temperatures in grocery store display areas range between 9 –12°C, while abuse temperatures at home may reach up to 10°C (Harris, 1989; Rhodhammel, 1992; Van Garde and Woodburn,

1987; Daniels, 1991). A temperature of 8°C was chosen to mimic a temperature at which fruits may be stored in retail stores and home refrigerators. According to Ryall and Pentzer (1982b), typical storage lives of blueberries, strawberries, and raspberries stored at 0°C and 90 – 95% relative humidity are ca. 2 weeks, 5 – 7 days, and 3 days, respectively. Therefore, storage time of 10 days at 8°C with evaluation sessions scheduled on days 0, 3, 7, and 10 represent appropriate storage conditions to determine the changes in sensory attributes of treated and untreated berries.

Control and treated (4.1 mg/L ClO<sub>2</sub>), uninoculated blueberries were determined by 31 untrained panelists to not be significantly different ( $\alpha = 0.05$ ) on each sampling day (Table 2.7). Untreated berries were rated between 5.38 – 6.93 for appearance, 5.75 – 6.99 for color, 5.15 – 5.44 for aroma, and 5.35 – 6.63 for overall quality during the 10-day storage period. For treated blueberries, ratings were 5.76 – 7.12, 5.96 – 7.17, 5.23 – 5.39, and 5.69 – 6.76, respectively. Specific attribute ratings for untreated and treated blueberries stored for the same length of time were not significantly different ( $\alpha = 0.05$ ). However, ratings for appearance, color, overall quality, and to a lesser extent, aroma decreased over time from the range of “like slightly” and “like moderately” (6 – 7) to “neither like nor dislike” and “like slightly” (5 – 6). This indicated that storage time affected the sensory attributes of blueberries, regardless of whether they were untreated or treated. Relative humidity of atmosphere surrounding untreated blueberries declined from 58% initially to 38% after 10 days, which may be due to removal of samples of blueberries from the bin at 3 and 7 days, thereby removing biomass that would otherwise contribute moisture to the air inside the bins over time (Figure 2.5). The atmospheric relative humidity surrounding treated blueberries declined from 48% to 38% during the 10-day storage period (Figure 2.5). The initial atmospheric relative humidity surrounding treated blueberries

Table 2.7. Mean hedonic ratings for sensory attributes of uninoculated blueberries exposed to 4.1 mg/L gaseous ClO<sub>2</sub>

Storage time (days)	Treatment (mg/L)	Attribute <sup>a</sup>			
		Appearance	Color	Aroma	Overall
0	0	A 6.93 X	A 6.99 X	A 5.44 X	A 6.63 X
	4.1	A 7.12 X	A 7.17 X	A 5.39 X	A 6.76 X
3	0	B 6.29 X	B 6.40 X	AB 5.29 X	B 6.01 X
	4.1	B 6.28 X	B 6.45 X	A 5.33 X	B 6.08 X
7	0	C 5.55 X	C 5.99 X	AB 5.25 X	C 5.54 X
	4.1	C 5.83 X	BC 6.10 X	A 5.23 X	B 5.69 X
10	0	C 5.38 X	C 5.75 X	B 5.15 X	C 5.35 X
	4.1	C 5.76 X	C 5.96 X	A 5.28 X	B 5.69 X

<sup>a</sup>Ratings were assigned by panelists using a 9-point hedonic scale with 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. Within treatment and attribute, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within the same storage time and attribute, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

was lower than that of untreated blueberries, possibly resulting from physiological changes in blueberries caused by ClO<sub>2</sub>.

The appearance, color, aroma, and overall ratings were not significantly different ( $\alpha = 0.05$ ) for untreated and treated (4.1 mg/L ClO<sub>2</sub>) strawberries on a given storage day (Table 2.8). Bleached spots on treated strawberries noted on day 0 were not as evident on days 3, 7, and 10. Storage time affected sensory attributes of treated and control strawberries significantly ( $\alpha = 0.05$ ), as shown by significant decreases in ratings on each successive day of analysis. Ratings show that the shelf life of strawberries, in general, is much shorter than blueberries. The

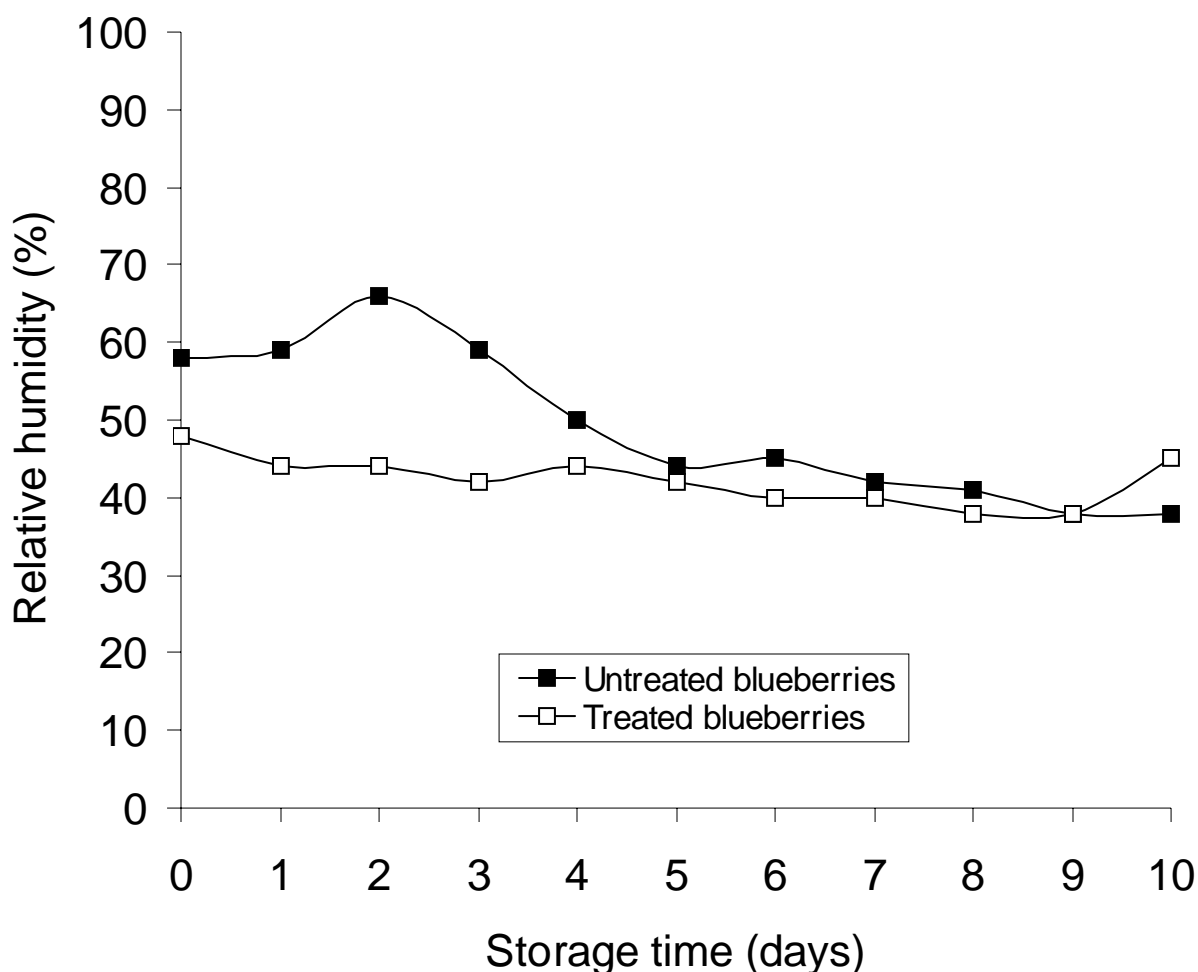


Figure 2.5. Atmospheric relative humidity in the plastic bin used to store blueberries at 8°C.

presence of gray mold, possibly *Botrytis cinerea*, on untreated strawberries on days 7 and 10 as well as on treated strawberries on day 10 indicates that storage of strawberries at 8°C does not prevent deterioration of the fruit. A higher water content of strawberries (89.9%) compared to that of raspberries (84.1%), and blueberries (82.3%) (Ryall and Pentzer, 1982b), may enhance the growth of molds on strawberries and may explain the low incidence of molds on blueberries and raspberries. Treated strawberries were less susceptible than untreated strawberries to mold

Table 2.8. Mean hedonic ratings for sensory attributes of uninoculated strawberries exposed to 4.1 mg/L gaseous ClO<sub>2</sub>

Storage time (days)	Treatment (mg/L)	Attribute <sup>a</sup>			
		Appearance	Color	Aroma	Overall
0	0	A 7.15 X	A 7.27 X	A 7.51 X	A 7.11 X
	4.1	A 6.46 Y	A 6.60 Y	A 6.89 Y	A 6.35 Y
3	0	B 5.03 X	B 5.74 X	B 6.94 X	B 5.58 X
	4.1	B 5.40 X	B 5.56 X	A 6.62 X	B 5.55 X
7	0	C 4.17 X	C 4.68 X	B 6.56 X	C 4.51 X
	4.1	C 4.59 X	C 5.01 X	A 6.52 X	C 4.82 X
10	0	D 2.06 X	D 2.32 X	C 3.75 X	D 2.18 X
	4.1	D 3.21 X	D 3.63 X	B 5.81 X	D 3.57 X

<sup>a</sup>Ratings were assigned by panelists using a 9-point hedonic scale with 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. Within treatment and attribute, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within same storage time and attribute, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

growth and had higher ratings for sensory attributes on day 7 and 10, possibly partly due to a lower moisture content in treated strawberries in comparison to untreated strawberries, as reflected by initial relative humidity (Figure 2.6) and also to the fungicidal effect ClO<sub>2</sub> has on the treated strawberries. Overall, sensory attribute ratings for untreated strawberries declined from “like moderately” to “dislike very much” (ca. 7 to 2), while ratings for treated strawberries declined from “like slightly” to “dislike moderately” (ca. 6 to 3), which indicates that although initially treated strawberries had significantly lower ratings than untreated strawberries, their shelf life is slightly longer.

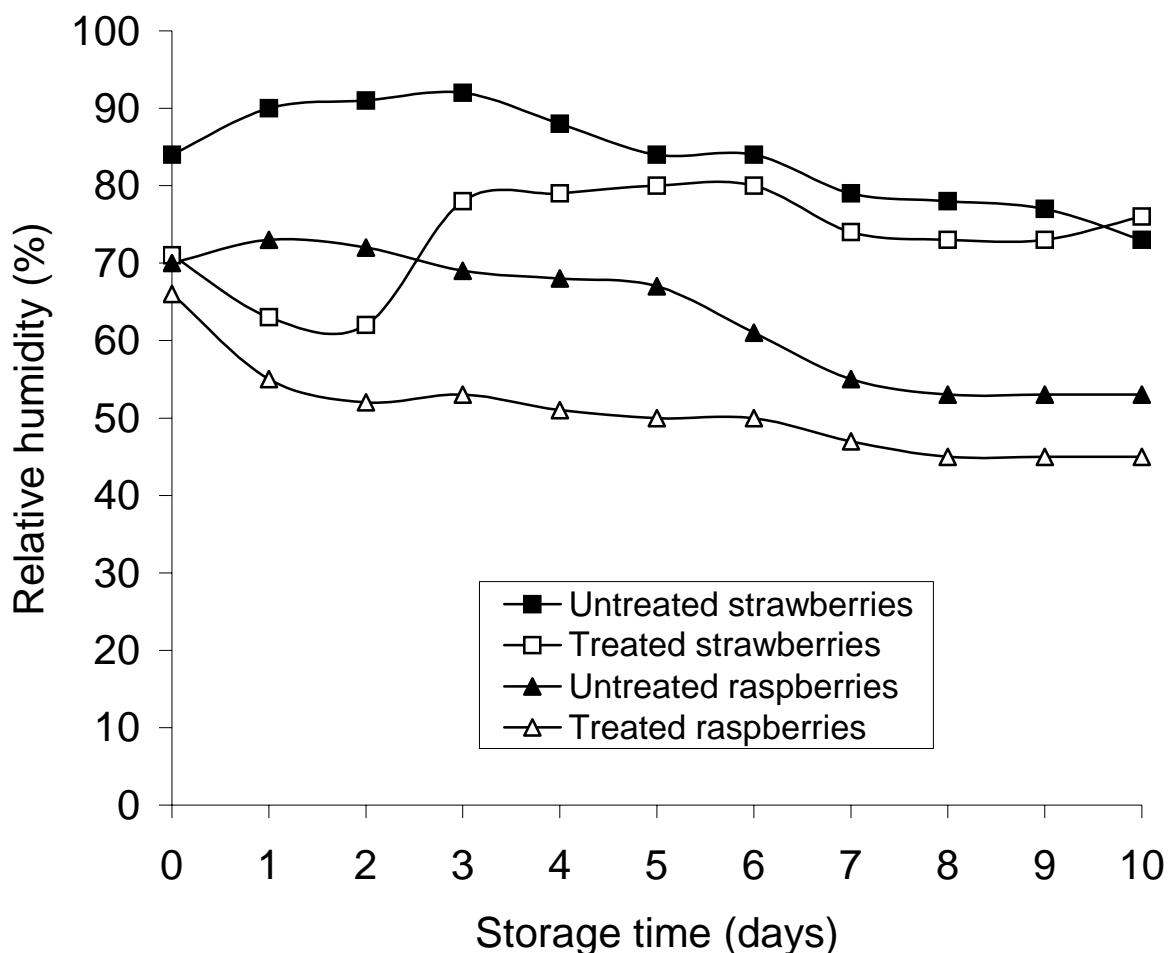


Figure 2.6. Atmospheric relative humidity in plastic bins used to store strawberries and raspberries at 8°C.

Sensory attributes of untreated and treated (4.1 mg/L) raspberries stored for 3, 7, or 10 days were not significantly different ( $\alpha = 0.05$ ) (Table 2.9). Ratings generally decreased significantly between 0 and 3 days and again between 7 and 10 days, regardless of treatment. The overall quality of untreated and treated raspberries on day 0 was rated as “liked slightly” or “like moderately” (6 to 7) by the panelists. On day 3, untreated and treated raspberries were



Table 2.9. Mean hedonic ratings for sensory attributes of uninoculated raspberries exposed to 4.1 mg/L of gaseous ClO<sub>2</sub>

Storage time (days)	Treatment (mg/L)	Attribute <sup>a</sup>			
		Appearance	Color	Aroma	Overall
0	0	A 6.91 X	A 6.88 X	A 6.00 X	A 6.66 X
	4.1	A 6.50 Y	A 6.61 X	A 6.21 X	A 6.35 Y
3	0	B 5.86 X	B 5.88 X	B 5.50 Y	B 5.58 X
	4.1	B 5.97 X	B 6.10 X	A 5.94 X	B 5.88 X
7	0	C 4.86 X	C 4.83 X	B 5.44 X	C 4.73 X
	4.1	C 4.63 X	C 4.51 X	B 5.37 X	C 4.48 X
10	0	C 4.50 X	C 4.54 X	B 5.57 X	C 4.49 X
	4.1	C 4.19 X	C 4.24 X	C 5.01 X	C 4.11 X

<sup>a</sup>Ratings were assigned by panelists using a 9-point hedonic scale with 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. Within treatment and attribute, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ). Within same storage time and attribute, mean values not followed by the same letter are significantly different ( $\alpha = 0.05$ ).

“neither liked nor disliked” or “liked slightly” (5 – 6), while panelists at day 7 and 10 were either neutral or disliked both the untreated and treated raspberries slightly (4 – 5). The shelf life of raspberries is limited (Ryall and Pentzer, 1982b), owing in part to its high respiration rate. The decline in sensory quality of raspberries within 10 days was therefore expected. The panelists did not distinguish between untreated and treated raspberries, except for certain attributes, appearance and overall quality on day 0. This indicates that treatment with 4.1 mg/L ClO<sub>2</sub> gas does not adversely affect the quality of treated raspberries compared to untreated raspberries. The relative humidity in the bin containing untreated raspberries was higher (53% –70%)

compared to that in the bin containing treated raspberries (45% – 66%) (Figure 2.6), a trend similar to that noted for blueberries and strawberries.

In summary, gaseous ClO<sub>2</sub> shows promise as a sanitizer for small fruits. Significant reductions in *Salmonella* populations of 1.7 – 4.1, 1.9 – 4.3, and 0.4 – 1.4 log<sub>10</sub> CFU/g on blueberries, strawberries, and raspberries, respectively, were achieved by treatment with 4.1 – 8.1 mg/L ClO<sub>2</sub> at elevated relative humidity. Treatment also reduced the yeast and mold populations by 1.2 – 2.8, 0.8 – 4.1, and 2.1 – 3.2 log<sub>10</sub> CFU/g of blueberries, strawberries, and raspberries, respectively. Sensory evaluations of appearance, color, and aroma of blueberries treated with gaseous ClO<sub>2</sub> were indistinguishable from respective attributes of untreated blueberries. Attributes of treated strawberries and raspberries were significantly lower than those of respective untreated fruits on day 0 only. Overall, reductions of *Salmonella* population without compromising or enhancing sensory quality of berries were achieved by treatment with gaseous ClO<sub>2</sub>. Research conducted on a larger scale is needed, however, to determine the effectiveness of application of gaseous ClO<sub>2</sub> on a commercial level.

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## CHAPTER 3

### EFFICACY OF GASEOUS CHLORINE DIOXIDE AS A SANITIZER FOR KILLING *SALMONELLA*, *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, AND YEASTS AND MOLDS ON FRESH AND FRESH-CUT PRODUCE

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## ABSTRACT

A study was done to determine the effectiveness of gaseous chlorine dioxide (ClO<sub>2</sub>) for its effectiveness in killing *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* on fresh and fresh-cut vegetables and *Salmonella*, yeasts, and molds on apples, tomatoes, onions, and peaches. Inoculum (100 µl, ca. 5.81 log<sub>10</sub> CFU) containing five serotypes of *S. enterica*, five strains of *E. coli* O157:H7, or five strains of *L. monocytogenes* was deposited on the skin surface of fresh-cut cabbage, carrot, or lettuce, dried for 30 min at 22°C, and held for 20 h at 4°C, followed by 30 min at 22°C before treatment. The skin surface of fresh, uncut produce was inoculated with 100 µl of cell suspension (ca. 7.00 log<sub>10</sub> CFU) containing five serotypes of *Salmonella* and dried for 20 – 22 h at 22°C before treatment. Sachets containing reactant chemicals were formulated to produce gaseous ClO<sub>2</sub> at concentrations of 1.4, 2.7, and 4.1 mg/L of air within 5.4 – 10.5, 10.4 – 20, and 20.5 – 30.8 min, respectively, at 21 – 25°C. Treatment with 4.1 mg of ClO<sub>2</sub>/L significantly ( $\alpha = 0.05$ ) reduced the population of pathogens on all produce. Reductions in populations of pathogens treated with 4.1 mg/L ClO<sub>2</sub> were 3.13 – 4.42 log<sub>10</sub> CFU/g of fresh-cut cabbage, 5.16 – 5.88 log<sub>10</sub> CFU/g of fresh-cut carrots, 1.53 – 1.58 log<sub>10</sub> CFU/g of fresh-cut lettuce, 4.21 log<sub>10</sub> CFU/apple, 4.33 log<sub>10</sub> CFU/tomato, 1.94 log<sub>10</sub> CFU/ onion, and 3.23 log<sub>10</sub> CFU/peach. The highest reductions in yeast and mold populations were 1.68 log<sub>10</sub> CFU/apple and 2.65 log<sub>10</sub> CFU/peach after treatment with 4.1 mg/L ClO<sub>2</sub>. Populations of yeasts and molds on tomatoes and onions were not significantly reduced by treatment with 4.1 mg/L ClO<sub>2</sub>. This may reflect the presence on these vegetables of genera with higher resistance to ClO<sub>2</sub>. Overall, results indicate that gaseous ClO<sub>2</sub> has promise as a sanitizer for both fresh and fresh-cut produce.

## INTRODUCTION

Fresh fruits and vegetables have been associated with several outbreaks of human infections. Causes for an increased frequency of outbreaks include changes in handling and production practices, increased consumption of fresh and lightly processed produce, including fresh-cut salad mixes, and increased importation of produce from countries where standard practices used to grow and handle produce may be compromised (Beuchat, 1998). Outbreaks of infections caused by bacteria, viruses, and parasites have been associated with or confirmed to have been caused by consumption of a wide range of fresh and fresh-cut produce (Olsen, 2000; Beuchat, 1998).

Treatment of fresh and fresh-cut fruits and vegetables with sanitizers often results in reductions in populations of pathogens not exceeding 2 – 3 log<sub>10</sub> CFU/g (Beuchat, 1998). This lack of effectiveness in killing high numbers of pathogens can be partially attributed to difficulties in delivering aqueous chemical sanitizers to areas on the surface of produce on which pathogens may be lodged. Treatment with aqueous chemical solutions can result in residual moisture on fruits and vegetables, which can promote the growth of yeasts and molds, thus reducing fresh-market shelf life. Infection with molds can in turn increase the pH of produce tissues and enhance the growth of *Salmonella* (Wade and Beuchat, 2003) and *Clostridium botulinum* (Draughon *et al.*, 1988). New and alternative interventions need to be developed to further improve produce safety.

Sanitizers such as gaseous chlorine dioxide (ClO<sub>2</sub>) are being explored as alternatives to aqueous chemicals for sanitizing fresh and minimally-processed, fresh-cut fruits and vegetables. Gaseous ClO<sub>2</sub> has bactericidal activity similar to its aqueous counterpart. Application of 4.0 mg/L of gaseous ClO<sub>2</sub> gas to the skin of apples for 10 min caused a 5.5-log<sub>10</sub> reduction in the

number of *Listeria monocytogenes* (Du *et al.*, 2002). With the exceptions of gaseous ClO<sub>2</sub> (Du *et al.*, 2002) and allyl isothiocyanate (Lin *et al.*, 2000), chemical treatments have not been reported to cause 5 log<sub>10</sub> reductions of bacteria on apples (Du *et al.*, 2002). Surface-injured green peppers treated with 1.24 mg/L ClO<sub>2</sub> gas for 30 min at 22°C under a relative humidity (RH) of 90 – 95% resulted in a reduction of 6.45 log<sub>10</sub> CFU/spotted site compared to 1.5 – 1.67 log<sub>10</sub> CFU/spotted site achieved by washing injured surfaces with water (Han *et al.*, 2000). Gaseous ClO<sub>2</sub> also has potential as a sanitizer for reducing yeast and mold populations in food processing plants and on fruits and vegetables. Inoculation of epoxy-coated stainless steel strips identical to those used in juice tanks with *Eurotium*, *Penicillium*, *Candida*, and *Saccharomyces cerevisiae* at populations > 4 log<sub>10</sub> CFU/ 2.5 x 7 cm area, followed by gaseous ClO<sub>2</sub> treatment, resulted in reductions to populations below detectable limits (Han, 1999).

In addition to its bactericidal activity, gaseous ClO<sub>2</sub> has other advantages, including its ability to break down phenolic compounds and remove phenolic tastes and odors from the water, not reacting with ammonia to form mutagenic by-products, exhibiting 2.5 times the oxidation capacity of chlorine, and being less corrosive than other sanitizers such as chlorine; it is not markedly affected by pH and it has high sporicidal activity (Dychdala, 1968; Reina, 1995; Cord and Dychdala, 1993). The lethality of ClO<sub>2</sub> against microorganisms may be caused by inhibition of protein synthesis (Bernade *et al.*, 1965), changes in outer membrane permeability (Olivieri *et al.*, 1984), and respiratory damage (Berg *et al.*, 1986), although the mode of action has not been clearly defined.

The objective of this study was to evaluate gaseous ClO<sub>2</sub> for its effectiveness in killing *Salmonella enterica*, *Escherichia coli* O157:H7, and *L. monocytogenes* inoculated onto the

surface of fresh-cut cabbage, fresh-cut carrot, and fresh-cut lettuce, and its effectiveness in killing *Salmonella*, yeasts, and molds on fresh, whole apples, tomatoes, onions, and peaches.

## MATERIALS AND METHODS

**Bacteria used and maintenance of cultures.** Five-serotype or five-strain mixtures of each of the three pathogens were prepared for inoculating fresh-cut vegetables. Isolates of *Salmonella enterica* from alfalfa sprouts (serotype Agona), human feces of patients in two tomato-associated outbreaks (serotype Baildon and serotype Montevideo), orange juice (serotype Gaminara), and a cantaloupe-associated outbreak (serotype Michigan) were used. Five strains of *E. coli* O157:H7 isolated from a patient in a cider-associated outbreak (C7927), calf-feces (E0018), and patients in outbreaks associated with alfalfa sprout (F4546), lettuce (H1730), and unpasteurized apple cider (SEA 13B88) were used. Strains of *L. monocytogenes* used in the study were isolated from celery (serotype 4b, F8027), peach and plum (serotype 1/2b, F8255), corn (serotype 1/2a, F8369), a patient in a coleslaw-associated outbreak (serotype 4b, G1091), and potato (serotype 1/2a, H0222).

All serotypes of *Salmonella* and strains of *E. coli* O157:H7 were grown in tryptic soy broth (TSB) (Difco/BBL, Sparks, Md.) supplemented with nalidixic acid (Sigma, St. Louis, Mo.) (TSBN) at a concentration of 50 µg/ml at 37°C for 24 h. All *L. monocytogenes* strains were grown in brain heart infusion broth (BHI) (Difco/BBL, Sparks, Md.) supplemented with nalidixic acid (BHIN) at a concentration of 50 µg/ml at 37°C for 24 h. Cultures were combined with glycerol (85:15, v:v, culture:glycerol) and stored as stocks at -20°C until used.

**Preparation of inoculum.** Frozen cultures of *S. enterica* and *E. coli* O157:H7 were thawed and streaked onto tryptic soy agar (TSA) (pH 7.3) (BBL/Difco) supplemented with

nalidixic acid (50 µg/ml) and sodium pyruvate (100 µg/ml) (TSANP). Frozen cultures of *L. monocytogenes* were thawed and streaked onto brain heart infusion agar (BHIA) (pH 7.4) (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (100 µg/ml) (BHIANP). The TSANP plates were incubated at 37°C for 24 h and the BHIANP plates were incubated at 37°C for 48 h before picking colonies to be transferred into 10 ml of TSBN or BHIN, respectively. A minimum of two consecutive 24-h transfers was made via loop inoculum (ca. 10 µl) into 10 ml of TSBN or BHIN. Cells were harvested by centrifugation at 2000 x g for 15 min (Centra CL2 centrifuge International Equipment Co, Needham Heights, Mass.). The supernatant was decanted and cells were resuspended in 5 ml of sterile 5% (v/v) horse serum (Sigma). Horse serum (5%) was used as a carrier for cells, in an attempt to mimic the presence of organic material in inoculum that might contaminate the surface of produce. Suspensions of each serotype or strain were combined to give 20 ml of a five-serotype mixture of *S. enterica*, 20 ml of a five-strain mixture of *E. coli* O157:H7, or 20 ml of a five-strain mixture of *L. monocytogenes*, each mixture containing approximately equal populations (ca. 8 or 9 log<sub>10</sub> CFU/ml) of each serotype or strain. Populations were determined by serially diluting suspensions in sterile 0.1% peptone and surface plating duplicate 0.1-ml samples on TSANP or BHIANP. Plates were incubated at 37°C for 24 h (TSANP) or 48 h (BHIANP) before colonies were counted.

**Fresh-cut produce tested.** Cabbage (*Brassica oleracea* L. var. *capitata* L.), carrot (*Daucus carota* subsp. *sativus*), and iceberg lettuce (*Lattuca sativa* L.) were purchased at a local market in Griffin, Ga. and stored at 4°C for a maximum of 2 days before using in experiments. Prior to inoculation with *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes*, two to four outer leaves of the heads of cabbage and lettuce were removed, while the outer skin of carrots was

removed with a sterile peeler. After removal of the outer leaves of cabbage, the inner leaves were cut into pieces (ca. 4 cm x 4 mm x 2 mm and weighing ca. 0.26 g). The inner leaves of lettuce were cut into pieces (4 x 4 cm) similar in size to those commercially marketed as pre-packaged salads. Carrots were cut with a sterile knife in Julian style (ca. 5 cm x 3 mm x 2 mm and weighing ca. 0.23 g), also resembling pieces used in pre-packaged salad mixes. Fresh-cut samples (20 g) of cabbage, carrot, and lettuce were placed in single layers on plastic weigh trays (14 x 14 x 2.5 cm) in preparation for inoculation.

**Fresh produce tested.** Golden Delicious apples (*Malus pumila*), tomatoes (*Lycopersicon esculentum* L.), Vidalia cv. onions (*Allium cepa* L.), and peaches (*Prunus persica*) were purchased at a local market in Griffin, Ga. and stored at 22°C for a maximum of 1 day before using in experiments. Samples consisting of one apple (ca. 6.5 x 6.5 x 7.5 cm and weighing ca. 150 g) placed stem-up, one tomato (ca. 8 x 8 x 6 cm and weighing 215 ± 15 g) placed stem-up, one onion (ca. 8 – 8.3 x 7 – 7.7 x 5.7 – 6.5 cm and weighing 180 ± 25 g) placed root-down, and one peach (ca. 5.5 x 5.5 x 6.0 cm and weighing 103 ± 17 g) placed stem-down were positioned in separate plastic weigh trays (14 x 14 x 2.5 cm) in preparation for inoculation.

**Inoculation of produce.** Fresh-cut vegetables at 22 ± 1°C in plastic weigh trays were spot-inoculated with 100 µl of a five-serotype/strain mixture of *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* using a micropipettor. Fresh produce at 22 ± 1°C in plastic weigh trays was spot-inoculated with 100 µl of a five-serotype mixture of *Salmonella*. For fresh-cut samples, inoculum was deposited on the surface of the vegetable, and for whole produce samples, inoculum was deposited on the skin surface. To prevent inoculum from passing through the fresh-cut vegetables or running off the side of un-cut produce and to facilitate drying, small approximately equal volumes of inoculum were applied at up to ten different locations on each

sample. All produce was inoculated in a biosafety hood. The fresh-cut vegetables were dried for 30 min at  $22 \pm 2^\circ\text{C}$ , followed by storing in plastic containers at 60% relative humidity for 22 h at  $4^\circ\text{C}$ . Prior to treatment with gaseous  $\text{ClO}_2$ , the fresh-cut vegetables were placed in a biosafety hood for 30 min at  $22 \pm 2^\circ\text{C}$ . After inoculation of whole produce, samples were stored in a biosafety hood for 20 – 22 h at  $22 \pm 2^\circ\text{C}$  prior to treatment with gaseous  $\text{ClO}_2$  (Figure 3.1).

**Relative humidity.** Three samples of either each fresh-cut vegetables or each fresh, uncut produce were placed in a Fisherbrand transparent plexiglass desiccator cabinet (45.7 x 30.5 x 30.5 cm) (Figure 3.2). Samples were placed on the bottom three shelves of the four-shelf cabinet. High (62 – 98%) relative humidity was achieved by placing 20 ml of hot water (initially at  $98 - 99^\circ\text{C}$ ) in a shallow plastic dish (8.6 x 8.6 x 2.2 cm) on the bottom shelf. A Radioshack Brushless 12VDC cooling fan (6.9 x 6.9 x 2.5cm) (Radioshack, Fort Worth, Tex.) was strategically placed on each of the four shelves to circulate the air. A Fisher Scientific Thermo-Hygro recorder (model no. 11-661-13) was used to monitor relative humidity and temperature inside the treatment cabinet.

**Gaseous  $\text{ClO}_2$  treatment.** Fresh-cut vegetables (1 sample/shelf) inoculated with ca.  $6.9 \log_{10}$  CFU of *Salmonella*/g, ca.  $6.7 \log_{10}$  CFU of *E. coli* O157:H7/g, or ca.  $6.7 \log_{10}$  CFU of *L. monocytogenes*/g, as well as fresh, uncut produce (1 sample/shelf) inoculated with *Salmonella* ca.  $8.0 \log_{10}$  CFU/piece, were placed in the cabinet and treated with either air (control) or gaseous  $\text{ClO}_2$  for 0, 5.4 – 10.5, 10.4 – 20, and 20.5 – 30.8 min (Figure 3.2). Sachets consisting of two compartments containing reactant chemicals, in the form of a granular porous solid impregnated with sodium chlorite, and an activator, a granular porous solid impregnated with an acid, ferric chloride were supplied by ICA TriNova, Inc. (Marietta, Ga.). Breakage of the septum between the two compartments, followed by mixing the chemicals initiated the release of



Figure 3.1. *Inoculated peaches drying under a biosafety hood for 20 – 22 h at  $22 \pm 2$  °C prior to treatment with gaseous  $ClO_2$ .*

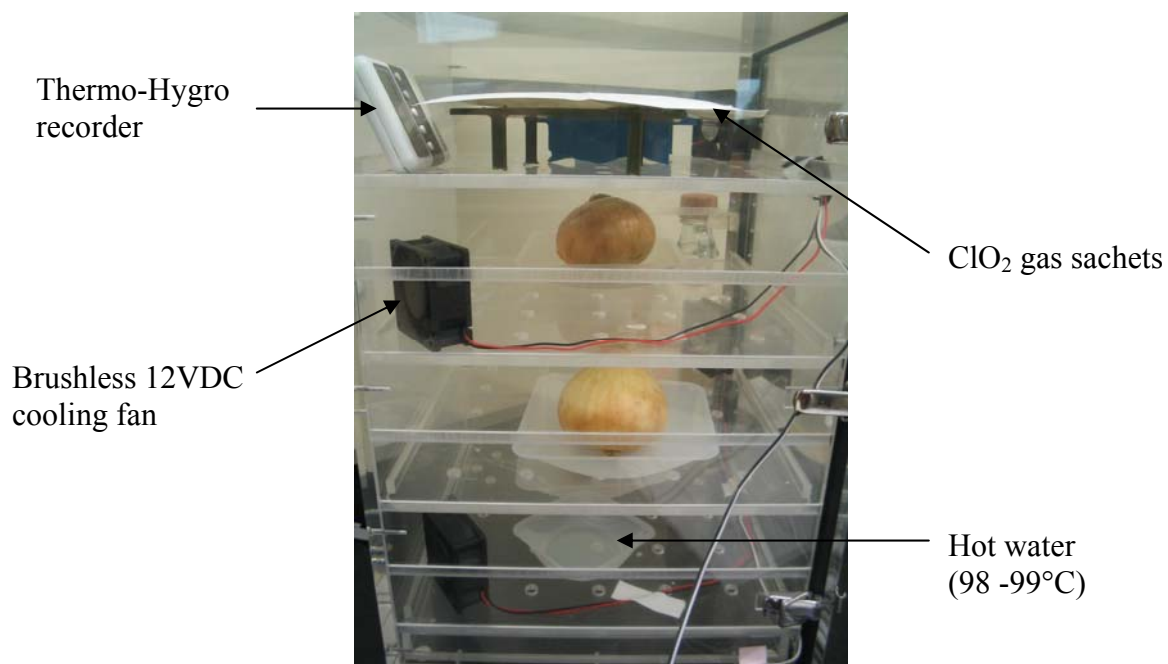


Figure 3.2. *Three samples of uncut onions treated with  $ClO_2$  gas in a plexiglass desiccator cabinet*



ClO<sub>2</sub> gas. The mixture of chemicals in three sachets was formulated to release gaseous ClO<sub>2</sub> into the cabinet (31.1 L) at concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L in 5.4 – 10.5, 10.4 – 20, and 20.5 – 30.8 min, respectively, at 22 – 25°C. The gaseous ClO<sub>2</sub> concentrations released into the cabinet can also be defined as ppmv, since a gas phase concentration of 1 mg/L is equivalent to 362 ppmv. Therefore, an alternative way to report the concentrations of gaseous ClO<sub>2</sub> released would be 507 ppmv, 977 ppmv, and 1,484 ppmv within 5.4 – 10.5, 10.4 – 20, and 20 – 30.8 min, respectively.

The amounts of ClO<sub>2</sub> released, as measured by mg/L versus time profiles (Figures 3.3, 3.4, 3.5, 3.6, and 3.7) were quantified through a series of titrations of potassium iodide (KI) buffer. Procedures and a description of chemical reactions are given in the Standard Methods (Anon, 1975; Aieta *et al.*, 1984). Immediately following placement of the produce samples on the bottom three shelves, hot water (20 ml) was placed on the bottom shelf and three sachets containing the reactant chemicals were simultaneously placed on an elevated mesh platform on the top shelf to deliver levels of relative humidity and gaseous ClO<sub>2</sub>. The control samples were handled in an identical manner, except ClO<sub>2</sub> sachets were not placed in the cabinet. Closing and securing the door to which a rubber gasket was affixed sealed the cabinet. This created a ClO<sub>2</sub> gas-sanitizing environment for the test produce.

**Microbiological analyses.** Uninoculated, inoculated, and untreated samples of fresh or fresh-cut produce not exposed to air or gaseous ClO<sub>2</sub> treatment in the cabinet, as well as samples held for up to 30.8 min in air or gaseous ClO<sub>2</sub> in the cabinet, were analyzed for populations of *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, or yeasts and molds.

Untreated and treated fresh-cut vegetable samples were placed in separate Stomacher 400 bags (Seward Medical Ltd., London, U.K.) and 100 ml of Dey-Engley (DE) broth (pH 7.6)

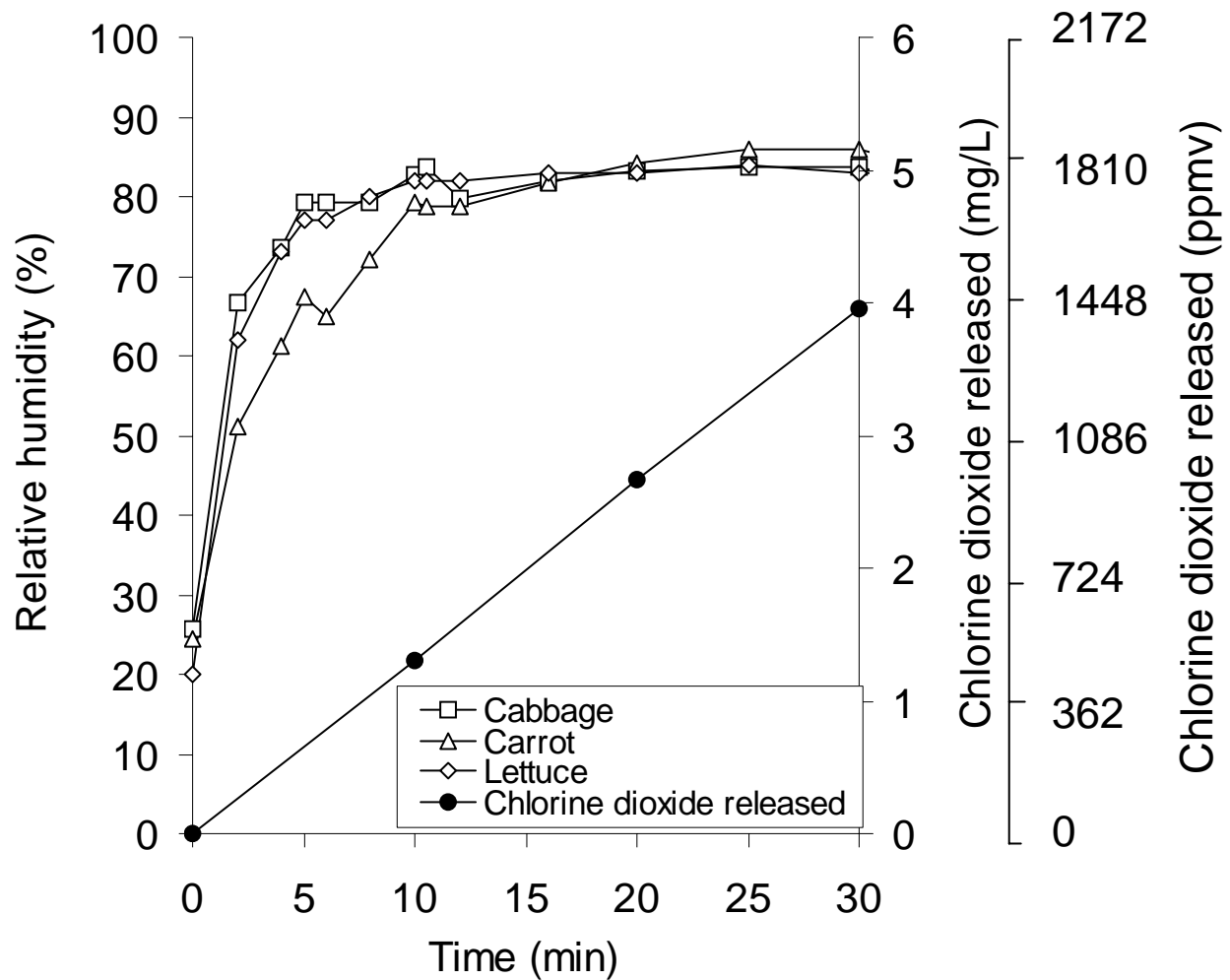


Figure 3.3. Atmospheric relative humidity in the cabinet used to treat fresh-cut cabbage, carrot, and lettuce inoculated with Salmonella, and amount of chlorine dioxide released into the atmosphere during treatment.

(BBL/Difco) was added to each bag. Samples were pummeled at normal speed for 1 min in a Stomacher 400 blender. Untreated and treated apples, tomatoes, onions, and peaches were individually placed in quart-size Ziploc® bags and 20 ml of DE broth were added. Samples were gently hand rubbed in DE broth for 1 min.

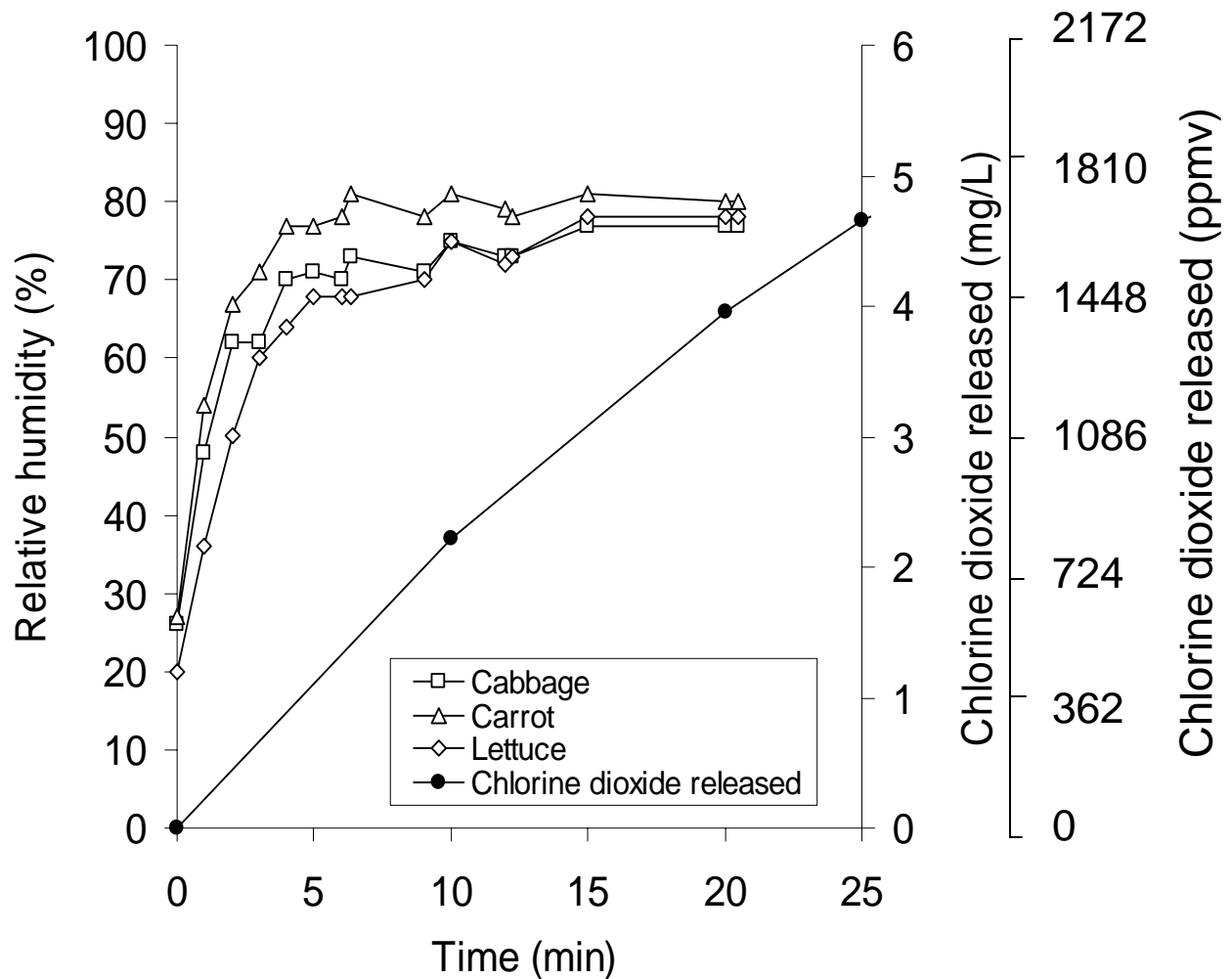


Figure 3.4. Atmospheric relative humidity in the cabinet used to treat fresh-cut cabbage, carrot, and lettuce inoculated with *E. coli* O157:H7, respectively, and amount of chlorine dioxide released into the atmosphere during treatment.

Undiluted DE broth from homogenates of fresh-cut vegetables or DE wash from uncut produce (0.25 ml in quadruplicate and 0.1 ml in duplicate), as well as samples (0.1 ml in duplicate) serially diluted in sterile 0.1% peptone, were surface plated on TSANP to enumerate *Salmonella* or *E. coli* O157:H7, on BHIANP to enumerate *L. monocytogenes*, and on dichloran rose bengal chloramphenicol (DRBC) agar (pH 5.6) (BBL/Difco) to enumerate yeasts and molds.

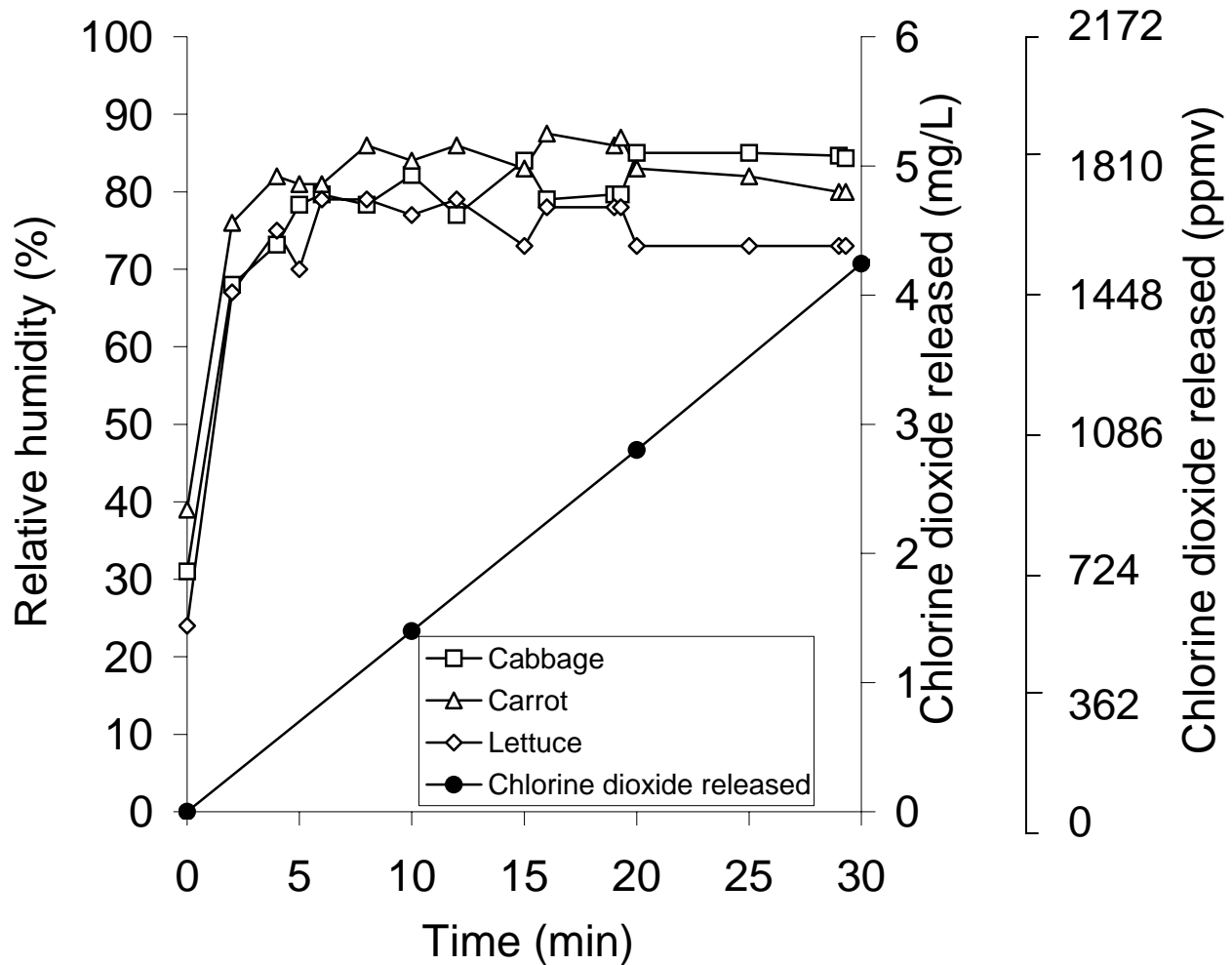


Figure 3.5. Atmospheric relative humidity in the cabinet used to treat fresh-cut cabbage, carrot, and lettuce inoculated with *L. monocytogenes*, respectively, and amount of chlorine dioxide released into the atmosphere during treatment.

The TSANP agar plates were incubated at 37°C for 24 h before presumptive-positive *Salmonella* or *E. coli* O157:H7 colonies were counted. Five to ten presumptive-positive *Salmonella* colonies from each sample were randomly selected for confirmation using a *Salmonella* latex test (Oxoid, Basingstoke, U.K.), lysine iron agar (LIA, pH 6.7) (BBL/Difco), and triple sugar iron (TSI) agar

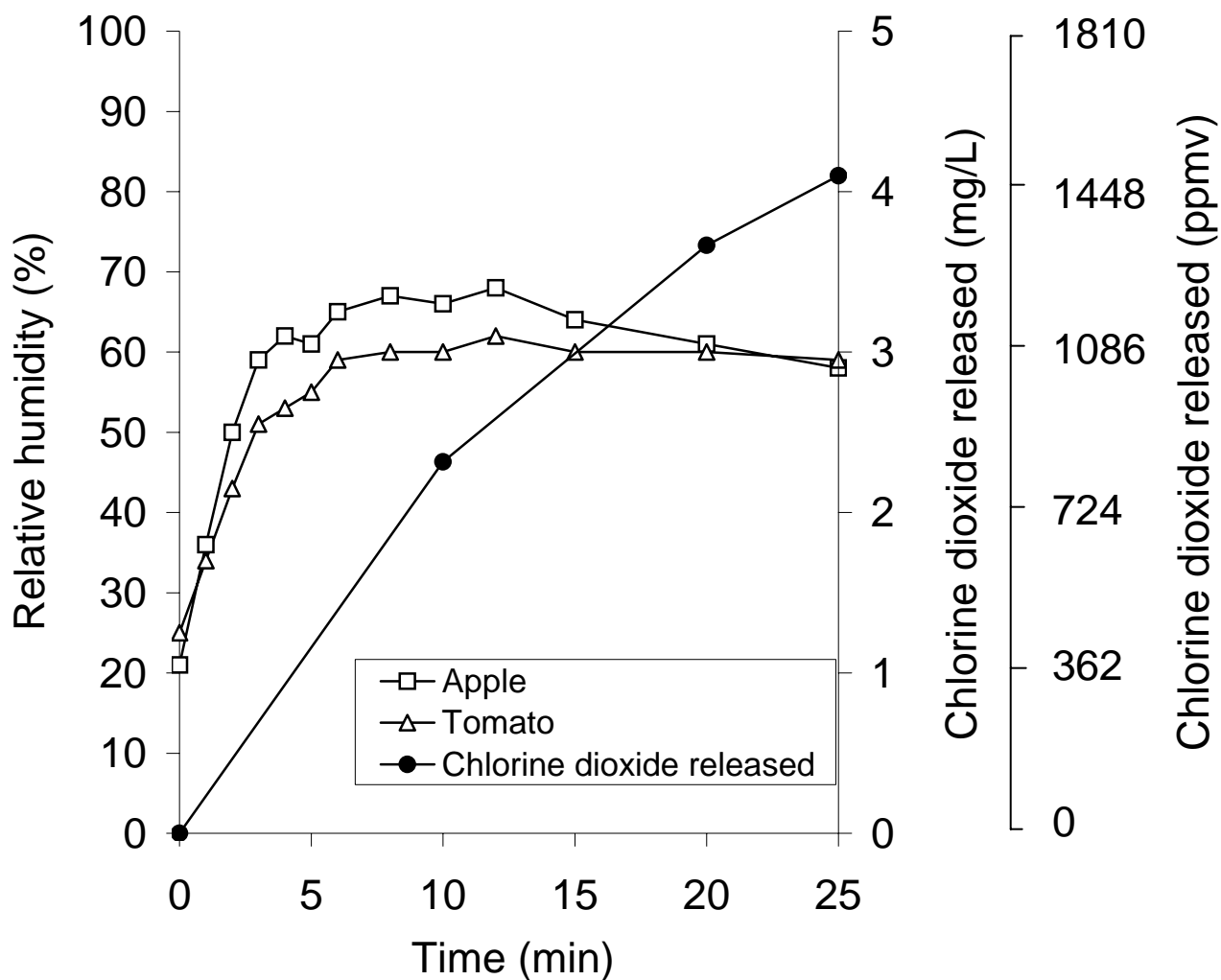


Figure 3.6. Atmospheric relative humidity in the cabinet used to treat whole apples and tomatoes, respectively, and amount of chlorine dioxide released into the atmosphere during treatment.

(BBL/Difco). Five to ten presumptive-positive *E. coli* O157:H7 colonies were randomly selected for confirmation using an *E. coli* O15:H7 latex test (Oxoid, Basingstoke, U.K.). The BHIANP plates were incubated at 37°C for 48 h before presumptive-positive *L. monocytogenes* colonies were counted. Five to ten presumptive-positive *L. monocytogenes* colonies from each

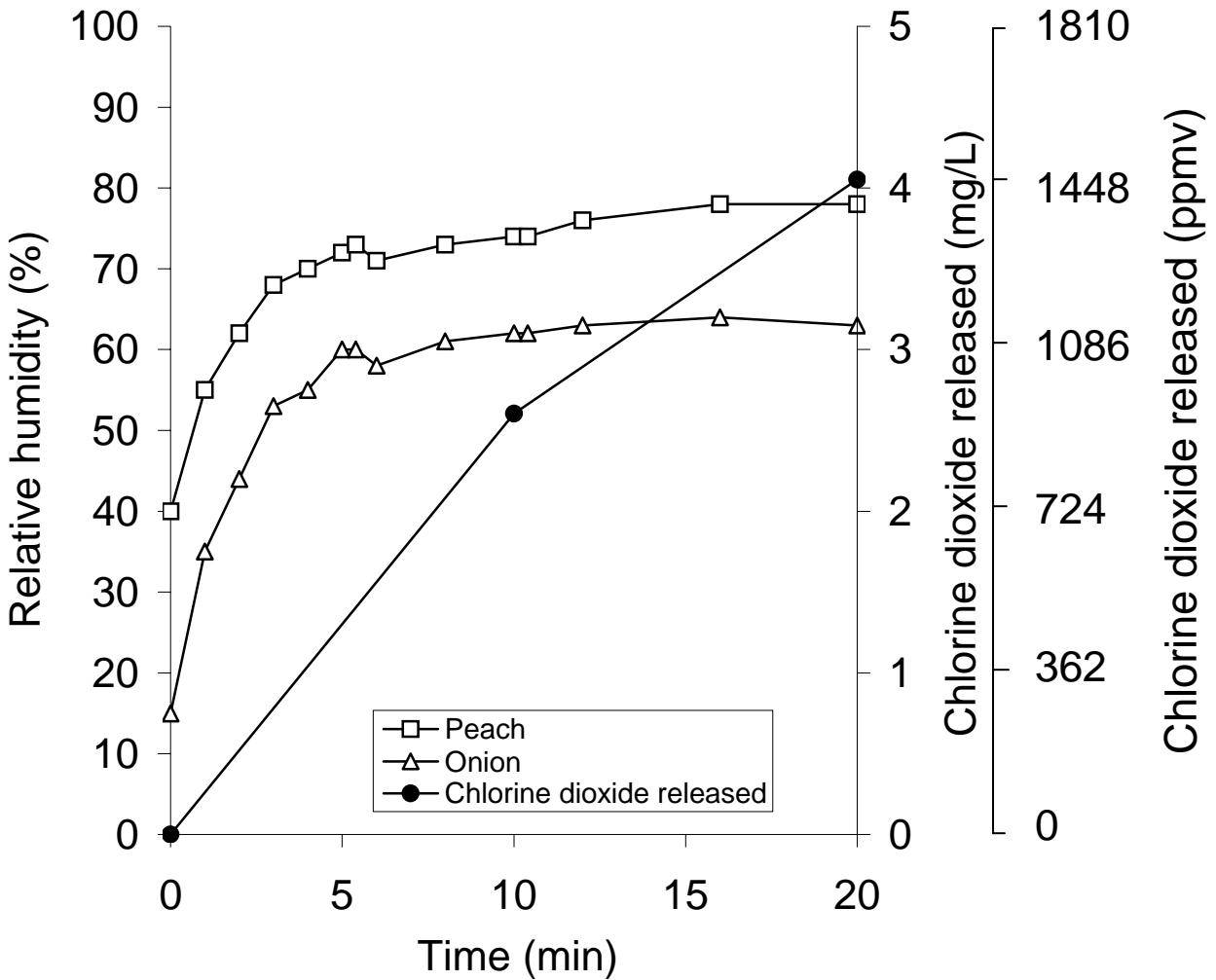


Figure 3.7. Atmospheric relative humidity in the cabinet used to treat whole onions and peaches, respectively, and amount of chlorine dioxide released into the atmosphere during treatment.

sample were randomly selected for confirmation using API *Listeria* diagnostic kit (BioMerieux Vitek Inc., Hazelwood, Missouri, U.S.A.). The DRBC agar plates were incubated at 25°C for 5 days before yeast and mold colonies were counted.

Following removal of homogenates from bags containing fresh-cut vegetables and DE wash broth from bags containing uncut produce for direct plating to enumerate *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, or yeasts/molds, 100 ml of 2X lactose broth supplemented

with nalidixic acid (100 µg/ml) and sodium pyruvate (200 µg/ml) (LBNP) was added to each bag containing DE wash broth and fresh-cut vegetables inoculated with *Salmonella*; 100 ml of 2X modified tryptic soy broth with cassamino acids (mTSB) (pH 7.0) supplemented with nalidixic acid (100 µg/ml) and sodium pyruvate (200 µg/ml) (mTSBNP) was added to each bag containing DE wash broth and fresh-cut vegetables inoculated with *E. coli* O157:H7; 100 ml of 2X *Listeria* enrichment broth (LEB) supplemented with nalidixic acid (20 µg/ml) and sodium pyruvate (200 µg/ml) (LEBNP) (pH. 7.3) (Oxoid) was added to each bag containing DE wash broth and fresh-cut vegetables inoculated with *L. monocytogenes*. Two-hundred milliliters of 1X LBNP was added to each bag containing fresh, uncut produce and DE wash broth. Mixtures of either fresh-cut vegetables or uncut produce with DE broth and LBNP, mTSBNP, or LEBNP were incubated at either 37°C for 24 h to enrich for *Salmonella* and *E. coli* O157:H7 or 37°C for 48 h to enrich for *L. monocytogenes*. If samples did not yield one or more colonies of *Salmonella* or *E. coli* O157:H7 on TSANP and one or more colonies of *L. monocytogenes* on BHIANP, pre-enriched mixtures were examined for the presence of pathogens. A loop (ca. 10 µl) of each DE broth/produce/LBNP mixture was streaked on xylose lysine desoxycholate (XLD) supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (100 µg/ml) (XLDNP) agar (pH 7.4) (BBL/Difco) to analyze for *Salmonella*, whereas a loop (ca. 10 µl) of each DE broth/produce/mTSBNP mixture was streaked on sorbitol MacConkey agar (SMAC) supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (100 µg/ml) (SMACNP, pH 7.1) (BBL/Difco) for *E. coli* O157:H7. To analyze for the presence of *L. monocytogenes*, a loop (ca. 10 µl) of DE broth/produce/LEBNP mixture was streaked on modified Oxford (MOX) agar supplemented with nalidixic acid (50 µg/ml) and sodium pyruvate (100 µg/ml) (MOXNP, pH 7.0) (Oxoid). Plates were incubated at 37°C for 24 h before examining for the presence of

presumptive *Salmonella* or *E. coli* O157:H7 colonies or at 37°C for 48 h before examining for presumptive *L. monocytogenes* colonies. In addition, 0.1-ml samples of the pre-enriched mixture in LBNP were inoculated into 10 ml of Rappaport-Vassiliadas (RV) enrichment broth (pH 5.1) (BBL/Difco). The RV broth was incubated at 42°C for 24 h and streaked on XLDNP agar if samples of pre-enriched mixtures did not yield *Salmonella* colonies on the XLDNP agar plates. XLDNP agar on which enriched samples were streaked was incubated at 37°C for 24 h before examining for presumptive *Salmonella* colonies, followed by confirmation.

**Statistical analyses.** All experiments were replicated three times and each replicate experiment consisted of three samples exposed to the same treatment conditions. Mean values were analyzed to determine significant differences ( $\alpha = 0.05$ ) in populations of *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, or yeasts/molds on produce subjected to various treatment concentrations. Data were subjected to SAS (Statistical Analysis Systems Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests.

## RESULTS AND DISCUSSION

**Treatment of fresh-cut cabbage.** Coleslaw or fresh-cut cabbage inoculated on the exposed surface with *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* was treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L within 6.4 – 10.5, 12.3 – 20, and 20.5 – 30.75 min, respectively, at 48 – 85% relative humidity and 21 – 25°C (Figures 3.3, 3.4, and 3.5). Log reductions (CFU/g) of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* resulting from these treatments are shown in Tables 3.1, 3.2, and 3.3, respectively. Compared to cabbage treated with air (control), all treatments with gaseous ClO<sub>2</sub> caused significant reductions ( $\alpha = 0.05$ ) in the number of viable cells of test pathogens. The



Table 3.1. Recovery of *Salmonella* from fresh-cut cabbage, carrot, and lettuce treated with gaseous ClO<sub>2</sub>

Fresh-cut Vegetable	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>a</sup>		
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>
Cabbage	0	0	A	6.85	
	10.5	1.4	B	5.61	1.24
	20	2.7	B	4.96	1.89
	30.8	4.1	B	2.43	4.42
Carrot	0	0	A	7.00	
	10.5	1.4	B	4.95	2.15
	20	2.7	B	3.99	3.11
	30.8	4.1	B	1.95	5.15
Lettuce	0	0	A	6.81	
	10.5	1.4	B	5.68	1.14
	20	2.7	B	5.58	1.21
	30.8	4.1	B	5.26	1.58

<sup>a</sup>Populations of *Salmonella* recovered on TSANP after treatment of cabbage, carrot, and lettuce with 0, 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 10.5, 20, and 30.8 min, respectively, at 21 – 25°C. Populations of *Salmonella* inoculated onto cabbage, carrot, and lettuce were 6.96 log<sub>10</sub> CFU/g, 7.00 log<sub>10</sub> CFU/g, and 6.86 log<sub>10</sub> CFU/g, respectively. The detection limit was 1 CFU/ml of DE wash (5 CFU/g of cabbage, carrot, or lettuce).

<sup>b</sup>Within each vegetable, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction (log<sub>10</sub> CFU/g) compared to the population recovered from cabbage, carrot, or lettuce receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, homogenized fresh-cut vegetable samples positive for *Salmonella*, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

Table 3.2. Recovery of *E. coli* O157:H7 from fresh-cut cabbage, carrot, and lettuce treated with gaseous ClO<sub>2</sub>

Fresh-cut Vegetable	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>a</sup>			
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>	
Cabbage	0	0	A	6.78		
	6.4	1.4	B	5.25	1.53	
	12.3	2.7	B	4.10	2.68	
	20.5	4.1	B	3.65	3.13	1/1
Carrot	0	0	A	6.81		
	6.4	1.4	B	4.78	2.03	
	12.3	2.7	B	3.63	3.18	2/2
	20.5	4.1	B	1.19	5.62	8/8
Lettuce	0	0	A	6.73		
	6.4	1.4	B	6.09	0.64	
	12.3	2.7	B	6.01	0.72	
	20.5	4.1	C	5.16	1.57	4/4

<sup>a</sup>Populations of *E. coli* O157:H7 recovered on TSANP after treatment of cabbage, carrot, and lettuce with 0, 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 10, 20, and 30 min, respectively, at 21 – 25°C. Populations of *E. coli* O157:H7 inoculated onto cabbage, carrot, and lettuce were 6.72 log<sub>10</sub> CFU/g, 6.72 log<sub>10</sub> CFU/g, and 6.64 log<sub>10</sub> CFU/g, respectively. The detection limit was 1 CFU/ml of DE wash (5 CFU/g of cabbage, carrot, or lettuce).

<sup>b</sup>Within each vegetable, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction (log<sub>10</sub> CFU/g) compared to the population recovered from cabbage, carrot, or lettuce receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, homogenized fresh-cut vegetable samples positive for *E. coli* O157:H7, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *E. coli* O157:H7 was recovered by direct plating were not analyzed by enrichment.

Table 3.3. Recovery of *L. monocytogenes* from fresh-cut cabbage, carrot, and lettuce treated with gaseous ClO<sub>2</sub>

Fresh-cut Vegetable	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/g) <sup>a</sup>			
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>	
Cabbage	0	0	A	6.71		
	10	1.4	B	4.95	1.76	
	19.3	2.7	B	3.40	3.31	1/1
	29.3	4.1	B	3.11	3.60	3/3
Carrot	0	0	A	6.48		
	10	1.4	B	3.20	3.28	1/1
	19.3	2.7	B	1.13	5.35	4/8
	29.3	4.1	B	0.60	5.88	1/9
Lettuce	0	0	A	6.41		
	10	1.4	B	5.60	0.81	
	19.3	2.7	B	5.18	1.23	1/1
	29.3	4.1	B	4.88	1.53	0/2

<sup>a</sup>Populations of *L. monocytogenes* recovered on BHIANP after treatment of cabbage, carrot, and lettuce with 0, 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 10, 20, and 30 min, respectively, at 21 – 25°C. Populations of *L. monocytogenes* inoculated onto cabbage, carrot, and lettuce were 6.75 log<sub>10</sub> CFU/g, 6.80 log<sub>10</sub> CFU/g, and 6.69 log<sub>10</sub> CFU/g, respectively. The detection limit was 1 CFU/ml of DE wash (5 CFU/g of cabbage, carrot, or lettuce).

<sup>b</sup>Within each vegetable, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction (log<sub>10</sub> CFU/g) compared to the population recovered from cabbage, carrot, or lettuce receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, homogenized fresh-cut vegetable samples positive for *L. monocytogenes*, as detected by enrichment (En), out of the number of samples analyzed. Samples on which *L. monocytogenes* was recovered by direct plating were not analyzed by enrichment.

highest log reductions on fresh-cut cabbage for *Salmonella* (4.42 log<sub>10</sub> CFU/g), *E. coli* O157:H7 (3.13 log<sub>10</sub> CFU/g), and *L. monocytogenes* (3.60 log<sub>10</sub> CFU/g) resulted from treatment with 4.1 mg/L ClO<sub>2</sub>, although reductions achieved using 1.4, 2.7, or 4.1 mg/L ClO<sub>2</sub> were similar within each pathogen. Compared to log reductions achieved by treatment of fresh-cut carrots, log reductions in fresh-cut cabbage were 1 – 2 logs lower. This may be attributable in part to the lower overall relative humidity in the chamber during treatment of cabbage compared to the relative humidity during treatment of carrots, which would retard the lethality of ClO<sub>2</sub>.

The fresh-cut cabbage showed very slight browning in appearance after treatment with 2.7 mg/L gaseous ClO<sub>2</sub> for 12.3 – 20 min at 73 – 83% relative humidity. Cabbage treated with 4.1 mg/L ClO<sub>2</sub> for 20.5 – 30.8 min at 77 – 84% relative humidity showed more brown discoloration, a condition resulting from biochemical reactions induced by ClO<sub>2</sub>. Objective evaluations of the sensory quality of cabbage treated with ClO<sub>2</sub> are warranted to determine consumer acceptability.

**Treatment of fresh-cut carrots.** Julian style, cut carrots inoculated with *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L within 6.4 – 10.5, 12.3 – 20, and 20.5 – 30.8 min, respectively, at 51 – 88% relative humidity and 21 – 25°C (Figures 3.3, 3.4, and 3.5). Populations of the three pathogens recovered from untreated and treated fresh-cut carrots are shown in Tables 3.1, 3.2, and 3.3. Inoculated fresh-cut carrots treated with 1.4 mg/L ClO<sub>2</sub> showed significant reductions ( $\alpha = 0.05$ ) in populations of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* of 2.14, 2.03, and 3.28 log<sub>10</sub> CFU/g, respectively. Larger but not significantly different reductions were caused by treatment with 2.7 mg/L and 4.1 mg/L ClO<sub>2</sub> compared to treatment with 1.4 mg/L. The highest reductions in population (5.15 log<sub>10</sub> CFU/g for

*Salmonella*, 5.62 log<sub>10</sub> CFU/g for *E. coli* O157:H7, and 5.88 log<sub>10</sub> CFU/g for *L. monocytogenes*) resulted from treatment of fresh-cut carrots with 4.1 mg/L ClO<sub>2</sub>.

The apparent effectiveness of ClO<sub>2</sub> gas in killing all three pathogens on fresh-cut carrots may in part reflect an additive or synergistic effect caused by natural antimicrobials present in carrot juice. Several studies have shown the lack of growth and even a reduction in populations of pathogens inoculated into carrot juice and on fresh-cut carrots. Beuchat and Brackett (1990) reported that raw carrot juice is lethal to *L. monocytogenes*. Abdul-Raouf *et al.* (1993) demonstrated that the number of *E. coli* O157:H7 inoculated on shredded carrots and stored at 5°C for 14 days decreased by 1 log<sub>10</sub> CFU/g after 3 days and 5 log<sub>10</sub> CFU/g after 7, 10, and 14 days of storage. In this same study, however, it was observed that *E. coli* O157:H7 inoculated on sliced cucumber followed by storage at 5°C was reduced by only 1.67 log<sub>10</sub> CFU/g after 14 days of storage, indicating that components unique to carrots may be responsible for lethality to the pathogen. According to Abdul-Raouf *et al.* (1993) and Kurosaki and Nishi (1993), 6-methoxymellein, a phytoalexin in carrot, inhibits the growth of several fungi and bacteria. The chemical may also have been inhibitory or toxic to *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* inoculated onto fresh-cut carrots in our study.

Following treatment with 1.4 mg/L gaseous ClO<sub>2</sub> for 6.4 – 10.5 min at 79 – 84% relative humidity, the treated fresh-cut carrots showed slight whitening in color. Higher concentrations (2.7 mg/L and 4.1 mg/L ClO<sub>2</sub>) within 12.3 – 20 and 20.5 – 30.8 min, respectively, at 78 – 87% and 80 – 85% relative humidity caused a greater whitening effect. This indicates that exposure of fresh-cut carrots to higher concentrations of gaseous ClO<sub>2</sub> and for longer time periods may result in more extreme biochemical changes and consequent deterioration in overall sensory quality.

**Treatment of fresh-cut lettuce.** Fresh-cut lettuce pieces inoculated with *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L within 6.4 – 10.5, 12.3 – 20, and 20.5 – 30.8 min, respectively, at 36 – 84% relative humidity and 21 – 25°C (Figures 3.3, 3.4, and 3.5). Reductions in populations of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* are shown on Tables 3.1, 3.2, and 3.3, respectively. Treatment with gaseous ClO<sub>2</sub> at a concentration of 1.4 mg/L significantly ( $\alpha = 0.05$ ) reduced the number of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* by 1.14, 0.64, and 0.81 log<sub>10</sub> CFU/g, respectively, compared to populations on untreated fresh-cut lettuce. There were no significant differences between the log reductions in *Salmonella* or *L. monocytogenes* with the three concentrations of ClO<sub>2</sub>. However, for fresh-cut lettuce inoculated with *E. coli* O157:H7, the highest concentration, 4.1 mg/L ClO<sub>2</sub>, not only caused the highest reduction (1.57 log<sub>10</sub> CFU/g), but was significantly more effective than treatment with 1.4 mg/L and 2.7 mg/L ClO<sub>2</sub> or the untreated control. The lower reductions in populations of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* on fresh-cut lettuce compared to reductions on fresh-cut cabbage and fresh-cut carrots treated with the same concentrations of ClO<sub>2</sub> are attributed in part to the protection of bacteria afforded by inaccessible, non-stressing sites in the cut tissue and openings in stomata on uncut surface (Garg *et al.*, 1990). Cut tissue facilitates microbial infiltration and exposes organic materials and nutrients that would have a neutralizing effect on ClO<sub>2</sub> and provide an environment for growth of microorganisms (Brackett, 1994). In a study done by Rodgers *et al.* (2004), several produce items (whole and sliced apples, uncut and shredded lettuce, strawberries, and cantaloupe) were inoculated with *L. monocytogenes* and *E. coli* O157:H7 and treated with a ClO<sub>2</sub> solution (3 µg/ml and 5 µg/ml chlorine) for 5 min. Only shredded lettuce and sliced apples showed recovery

in microbial counts. This supports the observation that an increase availability of nutrients resulting from breakage of cellular structures caused by cutting, slicing, or shredding enhances the survival of these pathogens. Abdul-Raouf *et al.* (1993) reported that *E. coli* O157:H7 survived at a higher population on shredded lettuce than on sliced cucumbers stored at 5°C for 14 storage days. Retention of higher numbers of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* on treated fresh-cut lettuce than on treated fresh-cut cabbage in our study suggests that differences in surface structure of the two vegetables, alone with factors associated with tissue juice composition, influences the lethality of ClO<sub>2</sub>. Another study reported the preference of microorganisms to initially attach to and enter stomata, broken trichomes, cracks in the cuticle, and the interior of cut edges of lettuce (Seo and Frank, 1999) and other produce (Burnett and Beuchat, 2001). Visualization of infiltration has been done using confocal scanning laser microscopy. Seo and Frank (1999) inoculated lettuce with *E. coli* O157:H7, followed by treatment with 20 mg/L of chlorine for 5 min. Viable cells of the pathogen were observed to be entrapped 20 – 100 µm below the surface in stomata and cut edges, while cells found on surface of leaf were dead.

Following treatment with 1.4 mg/L gaseous ClO<sub>2</sub> for 6.4 – 10.5 min at 68 – 82% relative humidity, the treated fresh-cut lettuce showed some browning in color. Higher concentrations (2.7 mg/L and 4.1 mg/L ClO<sub>2</sub>) within 12.3 – 20 and 20.5 – 30.8 min, respectively, at 73 – 83% relative humidity showed a greater browning discoloration, although relative to fresh-cut carrots that have received the same ClO<sub>2</sub> treatment, the degree of discoloration was less. Results indicate that, overall, visual sensory quality and viability of pathogens on fresh-cut lettuce are less susceptible to treatment with gaseous ClO<sub>2</sub>.

**Treatment of apples.** Fresh, uncut apples inoculated with *Salmonella* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L within 6, 12, and 25 min, respectively, at 35 – 68% relative humidity and 21 – 25°C (Figure 3.6). Reductions in populations of *Salmonella* are shown on Table 3.4. Compared to untreated apples, populations of *Salmonella* recovered from apples treated with all concentrations of ClO<sub>2</sub> were significantly lower ( $\alpha = 0.05$ ), although they were not significantly different from each other. The same was true for yeasts and molds.

The highest log reduction (4.21 log<sub>10</sub> CFU/apple) was achieved by treatment with 2.7 mg/L and 4.1 mg/L ClO<sub>2</sub>, while 1.4 mg/L ClO<sub>2</sub> caused a 3.21-log<sub>10</sub> CFU/apple reduction. These results are similar to those in a study conducted by Du *et al.* (2003) in which treatment of apples inoculated with *E. coli* O157:H7 and treated with 1.1 mg/L, 3.3 mg/L, and 4.8 mg/L of ClO<sub>2</sub> gas for 10 min resulted in reductions of 2.8, 3.9, and 4.8 log<sub>10</sub> CFU/spotted site, respectively. Treatment of apples with the same concentrations of ClO<sub>2</sub> for 20 min resulted in reductions of 4.7, 5.9, and  $\geq 6.3$  log<sub>10</sub> CFU/spotted site, respectively. In another study on inactivation of *L. monocytogenes* on apples, treatment with 1.0 mg/L, 3.0 mg/L, and 4.0 mg/L ClO<sub>2</sub> gas for 10 min caused reductions of 3.2, 3.3, and 5.5 log<sub>10</sub> CFU/spotted site, respectively (Du *et al.*, 2002). In our study, reductions in population of *Salmonella*, resulting from treating apples with 1.4 mg/L ClO<sub>2</sub> for 6 min or 2.7 mg/L ClO<sub>2</sub> for 12 min were slightly higher than reductions observed by Du *et al.* (2002, 2003) for *E. coli* O157:H7 and *L. monocytogenes* on apples treated with 1.1 mg/L – 3.3 mg/L and 1.0 mg/L – 3.0 mg/L ClO<sub>2</sub>, respectively, for 10 min. The reduction in population of *Salmonella* on apples treated with 4.1 mg/L ClO<sub>2</sub> for 25 min in our study was much lower than those of *E. coli* O157:H7 or *L. monocytogenes* reported by Du *et al.* (2002, 2003) that resulted from treatment of apples with 4.8 mg/L ClO<sub>2</sub> for 20 min or 4.0 mg/L ClO<sub>2</sub> for 30 min.



Table 3.4. Recovery of *Salmonella* and yeasts/molds from fresh, whole apple treated with gaseous ClO<sub>2</sub>

Microorganism	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/apple) <sup>a</sup>		
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>
<i>Salmonella</i>	0	0	A	5.49	
	6	1.4	B	2.28	3.21
	12	2.7	B	1.28	4.21
	25	4.1	B	1.28	4.21
Yeasts and molds	0	0	A	5.92	
	6	1.4	B	4.83	1.09
	12	2.7	B	4.44	1.48
	25	4.1	B	4.24	1.68

<sup>a</sup>Populations of *Salmonella* recovered on TSANP and yeasts/molds recovered on DRBC agar after treatment of apples with 0, 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 6, 12, and 25 min, respectively, at 21 – 25°C. The population of *Salmonella* inoculated onto apples was 8.00 log<sub>10</sub> CFU/apple. The detection limit was 1 CFU/ml of DE wash (20 CFU/apple).

<sup>b</sup>Within microorganism, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction (log<sub>10</sub> CFU/apple) compared to the population recovered from apples receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, washed apples positive for *Salmonella*, as detected by enrichment (En), out of the number of apples analyzed. Apples on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

Differences in results in the two studies are attributed in part to differences in resistance of pathogens to ClO<sub>2</sub> and also to differences in systems used to generate ClO<sub>2</sub>.

The yeast and mold populations detected on apples treated with 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L ClO<sub>2</sub> were significantly lower ( $\alpha = 0.05$ ) than those on untreated apples. The highest log reduction (1.68 log<sub>10</sub> CFU/apple) resulted from treatment with 4.1 mg/L ClO<sub>2</sub>. Compared to *Salmonella*, yeasts and molds naturally present on the skin of apples appear to be less susceptible to gaseous ClO<sub>2</sub>. Rodgers *et al.* (2004) reported that yeast populations on whole apples treated

with aqueous ClO<sub>2</sub> (3 µg/ml and 5 µg/ml) were reduced by 1.3 – 1.5 log<sub>10</sub> CFU/g; mold populations were reduced by 1.4 – 1.5 log<sub>10</sub> CFU/g, while *L. monocytogenes* or *E. coli* O157:H7 populations were reduced by approximately 5 log<sub>10</sub> CFU/g, indicating that lethality of ClO<sub>2</sub> to bacteria is much greater than to yeast and molds. These results agree with studies conducted by Beuchat *et al.* (1998), demonstrating that mold propagules on apples tend to be more resistant than bacteria to sanitizers used in the apple industry. Differences in sensitivity of various microbial cells may also be influenced by their hydrophobicity. Waxes in cutin on the surface of apples generally contains ingredients such as alcohols, morpholine, and surfactants that may enhance dispersion of spores, thereby resulting in an apparent increased number of colony-forming units dispersed in DE broth after washing apples (Kenney and Beuchat, 2002).

Following treatment with 4.1 mg/L gaseous ClO<sub>2</sub> for 25 min at 58% relative humidity, small brown spots on apples were evident. The appearance of apples treated with 1.4 mg/L and 2.7 mg/L ClO<sub>2</sub> at 65% and 68% relative humidity, respectively, was unaffected. This may be a result of the smaller amount of moisture present on the surface of apples to attract ClO<sub>2</sub>, thus allowing for minimal biochemical reactions that lead to brown discoloration, compared to fresh-cut vegetables in which greater amount of moisture from tissue juices released onto the surface would attract more ClO<sub>2</sub> and promote discoloration.

**Treatment of tomatoes.** Fresh, uncut tomatoes inoculated with *Salmonella* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L ClO<sub>2</sub> gas within 6, 12, and 25 min, respectively, at 34 – 62% relative humidity and 21– 24°C (Figure 3.6). Significant reductions ( $\alpha = 0.05$ ) in populations of *Salmonella* resulted from treatment with all concentrations of ClO<sub>2</sub>. The highest concentration, 4.1 mg/L ClO<sub>2</sub>, caused the highest log reduction (4.33 log<sub>10</sub> CFU/tomato), although this reduction was not significantly different than

those resulting from other ClO<sub>2</sub> treatments (Table 3.5). Log reductions on tomatoes were slightly lower than those on apples treated with 1.4 mg/L and 2.7 mg/L ClO<sub>2</sub>; however, treatment of apples and tomatoes with 4.1 mg/L ClO<sub>2</sub> resulted in similar reductions (ca. 4.2 log<sub>10</sub> CFU/fruit). It is possible that the smoother and harder surface of apples allows for easier access of ClO<sub>2</sub> gas to *Salmonella* inoculated on the surface. Log reductions of *Salmonella* (4.33 log<sub>10</sub> CFU/tomato) on tomatoes as a result of treatment with 4.1 mg/L ClO<sub>2</sub> for 25 min gas are similar to log reductions of *Salmonella* (3.67 log<sub>10</sub> CFU/cm<sup>2</sup>) on tomatoes treated for 30 min with neutral electrolyzed water solution (NEW) containing 89 mg/L active chlorine (Deza *et al.*, 2003); however, ClO<sub>2</sub> gas has a greater kill capacity than NEW, requiring a lower concentration to achieve approximately the same log reductions from treatment with NEW.

Yeast and mold populations on untreated and treated tomatoes were not significantly different from each other, although treatment of tomatoes with 4.1 mg/L of ClO<sub>2</sub> caused a reduction of 1.16 log<sub>10</sub> CFU/tomato (Table 3.5). Overall, reductions in yeast and mold populations on tomatoes treated with gaseous ClO<sub>2</sub> were less than those on treated apples, suggesting that interactions between fungal propagules and the epidermis of tomatoes may enhance their protection against penetration of ClO<sub>2</sub> gas (Burnett and Beuchat, 2001).

Following treatment of tomatoes with 1.4 mg/L, 2.7 mg/L, or 4.1 mg/L gaseous ClO<sub>2</sub> for 6, 12, and 25 min, respectively, at 34 – 62% relative humidity, subjective evaluation revealed no apparent visual differences in overall appearance and color compared to untreated tomatoes.

**Treatment of onions.** Fresh, uncut Vidalia onions inoculated with *Salmonella* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L within 5.4, 10.4, and 20 min, respectively, at 35 – 64% relative humidity and 21 – 24°C (Figure 3.7). Significant reductions ( $\alpha = 0.05$ ) in populations occurred, compared to populations on untreated

Table 3.5. Recovery of *Salmonella* and yeasts/molds from fresh, whole tomatoes treated with gaseous ClO<sub>2</sub>

Microorganism	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/tomato) <sup>a</sup>		
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>
<i>Salmonella</i>	0	0	A	5.90	
	6	1.4	B	4.79	1.11
	12	2.7	B	3.86	2.04
	25	4.1	B	1.57	4.33
Yeasts and molds	0	0	A	6.17	
	6	1.4	A	5.30	0.87
	12	2.7	A	5.35	0.82
	25	4.1	A	5.01	1.16

<sup>a</sup>Populations of *Salmonella* recovered on TSANP and yeasts/molds recovered on DRBC agar after treatment of tomatoes with 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 6, 12, and 25 min, respectively, at 21 – 24°C. The population of *Salmonella* inoculated onto tomatoes was 8.00 log<sub>10</sub> CFU/tomato. The detection limit was 1 CFU/ml of DE wash (20 CFU/tomato).

<sup>b</sup>Within microorganism, mean values not preceded by the same letter are significantly different ClO<sub>2</sub> ( $\alpha = 0.05$ ).

<sup>c</sup>Reduction (log<sub>10</sub> CFU/tomato) compared to the population recovered from tomatoes receiving no treatment (0 mg/L).

<sup>d</sup>Number of treated, washed tomatoes positive for *Salmonella*, as detected by enrichment (En), out of the number of tomatoes analyzed. Tomatoes on which *Salmonella* was recovered by direct Plating were not analyzed by enrichment.

onions (Table 3.6). The highest treatment concentration (4.1 mg/L ClO<sub>2</sub>) caused the highest log reduction (1.57 log<sub>10</sub> CFU/onion), although reductions resulting from all treatments were not significantly different. The log reductions of *Salmonella* achieved by treatment with gaseous ClO<sub>2</sub> were, overall, lower than those observed on treated apples, tomatoes, or peaches. This may be a result of several factors. The skin on onions may be more porous and less uniform compared to the smooth and waxy surfaces of apples and tomatoes and may harbor more soil. The porous surface of onions may enable easier infiltration of *Salmonella* and other

Table 3.6. Recovery of *Salmonella* and yeasts/molds from fresh, whole onions treated with gaseous ClO<sub>2</sub>

Microorganism	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/onion) <sup>a</sup>		
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>
<i>Salmonella</i>	0	0	A	6.95	
	5.4	1.4	B	6.12	0.83
	10.4	2.7	B	5.06	1.89
	20	4.1	B	5.01	1.94
Yeasts and molds	0	0	AB	6.24	
	5.4	1.4	B	5.88	0.36
	10.4	2.7	B	6.02	0.22
	20	4.1	A	6.46	-0.22

<sup>a</sup>Populations of *Salmonella* recovered on TSANP and yeasts/molds recovered on DRBC agar after treatment of onions with 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 5.4, 10.4, and 20 min, respectively, at 21 – 24°C. The population of *Salmonella* inoculated onto onions was 7.96 log<sub>10</sub> CFU/ onion. The detection limit was 1 CFU/ml of DE wash (20 CFU/onion).

<sup>b</sup>Within microorganism, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Within microorganism reduction (log<sub>10</sub> CFU/onion) compared to the population recovered from onions receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, washed onions positive for *Salmonella*, as detected by enrichment (En), out of the number of onions analyzed. Onions on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

microorganisms, a condition observed in produce with cut surfaces or presence of crevices, cracks, bruises, and natural openings (Dingman, 2002; Wells and Butterfield, 1997). The outer skins on onions occasionally overlapped and may provide protective sites by preventing direct exposure of *Salmonella* to ClO<sub>2</sub> gas. The presence of a soil, debris, and other organic and inorganic material may result in reduced effectiveness of ClO<sub>2</sub> gas as has been observed with chlorine (Dychadala, 1968) and ozone (Rajkowski and Rice, 2004; Moore *et al.*, 2000)

The yeast and mold populations recovered from untreated and treated were not significantly different (Table 3.6). Overall, reductions in populations were much lower on onions than on apples, tomatoes, or peaches. The same phenomenon affecting survival of *Salmonella* on onions may have also been partly responsible for survival of yeasts and molds. The greater initial population of yeasts and molds on onions compared to those on other produce, presence of different genera or species of yeasts and molds on the four types of produce, and the type, location, and number of mycelia, spores, and conidia may also be reflected in differences in reductions in counts resulting from ClO<sub>2</sub> gas treatment.

Following treatment with 1.4 mg/L, 2.7 mg/L, or 4.1 mg/L gaseous ClO<sub>2</sub> for 5.4, 10.4, and 20 min, respectively, at 36 – 64% relative humidity, subjective evaluations on onions were conducted. The appearance and color of untreated and treated onions were not different from each other as observed in tomatoes.

**Treatment of peaches.** Fresh, uncut peaches inoculated with *Salmonella* were treated with gaseous ClO<sub>2</sub> at release concentrations of 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L ClO<sub>2</sub> within 5.4, 10.4, and 20 min, respectively, at 55 – 78% relative humidity and 21 – 24°C (Figure 3.7). Significant reductions ( $\alpha = 0.05$ ), compared to populations recovered from untreated peaches, were observed (Table 3.7). The highest treatment concentration (4.1 mg/L ClO<sub>2</sub>) caused the highest log reduction (3.23 log<sub>10</sub> CFU/peach), although populations recovered from peaches treated with all concentrations of gaseous ClO<sub>2</sub> were not significantly different. Log reductions of *Salmonella* on treated peaches were slightly lower than those on apples and tomatoes, but higher than on onions. These results were not unexpected, since the skin of peaches is not smooth in comparison to that of apples and tomatoes. Numerous broken trichomes on peaches provide sites for harborage and protection of *Salmonella* against ClO<sub>2</sub>. The higher log reductions

Table 3.7. Recovery of *Salmonella* and yeasts/molds from fresh, whole peaches treated with gaseous ClO<sub>2</sub>

Microorganism	Treatment time (min)	Amount of ClO <sub>2</sub> released (mg/L)	Population (log <sub>10</sub> CFU/peach) <sup>a</sup>			
			Recovered <sup>b</sup>	Reduction <sup>c</sup>	En <sup>d</sup>	
<i>Salmonella</i>	0	0	A	7.19		
	5.4	1.4	B	6.19	1.00	
	10.4	2.7	B	5.67	1.52	2/2
	20	4.1	B	3.96	3.23	5/5
Yeasts and molds	0	0	A	4.84		
	5.4	1.4	AB	4.44	0.41	
	10.4	2.7	B	2.43	2.41	
	20	4.1	B	2.60	2.65	

<sup>a</sup>Populations of *Salmonella* recovered on TSANP and yeasts/molds recovered on DRBC agar after treatment of whole peaches with 1.4, 2.7, and 4.1 mg/L ClO<sub>2</sub> for 0, 5.4, 10.4, and 20 min, respectively, at 21 – 23°C. The population of *Salmonella* inoculated onto peaches was 8.03 log<sub>10</sub> CFU/peach. The detection limit was 1 CFU/ml of DE wash (20 CFU/peach).

<sup>b</sup>Within microorganism, mean values not preceded by the same letter are significantly different ( $\alpha = 0.05$ ).

<sup>c</sup>Within microorganism reduction (log<sub>10</sub> CFU/peach) compared to the population recovered from peaches receiving no ClO<sub>2</sub> treatment (0 mg/L).

<sup>d</sup>Number of treated, washed peaches positive for *Salmonella*, as detected by enrichment (En), out of the number of peaches analyzed. Peaches on which *Salmonella* was recovered by direct plating were not analyzed by enrichment.

of *Salmonella* on peaches treated with ClO<sub>2</sub>, compared to reductions on treated onions, may be attributed in part to the surface of peaches being less porous and containing less debris, soil, and other materials that may interact with ClO<sub>2</sub> gas and prevent contact with or inactivation of microorganisms. The yeast and mold populations on untreated peaches and peaches treated with 1.4 mg/L ClO<sub>2</sub> were not significantly different, but recovered populations on peaches treated with 2.7 mg/L or 4.1 mg/L ClO<sub>2</sub> were significantly lower than that on untreated peaches.

Overall, reduction of yeast and mold populations on peaches by treatment with gaseous ClO<sub>2</sub> were higher than those achieved by treatment of apples, tomatoes, and onions.

Following treatment with 1.4 mg/L, 2.7 mg/L, and 4.1 mg/L gaseous ClO<sub>2</sub> for 5.4, 10.4, and 20 min, respectively, at 55 – 78% relative humidity, peaches showed no apparent changes in appearance and color, possibly reflecting behavioral characteristics similar to those exhibited by apples and other fresh, whole produce.

In summary, gaseous ClO<sub>2</sub> shows promise as a sanitizer for fresh-cut cabbage, fresh-cut carrots, apples, tomatoes, peaches, and to a much lesser extent for fresh-cut lettuce and onions. Identification of structures and specific components of produce tissues that may enhance the survival of pathogens and yeasts/molds should be further studied. Objective evaluations of changes in appearance of fresh and fresh-cut fruits and vegetables caused by treatment with gaseous ClO<sub>2</sub> is warranted to ensure that treatment with ClO<sub>2</sub> at concentrations necessitating significant numbers of pathogens killed do not compromise sensory qualities.

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## CHAPTER 4

### SUMMARY AND CONCLUSIONS

The objectives of the studies documented in this thesis were:

1. To determine the efficacy of chlorine dioxide (ClO<sub>2</sub>) gas in killing or removing *Salmonella*, yeasts, and molds from small fruits.
2. To determine if the visual and olfactory attributes of blueberries, strawberries and raspberries are adversely affected by treatment with ClO<sub>2</sub> gas.
3. To evaluate the degree of inactivation of *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, yeasts and molds from fresh or fresh-cut produce by ClO<sub>2</sub> gas.

The research showed that log reductions of *Salmonella* from blueberries and strawberries were effectively reduced with treatment of gaseous ClO<sub>2</sub>. Pathogens inoculated on the skin compared to stem scar or calyx on blueberries were the most susceptible to the sanitizer.

Overall, reductions in yeast and mold populations were less.

Sensory evaluations of berries by consumers of fresh fruits and vegetables ascertained that treatment with 4.1 mg/L ClO<sub>2</sub> not only significantly reduced the population of pathogens by 2 log<sub>10</sub> CFU/g, but also did not adversely affect the appearance, aroma, color, or overall quality of blueberries. Treated strawberries and raspberries were only differentiated from controls on day 0 and were visually indistinguishable for the remainder of the storage shelf-life study.

Gaseous ClO<sub>2</sub> was most effective as a sanitizer for removal pathogens on fresh-cut carrots, fresh-cut cabbages, uncut apples, uncut tomatoes and uncut peaches. The ability of pathogens to attach and lodge in crevices, cracks, and stomata of fresh-cut lettuce aided in their ability to evade contact with ClO<sub>2</sub> gas, while porous surfaces of uncut onions allowed pathogens to infiltrate into the skin and thus protect *Salmonella* from being inactivated. Similar to observations on berries, yeasts and molds were also difficult to remove on fresh uncut apples and to a lesser extent peaches. Treatment of uncut onions and tomatoes with ClO<sub>2</sub> gas did not reduce

populations of fungi naturally present on the produce. This may be attributable in part to hydrophobic interactions occurring between waxes in cutin of produce that may enhance dispersion of spores, to the presence of different genera or species of yeasts and molds on the four types of produce, and the type, location, and number of mycelia, spores, and conidia present.