Journal of Food Protection, Vol. 70, No. 5, 2007, Pages 1088–1092

Efficacy of Aqueous Ozone for the Decontamination of *Escherichia coli* O157:H7 and *Salmonella* on Raspberries and Strawberries

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MS 06-366: Received 6 July 2006/Accepted 28 December 2006

ABSTRACT

The efficacy of ozone as a water additive for washing raspberries and strawberries was investigated. Pathogen-inoculated fruits were treated with aqueous ozone concentrations of 1.7 to 8.9 mg/liter at 20°C for 2 to 64 min, with an aqueous ozone concentration of 21 mg/liter at 4°C for 64 min, or with water as a control. Maximum pathogen reductions on raspberries were 5.6 and 4.5 log CFU/g for *Escherichia coli* O157:H7 and *Salmonella,* respectively, at 4°C, whereas reductions on strawberries were 2.9 and 3.3 log CFU/g for *E. coli* O157:H7 and *Salmonella,* respectively, at 20°C after 64 min. Washing with water (sparging with air as control) resulted in reductions of approximately 1 log CFU/g. The results presented here indicate that aqueous ozone may be useful as a decontaminant for small fruits.

Each year *Escherichia coli* O157 infections cost the United States approximately $405 million *(8)* and *Salmonella* infections result in $3 billion in medical expenses and lost productivity *(16).* Fresh produce has been increasingly implicated in many of these infections and is now the second leading source of pathogens associated with foodborne illness, having been responsible for 428 outbreaks between 1990 and 2003 *(1).*

Concern about the microbial safety of small fruits has arisen in recent years because of the implication of these fruits in several notable outbreaks. Strawberries have been responsible for three separate outbreaks of hepatitis A *(2),* and raspberries have been associated with five outbreaks of cyclosporosis *(3).* The production practices used with these small fruits make these fruits especially vulnerable to contamination. The fruits are not washed or treated prior to market and can become contaminated from a variety of sources; improper sanitation, infected pickers, contaminated irrigation water, and manure slurry used to fertilize fields are all potential culprits for contamination *(10).* Research has shown that pathogens such as *E. coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* are capable of surviving on the surfaces of strawberries for more than 7 days *(7, 13),* and a U.S. Food and Drug Administration survey revealed that 1 of 143 samples of imported strawberries were positive for *Salmonella* contamination *(17).*

Water and commercial sanitizers have had limited efficacy for decontamination of these fruits. Yu et al. *(18)* evaluated the efficacy of five different sanitizers for killing *E. coli* O157:H7 on strawberries: sodium hypochlorite, Tween 80, acetic acid, sodium phosphate, and hydrogen peroxide at various concentrations. The maximum reduction was reported as 2.2 log CFU/g with 3% hydrogen peroxide. When strawberries were treated with water alone, an 0.8-log reduction was achieved.

Previous research and commercial use suggest that ozone may be useful as an aqueous sanitizer. Ozone has been used to purify drinking water since the late 19th century *(9)* and is one of the most powerful oxidizers available. Ozone decays into oxygen in a relatively short time so no harmful residue is left behind, as occurs with some sanitizers such as chlorine. In 2001, ozone was granted approval for the treatment of raw commodities *(6).* Gaseous ozone can be generated on site with an ozone generator and dissolved into water to make aqueous ozone.

Aqueous ozone is an effective sanitizing agent for food-related microorganisms. Kim and Yousef *(11)* found that a 3.8-log reduction in *E. coli* O157:H7 could be achieved with 1.0 mg/liter aqueous ozone after only 10 s, and Dave et al. *(4)* attained a 6-log reduction in *Salmonella* Enteritidis with 1.5 mg/liter aqueous ozone. Aqueous ozone at very low concentrations is very efficient at killing suspended cells in water. However, ozone may not be as effective if microorganisms are on foods or in the crevices of the food surface. Therefore, the concentrations of ozone and treatment times may need to be increased. Research has shown that aqueous ozone is effective at inactivating *E. coli* O157:H7 on alfalfa sprouts and seeds at a concentration of 21 mg/liter *(15).*

Because of the results of previous research and the ineptitude of conventional sanitizers, this research was undertaken to determine whether aqueous ozone would be a suitable decontaminant for raspberries and strawberries.

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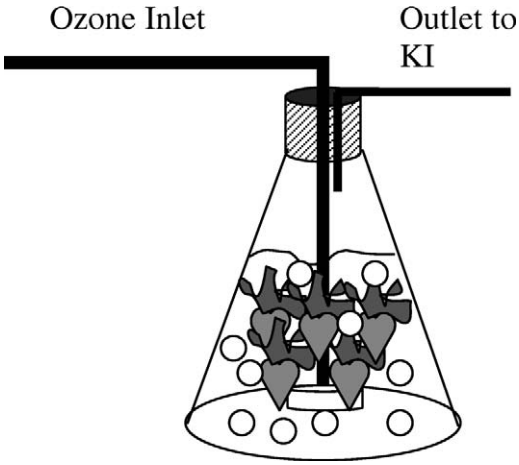

FIGURE 1. *Schematic of ozone treatment.*

MATERIALS AND METHODS

Preparation of inoculum. Five strains of nalidixic acid–resistant *E. coli* O157:H7 and *Salmonella* were obtained from the Center for Food Safety (University of Georgia, Griffin). The *E. coli* O157:H7 strains were 932 (human isolate), 994 (salami isolate), E0018 (calf fecal isolate), H1730 (human isolate from outbreak associated with lettuce), and F4546 (human isolate from outbreak associated with alfalfa sprouts). The *Salmonella* serotypes used were Agona (human isolate from outbreak associated with alfalfa sprouts), Baildon (human isolate from outbreak associated with diced tomatoes), Gaminara (orange juice isolate), Michigan (human isolate from outbreak associated with cantaloupe), and Montevideo (human isolate from outbreak associated with tomato). Cultures were grown in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.) supplemented with 50 μg/ml nalidixic acid (Fisher Scientific Co., Fair Lawn, N.J.) at 37°C for 24 h. Separate cocktails of *E. coli* O157:H7 and *Salmonella* strains were prepared by combining 10 ml of each strain culture and centrifuging for 15 min at 3,300 × *g* and 4°C. The supernatant was discarded, and the cells were resuspended in 10 ml of 0.1% peptone water (Difco, Becton Dickinson) to yield an approximate concentration of 10^8 CFU/ml.

Inoculation of small fruits. Fresh red raspberries (*Rubus idaeus*) and strawberries (*Fragaria ananassa*) were purchased from a local grocery store and left at room temperature for 1 h prior to inoculation. To inoculate the raspberries, 25 μl of either *E. coli* O157:H7 or *Salmonella* inoculum was deposited on the skin of each fruit. For strawberries, 50 μl of each inoculum was deposited on the skin of each fruit, approximately midway between the calyx and cap *(14).* The fruits were dried in a laminar flow hood for 24 h before further treatment to allow attachment of the microorganisms. The concentration of both *E. coli* O157:H7 and *Salmonella* on the inoculated raspberries and strawberries was approximately 10^5 CFU/g.

Production and delivery of ozone. Ozone gas was generated with a lab-scale ozone generator (model H-50, Hess Machines International, Ephrata, Pa.) equipped with an oxygen concentrator. Gas was delivered at a flow rate of 0.34 m^3/h and an ozone concentration of 5% (wt/wt), which was measured with a bench-top ozone analyzer (model 450H, Teledyne Technologies, Inc., Los Angeles, Calif.). A 500-ml flask containing sterile deionized water was sparged with ozone (Fig. 1) for various times and at various temperatures, and excess ozone was passed through a 2% (wt/vol) potassium iodide solution to prevent ozone from being released into the environment. The ozone treatments were performed in a fume hood for safety considerations.

Treatment with ozone. A sample of five inoculated strawberries or 18 inoculated raspberries was placed in a flask containing 500 ml of sterile deionized water and a 10-μm stainless steel sparger (Fig. 1). Fruits were subjected to ozone treatments at 20 and 4°C. Ozone was sparged into the water for 2, 4, 8, 16, 32, and 64 min at 20°C and for 64 min at 4°C. Water sparged with air for 64 min was used as a control for both treatment scenarios. Temperature was maintained by placing flasks in a water bath at the appropriate settings.

Aqueous ozone analysis. The final ozone concentration in a treatment flask without fruits was measured for each sparging time. Ozone concentrations in the actual treatment flasks were expected to be lower because of the degradation of ozone by microorganism and other organic matters. Ozone concentration was measured using direct UV absorption at 258 nm. The concentration was calculated using the following formula *(15)*:

$$c = \frac{48{,}000 \times A}{2{,}900}$$

where c is the concentration of ozone in water (milligrams per liter) and A is the absorbance value at UV 258 nm.

For a more in-depth description of the system, the volumetric mass transfer coefficient (k_La) was determined *(5).* This value is a measure of the rate of ozone use in the system and can be helpful when scaling up a process. The k_La value was determined using the following formula:

$$\frac{dC}{dt} = k_La(C^* - C) \quad (1)$$

where k_La is the volumetric mass transfer coefficient (min^{-1}), C^* is the liquid-phase ozone concentration at equilibrium (milligrams per liter), and C is the ozone concentration at a given time (milligrams per liter).

Microbial analysis. After treatment, strawberries and raspberries were placed in 50 and 25 ml of Dey-Engley neutralizing broth (Difco, Becton Dickinson), respectively, and pummeled for 1 min in a stomacher. The homogenate was then serially diluted in 0.1% peptone water (Difco, Becton Dickinson) and spiral plated (Autoplate 4000, Spiral Biotech, Norwood, Mass.) on tryptic soy agar (Difco, Becton Dickinson) supplemented with 50 μg/ml of nalidixic acid. Plates were incubated at 37°C for 24 h, and colonies were counted using Q-count (version 2.1, Spiral Biotech). Reductions in the concentration of bacteria per gram of fruit were calculated by subtracting the log values for a treated sample from those of an inoculated untreated sample. Random colonies of *E. coli* O157:H7 and *Salmonella* were confirmed serologically with the RIM *E. coli* O157:H7 latex test (Remel Microbiology Products, Lenexa, Kans.) and the *Salmonella* O Antiserum A-1 latex agglutination test (Remel). Enrichments were performed for samples with zero plate counts. For both *E. coli* O157:H7 and *Salmonella,* 1 ml of 0.1% peptone water was transferred to 9 ml of tryptic soy broth (Difco, Becton Dickinson) supplemented with 50 μg/ml of nalidixic acid (TSBN) and incubated at 37°C for 24 h. After incubation, 1 ml of the TSBN was transferred to 9 ml of either TT Broth Base Hajna (Difco, Becton Dickinson) or MacConkey broth (Difco, Becton Dickinson) for *Salmonella* and *E. coli* O157:H7 culture, respectively. TT Broth Base Hajna was incubated at 45°C for 48 h. A loopful of enrichment solution was then streaked onto xylose lysine desoxycholate agar (Difco, Becton Dickinson) and incubated for 24 h at 37°C. Colonies were

TABLE 1. *Reductions of* E. coli *O157:H7 and* Salmonella *populations on raspberries treated with ozone at 20°C*[a]

Organism	Treatment time (min)	Ozone concn (mg/liter)	Population reduction (log CFU/g)[b]
E. coli O157:H7	2	1.7	2.6 ± 0.0 A
	4	1.8	3.6 ± 1.4 AB
	8	3.7	4.3 ± 1.4 AB
	16	7.6	2.7 ± 0.6 A
	32	7.9	4.8 ± 0.3 B[c]
	64	8.9	4.8 ± 0.3 B[c]
Salmonella	2	1.7	1.3 ± 0.1 A
	4	1.8	1.4 ± 0.1 A
	8	3.7	2.2 ± 0.7 AB
	16	7.6	2.7 ± 0.9 ABC
	32	7.9	3.5 ± 1.1 BC
	64	8.9	4.4 ± 0.9 C[c]

[a] Mean weight of raspberries was 17.5 ± 2.5 g.
[b] Values are mean ± standard deviation. Within the same column and microorganism, values with different letters are significantly different ($P < 0.05$).
[c] Detection limit was $\sim 1.5 \times 10^1$ CFU/ml. Enrichments after treatment were positive for *E. coli* O157:H7 and *Salmonella*, respectively.

confirmed with the *Salmonella* O Antiserum A-1 latex agglutination test. MacConkey broth tubes were incubated for 24 h at 37°C, and a loopful of solution was streaked onto MacConkey agar plates, which were incubated for 24 h at 37°C. Colonies were confirmed as *E. coli* O157:H7 with the RIM *E. coli* O157:H7 latex test.

Quality analysis. To determine whether treatment with ozone had any negative effects on quality, color was evaluated for fruits from the treatment with the highest microbial reduction. A Chromo Meter CR200 colorimeter (Minolta, Ramsey, N.J.) was used to measure the L*a*b* color space based on the following parameters: L* indicates the lightness and a* and b* are the chromaticity coordinates, where −a* indicates a green color, +a* is a red color, −b* is a blue color, and +b* is a yellow color. Before use, the colorimeter was calibrated with a white tile. Three randomly selected spots were analyzed and averaged to get an overall measurement for each fruit, and this process was replicated three times.

Statistical analysis. All experiments were replicated three times, and MINITAB statistical software (version 13, State College, Pa.) was used to analyze the mean log reductions. A one-way analysis of variance with a 95% confidence level was used to compare the treatment times and scenarios. A Tukey's comparison was also used to determine significant differences based on $P \leq 0.05$.

RESULTS AND DISCUSSION

In this study, the efficacy of ozone for the decontamination of raspberries and strawberries inoculated with *E. coli* O157:H7 and *Salmonella* was evaluated. The effects of ozone as an additive to water were investigated at two different temperatures. The effect of ozone solubility was investigated by lowering the treatment water temperature because the solubility of a gas in a solution increases as the temperature decreases. Control fruits were treated similarly, except that the water was sparged with air.

TABLE 2. *Reductions of* E. coli *O157:H7 and* Salmonella *populations on raspberries after treatment with ozone or air at 20 and 4°C for 64 min*

Organism	Temp (°C)	Treatment	Ozone concn (mg/liter)	Population reduction (log CFU/g)[a]
E. coli O157:H7	20	Ozone	8.9	4.8 ± 0.3 A
		Air		1.2 ± 1.0 B
	4	Ozone	21	5.6 ± 0.2 A
		Air		1.3 ± 0.9 B
Salmonella	20	Ozone	8.9	4.4 ± 0.9 A
		Air		0.9 ± 0.6 B
	4	Ozone	21	4.5 ± 0.0 A
		Air		1.1 ± 0.4 B

[a] Values are mean ± standard deviation. Within the same column and pathogen, values with different letters are significantly different ($P < 0.05$).

Final ozone concentrations were measured in the treatment flask without fruits and were 1.7, 1.8, 3.7, 7.6, 7.9, and 8.9 mg/liter after 2, 4, 8, 16, 32, and 64 min, respectively, at 20°C and 21 mg/liter after 64 min at 4°C. The volumetric mass transfer coefficient (k_La) was determined at 20°C based on an equilibrium ozone concentration of 9 mg/liter to further characterize the system; k_La was determined to be 0.126 min^{-1} based on equation 1.

Treatment of raspberries. Raspberries inoculated with either *E. coli* O157:H7 or *Salmonella* were treated with ozone at 20 and 4°C. At 20°C, reductions of *E. coli* O157:H7 ranged from 2.6 to 4.8 log CFU/g for the 2- and 32-min treatment times, respectively (Table 1). Only the treatments of 32 and 64 min, which demonstrated reductions of 4.8 log CFU/g, resulted in significantly greater reductions than the 2-min treatment. By decreasing the treatment temperature to 4°C, the concentration of ozone increased from 8.9 to 21 mg/liter for the 64-min treatment.

TABLE 3. *Reductions of* E. coli *O157:H7 and* Salmonella *populations on strawberries treated with ozone at 20°C*[a]

Organism	Treatment time (min)	Ozone concn (mg/liter)	Population reduction (log CFU/g)[b]
E. coli O157:H7	2	1.7	1.0 ± 0.2 A
	4	1.8	1.3 ± 0.2 AB
	8	3.7	1.5 ± 0.2 B
	16	7.6	1.8 ± 0.3 B
	32	7.9	1.7 ± 0.1 B
	64	8.9	2.9 ± 0.7 C
Salmonella	2	1.7	0.5 ± 0.2 A
	4	1.8	1.4 ± 0.4 AB
	8	3.7	1.5 ± 1.1 AB
	16	7.6	1.8 ± 0.2 B
	32	7.9	2.1 ± 0.4 B
	64	8.9	3.3 ± 0.6 C

[a] Mean weight of strawberries was 110 ± 5 g.
[b] Values are mean ± standard deviation. Within the same column and microorganism, values with different letters are significantly different ($P < 0.05$).

TABLE 4. *Reductions of* E. coli *O157:H7 and* Salmonella *populations on strawberries after treatment with ozone or air at 20 and 4°C for 64 min*

Organism	Temp (°C)	Treatment	Ozone concn (mg/liter)	Population reduction (log CFU/g)[a]
E. coli O157:H7	20	Ozone	8.9	2.9 ± 0.7 A
		Air		1.6 ± 0.6 B
	4	Ozone	21	2.6 ± 0.1 A
		Air		1.3 ± 0.5 B
Salmonella	20	Ozone	8.9	3.3 ± 0.6 A
		Air		0.9 ± 0.1 B
	4	Ozone	21	2.4 ± 0.5 C
		Air		1.4 ± 0.2 B

[a] Values are mean ± standard deviation. Within the same column and pathogen, values with different letters are significantly different ($P < 0.05$).

This higher concentration increased reductions to 5.6 log CFU/g after treatment for 64 min compared with 4.8 log CFU/g at 20°C after 64 min (Table 2). However, these reductions were not significantly different. The reduction was significantly greater with ozone than with air at 4°C (5.6 versus 1.3 log CFU/g) and at 20°C (4.8 versus 1.2 log CFU/g).

Reduction of *Salmonella* at 20°C ranged from 1.3 to 4.4 log CFU/g after 2 and 64 min, respectively (Table 1). The 64-min treatment resulted in a significantly greater population reduction than did the 2-, 4-, and 8-min treatments (1.3, 1.4, and 2.2 log CFU/g compared with 4.4 log CFU/g). When the temperature was lowered to 4°C, which increased the ozone concentration to 21 mg/liter, the *Salmonella* population reduction was 4.5 log CFU/g, which was not significantly different from that for the 64-min treatment at 20°C (4.4 log CFU/g; Table 2). As a control, raspberries were treated with air instead of ozone at both 20 and 4°C to determine whether bacterial reduction was due to ozone presence or to the agitation supplied by the gas. The resulting reductions were 0.9 and 1.1 log CFU/g for 20 and 4°C, respectively. Statistical analysis indicated that these values were significantly lower than reductions resulting from ozone treatment.

Treatment of strawberries. Strawberries inoculated with *E. coli* O157:H7 and *Salmonella* were treated with aqueous ozone at both 20 and 4°C. Reductions of *E. coli* O157:H7 were 1.0 to 2.9 log CFU/g after 2 to 64 min at 20°C (Table 3). The 64-min treatment resulted in significantly greater reductions than did the shorter treatment times. At 4°C, the reduction at 64 min was 2.6 log CFU/g. Consistent with the findings for raspberries, the lethal part of the treatment was determined to be ozone exposure rather than gas agitation; ozone treatments produced significantly greater reductions than did air treatment (Table 4). A reduction of 1.6 log CFU/g resulted after 64 min of air treatment, whereas 64 min of ozone treatment resulted in a reduction of 2.9 log CFU/g.

Reductions of *Salmonella* were between 0.5 and 3.3 log CFU/g for 2 to 64 min of treatment at 20°C (Table 3). The 64-min treatment resulted in significantly greater reductions than did shorter treatment times. As with *E. coli* O157:H7, reductions obtained at 4°C after 64 min did not result in significantly greater reductions than those at 20°C for 64 min. The mean reduction at 4°C was 2.4 log CFU/g compared with 3.3 log CFU/g at 20°C. The data presented here indicate that the significant effects were the result of the ozone itself rather than the agitation created by sparging with a gas. When fruits were treated with air sparging, a significantly lower reduction of *Salmonella* was observed. Air alone resulted in a 0.9- and 1.4-log reduction in *Salmonella* at 20 and 4°C, respectively.

Color measurement. Fruits from the most effective treatments were analyzed to determine whether ozone treatment had any negative effects on color. The most effective antimicrobial treatment for both raspberries and strawberries was the 64-min treatment at 20°C. Treated raspberries had mean L*, a*, and b* values of 29.64, +21.94, and +11.77, whereas the untreated raspberries had values of 29.20, +20.25, and +11.66, respectively (Table 5). None of the differences were significant. For treated strawberries, the mean L*, a*, and b* values were 34.10, +30.08, and +19.36, which were not significantly different from the values for untreated strawberries, which were 33.17, +25.94, and +17.30, respectively.

In previous studies concerning the efficacy of aqueous ozone, the ability of this gas to inactivate microorganisms is very dependent on the food product involved, and results have been somewhat inconsistent. In studies on the efficacy of aqueous ozone for treating lettuce, reductions ranged from 4.6 to just 1.5 log CFU/g *(12, 14).* Kim et al. *(12)* reported a 4.6-log reduction in aerobic microorganisms af-

TABLE 5. *L*, a*, and b* color readings for raspberries and strawberries after ozone treatment*[a]

Color parameter	Raspberries[b]		Strawberries[c]	
	Treated	Untreated	Treated	Untreated
L*	29.64 ± 2.93 A	29.20 ± 1.85 A	34.10 ± 1.96 A	33.17 ± 1.94 A
a*	21.94 ± 3.07 A	20.25 ± 2.22 A	30.08 ± 1.77 A	25.94 ± 4.15 A
b*	11.77 ± 2.18 A	11.66 ± 1.68 A	19.36 ± 2.71 A	17.30 ± 3.19 A

[a] Treatments were conducted at 20°C for 64 min. Values are mean ± standard deviation. within the same row, means with the same letter are not significantly different ($P > 0.05$).

[b] Each replication consisted of nine samples with two readings per sample.

[c] Each replication consisted of three samples with two readings per sample.

ter a 5-min treatment at a lower concentration (1.3 mg/liter), and Koseki et al. *(14)* reported a 1.5-log reduction after treatment with 5 mg/liter aqueous ozone for 10 min.

The roughness of the surface and presence of achenes on strawberries and other small fruits *(18)* and the space between the druplets of raspberries make these fruits difficult to decontaminate. The research presented here indicates that aqueous ozone is a promising sanitizer for small fruits. Ozone was applied to raspberries at two different temperatures with a maximum reduction of 5.6 log CFU/g for *E. coli* O157:H7 at 4°C and 4.5 log CFU/g for *Salmonella* at 4°C. The reductions obtained with aqueous ozone were significantly greater than those for washing with water alone, which produced a maximum reduction of 1.3 and 1.1 log CFU/g for *E. coli* O157:H7 and *Salmonella,* respectively. Reductions on strawberries were slightly lower, with maximum reductions of 2.9 and 3.3 log CFU/g for *E. coli* O157:H7 and *Salmonella,* respectively, at 20°C but still were significantly greater than those obtained by washing with water alone. The treatment temperature made no significant difference on the efficacy of the sanitizer; therefore, treatment could be performed at room temperature (20°C), resulting in lower energy costs. This research indicates that aqueous ozone could be an effective sanitizer for small fruits.

ACKNOWLEDGMENTS

This research was funded by the Pennsylvania Experiment Station. We are thankful to Hess Machine International (Ephrata, Pa.) for providing the ozone generator used in this study. We also thank Dr. Larry Beuchat (Center for Food Safety, University of Georgia, Griffin) for supplying the cultures used in this study.

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