Journal of Food Protection, Vol. 72, No. 5, 2009, Pages 952–958

Chlorine Dioxide Gas from an Aqueous Solution: Reduction of *Salmonella* in Wounds on Tomato Fruit and Movement to Sinks in a Treatment Chamber

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MS 08-556: Received 5 November 2008/Accepted 2 January 2009

ABSTRACT

Chlorine dioxide (ClO_2) off-gassed from an aqueous solution and reacted incrementally with potassium iodide solutions (sinks). After 30 min, 45% of the initial dose was detected as chlorite ion in the sink, whereas 35% of the initial dose was still in the source. Aqueous solutions of ClO_2 can be used as a source of ClO_2 gas in various laboratory experiments involving treatment of fruits or vegetables. Movement from source to sink is continuous, which precludes the development of large headspace concentrations and the need for a tight chamber seal. When the source solution has dissipated, the chamber can be opened safely as there is little free ClO_2 remaining in the headspace. In tests with whole, wound-inoculated tomato fruit, at both green and pink stages of ripeness, the control of *Salmonella enterica* serotype Typhimurium in wounds varied with the weight of gas used. The number of viable cells of Typhimurium recovered was reduced by >5 log units when ≥ 0.5 mg of ClO_2 was applied to three pieces of fruit during a 2-h treatment.

Chlorine dioxide (ClO_2) gas has been used effectively to sanitize strawberries *(8),* apples *(4, 5),* and green peppers *(6, 7)* that were contaminated with *Escherichia coli,* and blueberries, strawberries, and raspberries contaminated with *Salmonella (12).* In the latter report *(12),* the gas treatment also reduced naturally occurring populations of yeasts and molds. In most of these tests, ClO_2 was produced by equipment that mixed chlorine gas with solid chlorite (ClO_2^-) *(10).* The ClO_2 gas was applied to the fruits or vegetables as a batch treatment (gas was injected into a chamber containing the commodity) or continuous injection (headspace concentration in chamber was maintained based on spectroscopic measurements). In both types of treatments, the period of treatment was relatively short and excess ClO_2 was eliminated from the headspace at the end of the treatment. The amount of ClO_2 applied reflected treatment conditions and was reported as the weight or volume of ClO_2 per volume of the treatment chamber *(8, 10, 12, 13).*

Treatments involving the continuous introduction of small quantities of chlorine dioxide gas into the space surrounding contamination have also been reported. Small amounts of ClO_2 gas produced continuously from self-activating packets eliminated mildew on library books *(16).* The packets were designed to produce ClO_2 over a period of 15 to 30 days depending on the relative humidity of the atmosphere in which they were deployed. Higher humidity led to faster production. The books were free of mildew at a 6-month posttreatment inspection. Only a slight odor was observed during the treatment (odor threshold, 0.1 ppm, vol/vol).

The incidence of bacterial soft rot during storage of wound-inoculated (*Erwinia carotovora*) tomato (*Lycopersicon esculentum* Mill.) fruit was greatly reduced if the fruit had been exposed to ClO_2 gas in a sealed aluminum container *(11).* The gas was produced over 2 or 24 h by a dry mixture of sodium chlorite and ferric chloride contained in a gas-permeable Tyvek envelope. When the chamber was opened at the completion of these experiments, the odor of ClO_2 was not observed if small doses of ClO_2 were generated, whereas with large doses, the odor was faint. The large doses produced phytotoxicity on the fruit in the form of bleached stem scars and wound surfaces.

The treatment interval (2 to 24 h) used for applying ClO_2 gas to tomatoes for the control of bacterial soft rot *(11)* was considered compatible with commercial tomato handling. Freshly harvested fruit are sometimes held overnight before processing, whereas palletized stacks of packed fruit are stored up to 8 days before shipment.

Problems associated with the use of packets of dry reactants to produce ClO_2 gas in the laboratory include the effect of storage environment, where increased humidity leads to enhanced production *(16)* and efficacy *(6).* If the reactants are enclosed in a Tyvek envelope, their weight and composition are not under control by the laboratory technicians. Variation in either weight or composition affects production of ClO_2 gas *(10, 11).* The reactants must be mixed uniformly and kept together *(14).* Finally, the literature does not clearly indicate that small amounts of ClO_2 continually impinging on microbial cells would cause incremental damage leading to cell death. The report on its

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use in the library suggests that incremental damage occurred to the mildew, since strong odors were not observed and there was no evidence for a bleaching of the book bindings *(16)*.

For laboratory tests with ClO_2 gas, the physical and chemical nature of the compound enables a reproducible method for varying the dose while maintaining a standard environment. In aqueous solutions, ClO_2 exists as a dissolved gas and does not dissociate like Cl_2 *(10, 17)*. It has an appreciable vapor pressure with a concentration in water at 23 times that in the air immediately above the solution *(17)*. Therefore, according to Henry's law *(9)*, ClO_2 should off-gas at a rate that is in part proportional to its concentration in the atmosphere above a solution. If ClO_2 gas is continually removed from the headspace over a solution such as by reactions with various reducing agents (i.e., sinks), then the amount dissolved in the solution should eventually disappear. If this process is continuous and incremental, then a positive headspace air pressure should not develop and the treatment chamber would not require an airtight seal. Therefore, the source of ClO_2 gas for laboratory treatments could be an aqueous solution. The solution could be tested for concentration of ClO_2 gas immediately prior to an experiment. A desired amount of the solution could be added to water to provide a standardized source volume, where ClO_2 stock plus makeup water equal standard volume. The surface area of this solution, volume of the headspace, and laboratory temperature provide for a reproducible level of relative humidity. As a precaution against loss of ClO_2 during the experiment setup, the ClO_2 stock solution could be introduced underneath the makeup water via pipette.

A chemical sink can be placed in the reaction chamber to monitor the movement of ClO_2. The gas dissolves in an aqueous solution of KI buffered to a neutral pH and reacts with the I^- to produce I_2 and ClO_2^- *(14)*. The concentration of these compounds can be determined by titration with a standard thiosulfate solution in which the iodine is reduced to the colorless I^- by titration at a neutral pH *(2, 11)*. The endpoint is sharpened by the addition of a few drops of starch solution. The solution is then acidified to a pH of <2.0 and allowed to stand in the dark for 5 min. At this pH, the acidity converts the ClO_2^- to chlorous acid ($HClO_2$), which reacts with I^- to produce I_2 plus Cl^- plus H_2O. The I_2 is again reduced to the colorless I^- by titration with the thiosulfate. The initial titration involves a one-electron transfer, whereas the second titration involves a four-electron transfer. The amount of standard thiosulfate used for the titrations as adjusted by the number of electrons transferred yields the number of moles of ClO_2 that reacted with I^- in the solution. The number of moles of ClO_2 hereby determined can be converted either to weight or volume. Similarly, an addition of KI to a source solution followed by mixing and titration can be used to determine the amount of ClO_2 that has off-gassed. There is some question about the stability of I_2 formed in the sinks. The element has appreciable vapor pressure (i.e., it will off-gas from aqueous solutions) and can be readily oxidized to a higher oxidation state, which introduces errors in the iodometric titration *(2)*. By contrast, in a neutral pH solution the ClO_2^- reaction product is relatively stable, is not subject to off-gassing, and will not oxidize I^-. Thus, the most accurate method to check for sink absorption of ClO_2 may be to eliminate the color of the solution (I_2) with thiosulfate, acidify the solution, and then titrate the I_2 formed as the ClO_2^- is reduced to Cl^-. Herein, we report an incremental mass transfer of ClO_2 gas from an aqueous solution to sinks within a treatment chamber and then demonstrate that this mass transfer can be used to inactivate *Salmonella enterica* placed in wounds on tomato fruit to an extent predicted by varying initial doses of ClO_2. Portions of this report were published previously as an abstract *(1)*.

MATERIALS AND METHODS

Production of aqueous solutions of ClO_2. A concentrated aqueous solution of ClO_2 was produced in deionized water from a Tyvek (DuPont Corporation, Wilmington, DE) sachet that contained dry granules of $NaClO_2$ and a formulation of $FeCl_3$ that served as an acid activator (ICA Tri-Nova LLC) *(10)*. Folded sachets with chlorite in one side and activator in the other were unfolded, vigorously shaken, and then submerged in the water for up to 72 h. The resulting solution was stored at 4°C in an amber-colored glass bottle, which was closed with a ground glass stopper. Prior to each test, the ClO_2 concentration in this stock solution was determined by iodometric titration *(2, 11)*. A similar titration was used to determine the amounts of ClO_2 and chlorite ion in the various aqueous solutions.

Treatment chambers. A Lexan (SABIC Innovative Plastics, Houston, TX) polycarbonate lid was placed over a 19-liter rectangular clear polycarbonate bin (model 6318, Rubbermaid, Inc., Fairlawn, OH). A plastic box fan (12 V; diameter, 72 mm) was attached to the undersurface of the lid to keep the air within the chamber uniformly distributed (Fig. 1). The power wires for the fans were threaded through a sealed hole in the lid and were each connected in parallel to a single DC power supply that was plugged into a standard outlet. Preliminary tests were conducted to determine if the lid had to be sealed to the bin in order to retain the ClO_2. Tested sealants included silicone caulk (Silicone II Kitchen and Bath, GE Silicones, Waterford, NY) and weather stripping (high-density, closed-cell, PVC Foam Tape Weatherstrip, ¼ in. thick by ½ in. wide; M-D Building Products, Inc., Oklahoma City, OK).

Inoculum. *S. enterica* serovar Typhimurium LT2 (ATCC 15277) that was adapted for resistance to rifamycin (Rif) was cultured on nutrient agar plates plus 200 μg of L-1 Rif. Cells were transferred to nutrient broth with 2% dextrose and 200 mg of L-1 Rif and then incubated for 16 to 20 h on a shaker (~50 rpm) at 30°C. Cells were removed from the broth by centrifugation (IEC Centra MP4R, International Equipment Co., Needham Heights, MA) at 450 × *g* for 10 min. The supernatant was discarded, and then the pellet was resuspended in sterilized tap water (STW, pH 8.3) as a wash step. Cells were treated three times in this manner (broth recovery plus two wash steps). The concentration of bacteria in the final suspension was estimated from the optical density in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY) set at 600 nm, based on a previously determined regression of optical density versus CFU per milliliter. The stock solution was diluted as needed. Samples of the LT2 cultures were serially diluted and pour plated for verification of initial inoculum density.

FIGURE 1. *Treatment chambers with fan (A), dry $FeCl_3$ plus $NaClO_2$ in petri dish (B) or sachet (C), and aqueous solutions of KI as ClO_2 sinks (D).*

Fruit. Green tomato fruit (Florida-47) of either 6×6 or 5×6 size was obtained from a commercial fruit packer. The fruit was stored at 12.5°C and 90% relative humidity prior to use. For trials on pink fruit, green fruit was stored at 20°C and 90% relative humidity until it reached the desired stage of ripeness. Before each experiment, fruit was removed from storage and allowed to warm to room temperature (~22°C). Ripeness levels were estimated based on a commercial fruit color chart *(15).* The fruits utilized in this study were selected to be free of blemishes and defects.

Inoculation. A scalpel was used to remove five sections of epidermis that were each ca. 2 cm in diameter and about 1 mm deep. The wounds were evenly spaced in a radial pattern about halfway between the equator and the blossom scar *(11).* A 10-μl sample of an aqueous cell suspension of bacteria was then applied to each wound. The inoculated wounds were allowed to dry for 20 min before treatment.

Treatments. The movement of ClO_2 gas within the treatment chambers was evaluated by comparing the loss of the chemical from an aqueous solution (off-gassing) with the appearance of I_2 and ClO_2^- in nearby aqueous solutions of iodide ion in 0.5 M phosphate buffer with a pH of 7.0. The solutions were placed in 90-mm-diameter petri dish bottoms (surface area, ca. 64 cm^2). Treatments were initiated by using a pipette to deliver a sample of the stock solution to the bottom of a container of deionized water. The final solution had 2.2 to 6 mg of ClO_2 in 30 ml of water, depending on the test. Next, a lid was placed on the chamber and the fans were activated. Several chambers were prepared in this manner such that one could be opened at 0.5, 1, 2, 4, 6, or 24 h after treatment initiation. At each time, a buffered solution containing 2 g of KI was quickly mixed with the source solution to react with any remaining ClO_2. Both the source and the sink dishes were removed from the chamber, and their contents were titrated for I_2 and ClO_2^-. All calculations were converted to milligrams of ClO_2 or as a percentage of the milligrams of ClO_2 applied.

In a series of tests, one to five 30-ml containers of KI solution were placed in the same chamber to find if the sink surface area (64 to 320 cm^2) or volume (30 to 150 ml) affected the rate of off-gassing of ClO_2 from the aqueous solution or the amount of ClO_2 captured. In a second series of tests, the amounts of ClO_2 captured by KI sinks were compared for three sources of ClO_2: (a) off-gassing from an aqueous solution, (b) production by a two-dry-component mixture placed in a Tyvek envelope, or (c) two dry components mixed together in a glass beaker. The chambers were prepared as above, an average of 5.5 mg of ClO_2 was used in the aqueous source treatment, 1.2 g of each dry reactant (activator and chlorite) was placed in the envelope, and a similar amount was placed in the glass beaker. According to the supplier, equal amounts of chlorite plus activator produce 5 mg of ClO_2 gas/g of chlorite within 2 h *(14).* Thus, the two dry systems had the potential to produce 6 mg of ClO_2 gas over a period of 2 h. The chambers were opened after 2 h, and the contents of the sinks were titrated as described. The amount of ClO_2 gas captured by the sinks was expressed as a percentage of the initial dose.

For tests on the efficacy of ClO_2 off-gassed from an aqueous solution for sanitizing tomato fruit contaminated with *S. enterica* serotype Typhimurium, three fruits of green or pink ripeness stages were weighed, wounded, inoculated with bacteria, and then placed into treatment chambers. A sample of the aqueous stock solution of ClO_2 was delivered to an open petri dish as above. After 2 h, the fans were stopped and the chambers were opened. For each trial, a set of fruit was wounded (but not inoculated) and treated (negative control), and a second set of fruit was wounded and inoculated but not treated (positive control) for comparison with the inoculated and then treated fruit.

For recovery of surviving bacteria, individual fruits were immediately placed in sterile stomacher bags (Secure T, no. 01-002-55, Fisherbrand, Fisher Scientific Co., Ottawa, Ontario, Canada) containing 100 ml of sterilized 0.1% peptone broth. The bags were sealed and then treated with the rub-shake-rub method for removing bacteria from produce surfaces, as described by Burnett and Beuchat *(3).* A 500-μl sample of the broth was removed from the bags and then serially diluted 1:10 a total of three times (3-log maximum dilution) in test tubes containing 4.5 ml of sterile 0.1% peptone broth. A 1-ml sample was then taken from each dilution and pour plated in molten nutrient agar containing 100 mg of L-1 Rif. Plates were stored at 30°C for 72 h, and then Rif-resistant colonies were counted. The counts of plates containing 25 to 250 CFU were used to determine total CFU per fruit. Data from the three separate experiments were averaged, expressed as milligrams of ClO_2 per kilogram of fruit (to equalize the mass of fruit treated with each dose), and the standard deviation was used to provide 95% confidence internals.

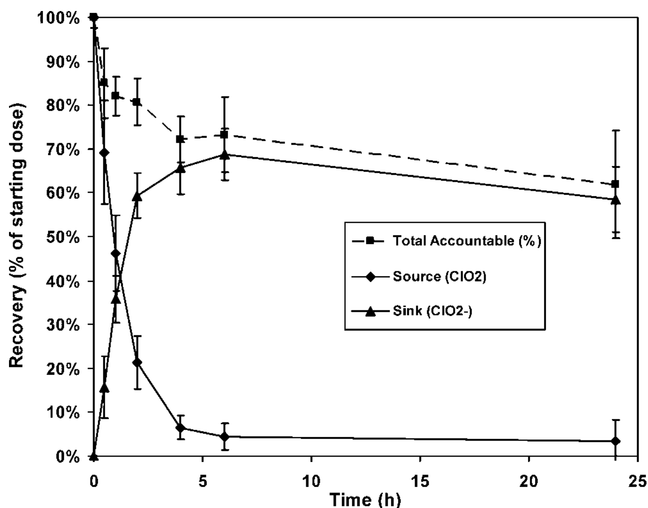

FIGURE 2. *Off-gassing of ClO_2 from an aqueous solution in polycarbonate chambers containing an aqueous solution of KI as a sink. Recovery in sink is in milligrams of ClO_2^-; source is in milligrams of ClO_2. All values are given as percentage of source.*

RESULTS

The amount of ClO_2 detected in the freshly prepared solutions that had been placed in the treatment chambers (Fig. 1) equaled the calculated quantity in the stock solution that had been added to those solutions (data not shown). Thus, the dilution of the stock solution, followed by the immediate addition of KI and then titration as described above did not lead to a loss of a significant amount of ClO_2.

When the lids were sealed to the polycarbonate chambers with silicone caulk, the ratio of the thiosulfate used in the neutral (for ClO_2) and acid (for ClO_2^-) titrations of the source solutions decreased from 1:4 at the start of a test to 1:38 to 1:49 after 2 h (data not shown). By contrast, when weather stripping was placed around the edge of the lid, the ratios were 1:6 to 1:9. When the lids were held in place by weights, the ratio remained approximately 1:4. This was evidence that volatile gasses from the caulk and weather stripping were being oxidized by the ClO_2 in the source solution. The capture of ClO_2 by the KI sinks in the caulk-sealed chambers was about 40% less than that in chambers without sealant (lids held down with weights), whereas when weather stripping was used, the amount captured was about 20% less than that occurring in the nonsealed containers. Subsequently, the lids were simply held in place with weights.

The off-gassing of ClO_2 from an aqueous source of the oxidizer was nearly complete within 4 h (Fig. 2). A violet color typical of iodine began appearing in the sink solutions within minutes after the chambers were closed. By 15 min, the source had lost 30% of the initial dose, whereas the sink had captured 50% of that off-gassed ClO_2 (Fig. 2). As the treatment progressed, the amount of I_2 detected in the sinks decreased, whereas the chlorite concentrations increased or remained relatively stable. The apparent capture of ClO_2 lagged behind the off-gassing at all time intervals. For example, during the period of 30 to 60 min after start of a test, the rate of off-gassing was 0.051 mg/min, whereas the rate of capture in the KI sink was 0.024 mg/min. For the period of 60 to 120 min, the respective values were 0.021 and 0.013 mg/min. When off-gassing was complete or nearly so, capture also ended. At the initial observations, the sum of the quantity of ClO_2 in the source and the ClO_2^- in the sink at 30, 60, or 120 min equaled $>$80% of the starting dose. By 6 h, that total had decreased to about 70%, and by 24 h, only 62% of the initial dose could be detected and virtually all of this was ClO_2^- as the I_2 disappeared from the sink based on not only the titration but also loss of visible color.

With multiple sinks and a single source, the amount of ClO_2 detected in the source solution at 1 h ranged from 48 to 50% of the calculated initial dose (data not shown). In a second test, these values ranged from 51 to 43%, respectively (not shown). The surface area of the sinks used in these tests ranged from 64 to 320 cm^2, whereas the volume of the buffered KI ranged from 30 to 150 ml, respectively. In a third test with a 2-h exposure, the amount of ClO_2 detected in the source solution for one, two, or four sinks averaged 23% (Fig. 3). The amount of ClO_2 captured averaged about 88% of the amount off-gassed and did not

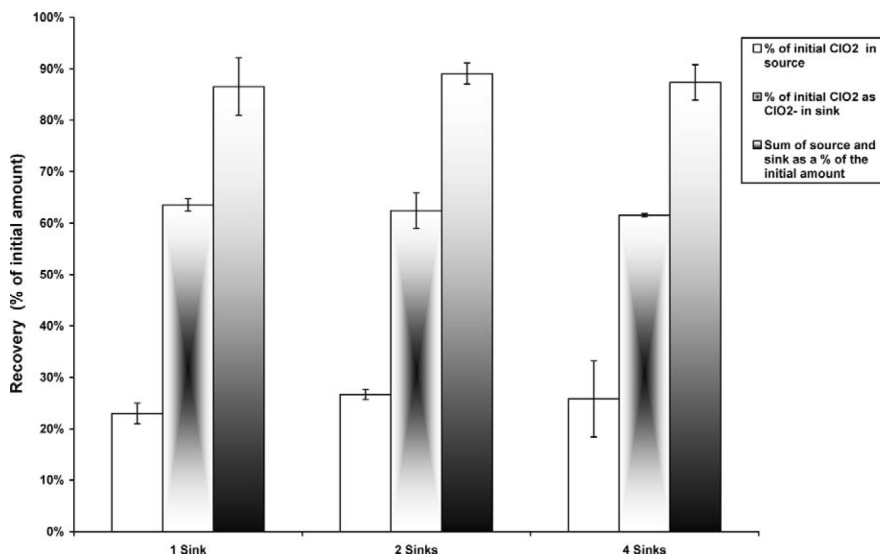

FIGURE 3. *Off-gassing of ClO_2 from an aqueous source and recovery by 1 to 4 sinks in a closed container with closed air circulation after 2 h.*

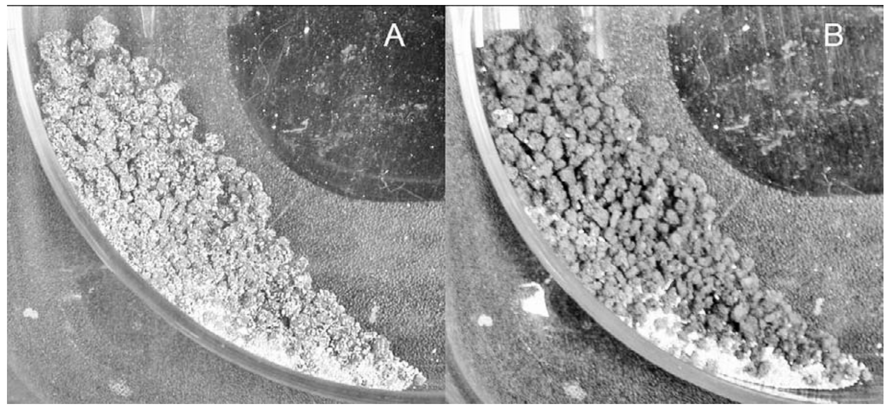

FIGURE 4. *Dry materials in petri dish before (A) and after (B) a 2-h treatment.*

change significantly as the number of sinks increased. Thus, with these treatment chambers, the rate of off-gassing of ClO_2 from an aqueous solution and subsequent incremental flow from that source to a sink was not affected by the volume or surface area of the sinks.

When ClO_2 was produced over a 2-h period from dry materials (Figs. 4 and 5), the amount found in a sink was 59 to 83% of the amount of ClO_2 that was calculated to be yielded by the weight and ratios of the reactants used (Fig. 6). By contrast, 69% of the amount placed in the aqueous source was detected in the sink. The highest value occurred when the generation came from a mixture that was enclosed in a packet (Fig. 5), whereas the lowest value occurred when the materials were mixed together in a glass dish (Fig. 6). In the initial mixture, the light-colored granules of sodium chlorate were uniformly mixed with the dark red granules of the $FeCl_3$ formulation (Fig. 6A). After the treatments, those enclosed in the closed packet remained uniformly mixed (Fig. 5), whereas those in the glass dish had separated (Fig. 6B).

With fruit inoculated with *Salmonella* Typhimurium LT 2, treatment for 2 h with doses expressed as weight of ClO_2 gas applied per kilogram of fruit led to a decrease in recoverable bacteria as the dose increased for both green and pink fruit (Fig. 7). The different doses were provided by off-gassing from aqueous solutions. There was no apparent odor of ClO_2 gas when the chambers were opened at the end of the 2-h treatment. Thus, an incremental mass flow of ClO_2 gas from an aqueous solution as modeled in the source/KI sink tests produced an effective treatment for tomatoes that were contaminated with *Salmonella* Typhimurium.

DISCUSSION

Weight of ClO_2 gas produced by dry ingredients or off-gassed from aqueous solutions in the tests reported here ranged from 2.2 to 6 mg/19-liter treatment chamber. On a chamber volume basis, if the gas flashed off immediately, this converts to a maximum of 0.12 to 0.32 mg/liter (wt/vol) or 42 to 116 ppm (vol/vol). Obviously, this did not occur since no loss was detected when a source solution was prepared and then a solution of KI was immediately mixed with it. Similarly, regressions of weight of ClO_2 produced over time by different ratios of the dry ingredients involved moving an activated sachet from one glass jar to another with each containing a KI sink *(11)*. The regressions were smooth with no evidence of a significant loss of ClO_2 gas during the sachet transfers. The highest rates of gas production for both the aqueous solution and the activated sachet should be at the start, when the concentration in water is the highest and the amounts of the two reactants were highest, respectively. However, the most rapid off-gassing occurred between 60 and 120 min, apparently due to a slow equilibration of the dissolved ClO_2 within the solution (stock was introduced under the surface of the

FIGURE 5. *Sachet containing dry materials, opened after a 2-h treatment.*

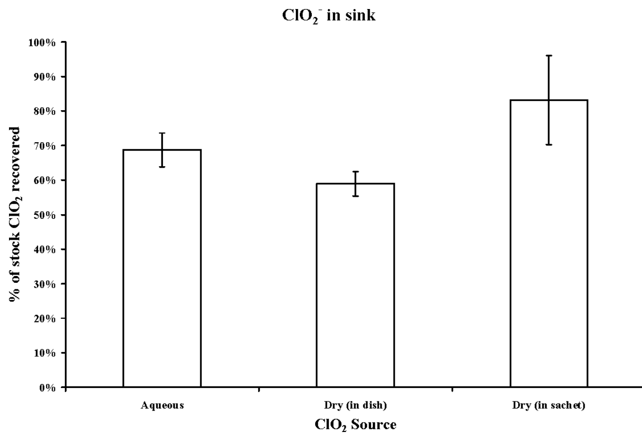

FIGURE 6. *Recovery of ClO_2 by an aqueous solution of KI. The ClO_2 had been off-gassed from an aqueous solution (average amount supplied, 5.5 mg) or produced by a dry source (theoretical amount produced, 6.0 mg) over a period of 2 h in a sealed chamber with closed air circulation. Values are given as percentages of initial doses.*

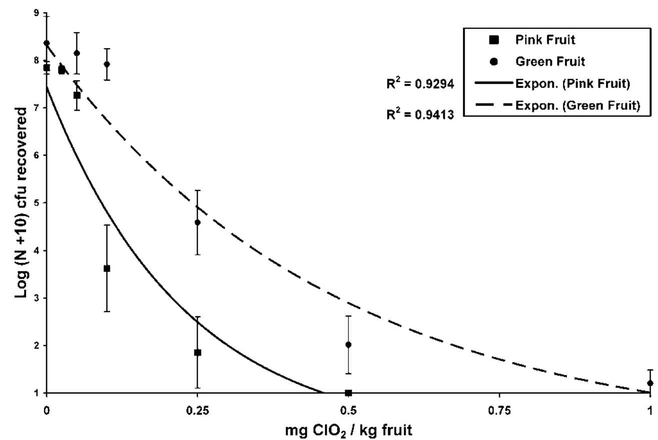

FIGURE 7. *Recovery of* S. enterica *serovar Typhimurium strain LT 2 cells from wound inoculated red or green tomato fruit that had been treated with ClO_2 gas for 2 h. Detection limit, 3 log.* P *< 0.05.*

deionized water used to dilute the solution to a standard volume).

Sealing the lids to the chamber proved unnecessary. Moreover, the two sealants, silicone caulk or weather stripping, released volatiles that dissolved in the source and were oxidized by ClO_2. In the absence of sealing the lids to the chambers, over 65% of the ClO_2 placed in the source solution was detected as ClO_2^- in the KI sink at 24 h after the treatment began. The fate of the remaining ClO_2 is unclear. Virtually none was left in the source solution by 6 h. The accumulation of ClO_2^- in the sink solutions also ceased after 6 h. Some leakage around the unsealed lids might have occurred. Alternatively, some of the chlorine dioxide may have dissolved in, or reacted with, the polycarbonate walls of the chamber. The failure of the chlorite ion concentrations in the sinks to keep increasing at longer exposures was evidence that little gas remained in the headspace.

The amount of ClO_2 gas used in each treatment was controlled more precisely when aqueous solutions were used than when produced from dry ingredients. Moreover, the water in these solutions would provide increased relative humidity, which has been reported to enhance the activity of ClO_2 gas against microbes *(6)*. Two types of dry ingredients were used as sources of ClO_2 gas. The production was more efficient when the dry materials were held together in an envelope than when mixed together in a glass dish. Visually, the mixture of two ingredients appeared to separate in the dish (Fig. 4). Apparent desiccation of the previously moist activator (dark grains) has allowed the smaller white grains of solid ClO_2^- to separate out, disrupting contact and thus chemical reactions between the two.

The titration of I_2 formed in a solution of KI appeared to be an accurate measurement of the total amount of ClO_2 that dissolved in that solution if the titration occurred immediately *(11)*. However, during longer treatments, the concentration of the ClO_2^- ion appeared to be a more accurate parameter for ClO_2 captured. The I_2 produced by the ClO_2 oxidation of I^- has an appreciable vapor pressure *(2)* and is likely to off-gas. In the tests reported here, I_2 concentrations as denoted by color of the solution and by titration at a neutral pH disappeared with treatment intervals of 24 h.

Providing ClO_2 gas by off-gassing from an aqueous solution effectively eliminated *Salmonella* Typhimurium LT2 from wounds on tomato fruit (Fig. 7). The log reduction increased as dose increased in an exponential manner with a highly significant R^2 value. The total amount applied was lower than doses that have been reported previously (*4–7, 9*). Based on the source-sink mass flow tests reported here, the tomato fruit had clearly received an incremental exposure to ClO_2 gas. High headspace concentrations were not required to disinfect tomato fruit. If incremental exposure is sufficient to sanitize tomato fruit, then doses may be based on the strength of the various sinks in the area being treated. If tomato fruit is the strongest sink in a treatment chamber, then it may be possible to base doses on fruit weight. Aqueous solutions of ClO_2 gas provide a reproducibly efficient way to test the effect of the gas as a sanitizer on various fresh fruits or vegetables. The dose can be varied as needed for a particular test. The stock solution is produced without concerns over use and maintenance of equipment that mixes concentrated acids and chlorine gas. The stock is easily tested prior to the start of a test as a measure of quality control. The technique provides a source of the gas while maintaining optimum relative humidity for fresh products.

ACKNOWLEDGMENTS

We thank the Florida Tomato Committee for their continued financial support. We also thank the DíMare Fresh Corporation of Tampa, Florida, for supplying fresh fruit.

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