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ORIGINAL PAPER

Evaluation of Steady-State Gaseous Chlorine Dioxide Treatment for the Inactivation of Tulane virus on Berry Fruits

David H. Kingsley[1] · Bassam A. Annous[2]

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Abstract

The effectiveness of steady-state levels of gaseous chlorine dioxide (ClO_2) against Tulane virus (TV), a human norovirus surrogate, on berries was determined. The generated ClO_2 was maintained at 1 mg/L inside a 269 L glove box to treat two 50 g batches of blueberries, raspberries, and blackberries, and two 100 g batches of strawberries that were immersion coated with TV. The standardized/normalized treatment concentrations of ClO_2 ranging from 0.63 to 4.40 ppm-h/g berry were evaluated. When compared to untreated TV contaminated berries, log reductions of TV were in excess of 2.9 log PFU/g for all berry types and conditions tested, indicating that ClO_2 was highly effective. In general, the efficacy of all ClO_2 treatments on log reductions of TV on all berries was not significantly different ($p < 0.05$). The average log reduction with strawberries, raspberries, blueberries, and blackberries, treated with the lowest ClO_2 concentration, 0.63 ppm-h/g, were 2.98, 3.40, 3.82, and 4.17 log PFU/g, respectively. Overall results suggest that constant levels of ClO_2 could be quite effective against foodborne viruses.

Keywords Norovirus · Tulane virus · Gaseous chlorine dioxide · Blueberry · Raspberry · Blackberry · Strawberry

Introduction

Human norovirus (HuNoV) is thought to cause approximately 700 million illnesses and 220,000 deaths worldwide every year (Bartsch et al. 2016), 14% of which is thought to be foodborne (Verhoef et al. 2015). Foodborne transmission of HuNoV is via the fecal-oral route or by consumption of food exposed to aeorsolized vomit (Kirby 2016). Produce can pose substantial foodborne illness risk since it is often consumed raw (Hall et al. 2013). As a result, norovirus and other foodborne viruses (i.e., hepatitis A) are a primary concern for berry producers and consumers which can be contaminated as a result of irrigation, soil, animal manure, mixture of fertilizers or pesticides with non-potable water, unsanitary ice or rinse water, and by unclean harvest equipment or human handling (Olaimat and Holley 2012). These non-enveloped viruses are environmentally persistent and resilient, resisting ethanol and most disinfectants (Fraisse et al. 2011; Kingsley et al. 2014; Nowak et al. 2011; Tung et al. 2013). Freezing and freeze-drying is not effective against foodborne viruses (Butot et al. 2009; Richards et al. 2012) and post-harvest processing options for berries are limited, especially dry technologies which are preferred by the berry industry since water can potentially contribute to mold growth which could results in product spoilage.

One prospective intervention is gaseous chlorine dioxide (ClO_2). Chlorine dioxide is a true gas (greenish yellow) at room temperature with effective biocidal activity over a wide range of pH 3–8 and temperature (Bernarde et al. 1976; Keskinen and Annous 2011; Saade et al. 2018). Chlorine dioxide received FDA approval in 2001 to reduce or eliminate microorganisms in a wide variety of food products such as fruits and vegetables (Rulis 2001). The use of ClO_2 in the gas form is generally more effective than the liquid disinfectants including the liquid ClO_2 form (Banach et al. 2015; Keskinen and Annous 2011; Montazeri et al. 2017; Parish et al. 2003) since it has the potential to reach areas on product

✉ Bassam A. Annous
bassam.annous@ars.usda.gov

1 Food Safety and Intervention Technologies Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Delaware State University, 1200 DuPont Hwy, Dover, DE 19901, USA

2 Food Safety and Intervention Technologies Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

surfaces that liquid disinfectants cannot due to hydrophobic fruit coatings and possible surface air pockets (Keskinen and Annous 2011; Olaimet and Holley 2012). Chlorine dioxide gas readily diffuses into inaccessible sites and microbial biofilms to inactivate human pathogens attached to produce surfaces (Annous and Burke 2015; Prodduk et al. 2014). Chlorine dioxide is a selective and strong oxidizing agent, which unlike chlorine does not chlorinate organic compounds to produce carcinogenic trihalomethanes (Annous and Burke 2015; DiCristo et al. 2013; Fan and Sokorai 2015; Keskinen and Annous 2011; Saade et al. 2018). Also, it does not react with ammonia to form chloramines (Annous and Burke 2015; Keskinen and Annous 2011; Oxenford 1995), which makes it very attractive for use as an antimicrobial in foods. High humidity is a critical parameter for ClO_2 with significant differences in bacterial inactivation observed between 70 and 90% (Park et al. 2018).

Chlorine dioxide is highly effective in inactivating *Escherichia coli* O157:H7, *Salmonell*a, and *Listeria monocytogenes* (Annous and Burke 2015; Prado-Silva et al. 2015), some parasites (Ortega et al. 2008), and yeasts, mold and bacillus spores on berry fruits (Popa et al. 2007; Shiraski et al. 2016, Sy et al. 2005; Wu and Kim 2007). Chlorine dioxide fumigation has been shown to effectively inhibit microbial growth and improve shelf-life of strawberries and blueberries (Sun et al. 2014; Yang et al. 2018). Also, pathogens on sprouting seeds and sprouts can be eliminated by ClO_2 (Annous and Burke 2015; Produduk et al. 2014). Evaluation of sensory qualities of ClO_2-treated blueberries, strawberries, and red raspberries generated by sachet treatment of 4.1 mg/l for 30 min indicated that the treatment did not compromise the sensory quality of berries (Sy et al. 2005), although some "bleaching spots" were observed on strawberries that subsequently disappeared after 3 days of refrigerated storage.

Kingsley et al. (2018) reported that blueberries treated with ClO_2 generated in a small chamber with acidified sodium chlorite solution inactivated the Tulane virus (TV), the human norovirus surrogate, without ostensibly altering the appearance and quality of the blueberries. That previous study evaluated application of a bolus of ClO_2 which then rapidly decayed over time and the previous treatment was only applied to a small number of blueberries. Since different amounts and types of produce can potentially have different chlorine dioxide oxidation burdens, it is difficult to directly compare batch applications of ClO_2. Application of constant levels (steady state) of ClO_2 is independent of different oxidative burdens presented by different fruits, amounts and commodity types offering a key advantage over ClO_2 producing sachets which produce a pulse specific quantity of ClO_2.

The objective of this study was to investigate the efficacy of steady-state ClO_2 treatments on inactivation of Tulane virus, a human norovirus surrogate, on four different berries, raspberries, blackberries, blueberries, and strawberries.

Materials and Methods

Cells and Virus

Tulane virus (TV) was provided by Dr. Haiqiang Chen, University of Delaware. Tulane virus was propagated using monolayers of the monkey kidney cell line MK2-LLC cultured at 37 °C in low serum Eagle's minimum essential medium (Opti-MEM; Gibco, Paisley, PA), supplemented with 2% fetal bovine sera (FBS; Atlanta Biologicals, Flowery Branch, GA), 100 U/ml penicillin/streptomycin and Glutamax (Gibco), under a 5% CO_2 atmosphere. Virus stocks were prepared by freezing and thawing, pelleting of debris by centrifugation at 1000×*g* for 30 min, followed by 0.2 µM filtration three days post-infection. Titers of TV stocks were approximately 6 log PFU/ml.

Berry Contamination and Virus Extraction

Fresh strawberries, raspberries, blackberries, and blueberries were purchased from a local supermarket. Approximately two 50 gm each of raspberries, blackberries, and blueberries, and two 100 gm of strawberries were contaminated with 10 ml of TV inoculum using sterile sealed beakers. The beakers containing the sample and the TV inoculum were rotated and inverted several times for approximately 1 min. Inoculum was then decanted, and berry fruits were placed in Safe-T-Fresh clamshell containers (TS-12, Inline Plastics Corp., Shelton, CT) with paper towel for several minutes after which the paper towel was removed, and berries were then allowed to dry inside a biosafety cabinet for 1 h prior to treatment.

Chlorine Dioxide Treatment

Chlorine dioxide treatments were conducted as previously described by Alicea et al. (2018) using ClorDiSys Minidox-L chlorine dioxide generation system (ClorDiSys Solutions inc.; Lebanon, NJ). Briefly, the generated ClO_2 gas was pumped into a 269-L sealed glove box (815-PGB; Plas-Labs Inc, Lansing, MI) and the concentration of the gas inside the chamber was controlled by the on board electronics. Two clamshells of each inoculated berries were treated inside the chamber at room temperature (RT = 21 °C) and 90–95% humidity. Chlorine dioxide gas was generated and continuously pumped into the chamber and maintained at 1 mg/L of air for the required time to obtain the desired treatment concentrations (ppm ClO_2-h per gm of treated berry). Two trials were conducted using two 50 gm of blueberry, raspberry,

and blackberry, and two 100 gm of strawberry for each treatment group. Samples were then analyzed for residual TV populations following treatment.

Tulane Virus Quantification

Following ClO_2 treatment, the TV was eluted from the berries using 10 ml of the elution buffer (100 mM Tris-HCl, 50 mM glycine, 50 mM $MgCl_2$ and 1% soy protein at pH 9.5) and gently agitating the berries in sterile beakers for approximately 1 min. The eluted virus was removed, and the pH was neutralized to 7.0 with 2N HCl (approximately 300 µl). Extracted TV was quantified by infection of confluent monolayers of MK2-LLC cells with 10-fold serial Earle's balanced salt solution (Gibco) dilutions. Cell inoculation was performed for 2 h, manually tilting cell monolayers plates every 15 min at 37 °C to ensure uniform infection. Overlay with a 1:1 mix of 2X Opti-MEM (37 °C) and 3% low melting agarose at 42 °C (Fisher; Waltham, MA) was subsequently performed after inoculum removal. Infected cells were incubated for 4 days in a tissue culture incubator at 37 °C, followed by staining of virus plaques with 1 ml of a 1:10 dilution of a 0.33% neutral red (Fisher) stock for 4 h. Log reductions of TV plaques were calculated based on the amount of TV extracted from inoculated samples without ClO_2 treatment in comparison to treated samples. Where no TV plaques were observed in treated samples, the assumed maximum reduction was determined by subtracting the amount of TV extracted from untreated berries minus the limit of detection (0.67 log PFU/ml).

Statistical Analysis

The experiments were conducted twice as a randomized complete block with two replicates for each trial. Analysis of variance using general linear model was performed using SAS/STAT software version 9.1 (SAS Institute, Cary, NC). The data were analyzed to determine significant differences ($p \leq 0.05$) among mean log reductions in TV levels in response to ClO_2 treatments.

Results and Discussion

The goal of this study was to determine the feasibility of using steady-state ClO_2 treatment to inactivate Tulane virus (TV), a human norovirus (HuNoV) surrogate, on the surface of berry fruits. Currently, HuNoV propagation is very challenging (Ettayebi et al. 2016; Costantini et al. 2018) making direct assessment of its inactivation difficult and as a result many of its properties remain largely uncharacterized. As a result, TV was chosen as surrogate based on its genetic similarities to the HuNoV, its ease of assay and propagation, its robustness (Cromeans et al. 2014; Hirneisen and Kniel 2013; Tian et al. 2013), and its ability to interact with porcine gastric mucin (Dancho et al. 2012; Li and Chen 2015).

The concentration of ClO_2 was calculated and reported in this manuscript as ppm ClO_2-h per g treated product. This method of reporting ClO_2 concentration would allow normalization / standardization of the reported data for the purpose of comparing different treatments, taking into account the total amount of ClO_2 needed to treat set weight of product, and their efficacies. Currently, almost all data reported in the literature refer to ClO_2 concentration as mg/L air or ppm without reporting the weight of the treated products and/or the required treatment time. Thus, the data reporting method, reported here, allows for the ability to compare different treatments using similar (standardized) scale. Also, this method of data reporting allows for the use of reproducible concentrations based on product weight to be treated and the total concentration of ClO_2 needed.

Here, we demonstrate that ClO_2 is an effective intervention for virus-contaminated blueberries, strawberries, raspberries, and blackberries. Two 50 g each of blackberries, blueberries, and raspberries, and two 100 g strawberries were contaminated by immersion in 10 ml of TV, placed in commercial clamshell containers and treated with ClO_2 at a constant level (1 mg/L air) of ClO_2 for the required time to obtain the desired treatment concentration of ppm ClO_2-h per g treated product. Log reductions in TV titer of treated samples (Tables 1, 2, 3, 4) are based on virus extracted from untreated samples (Tables 1, 2, 3, 4) as compared to those from treated samples. Chlorine dioxide treatments of all berries were in the range of 0.63 to 4.4 ppm-h/g.

Log reductions in TV on all berries under different ClO_2 concentrations are shown in Tables 1, 2, 3 and 4. Overall it is evident that constant ClO_2 is quite effective against TV with all treatments resulting in as much as 4.75 log reductions. The average log reductions with strawberries (Table 1), raspberries (Table 2), blueberries (Table 3), and blackberries (Table 4), treated with 0.63 ppm-h/g, the lowest concentration tested, were 2.98, 3.40, 3.82, and 4.17 log PFU/g, respectively. There was no clear pattern which might indicate that TV is more difficult to inactivate on a specific berry type under all ClO_2 treatments tested. Further, it is evident that treatments of 1.25 ppm-h/g which resulted in at least a 4-log reduction for all berries are sufficient for TV inactivation (Tables 1, 2, 3, 4). While 2.35 and 3.01 ppm-h/g did not give reductions that were as substantial as 1.25 ppm-h/g, this less impressive reduction may be due in part to the lower virus titer extracted from the untreated samples for those treatment groups (Tables 1, 2, 3, 4). The effect of ClO_2 concentrations on log reductions in TV on all berries was not significantly different ($p \leq 0.05$) within each berry treated. Overall analysis of data across all berries treated showed that the lowest ClO_2 treatment of 0.63 ppm-h/g could be an

Table 1 Efficacy of chlorine dioxide gas (ClO_2) treatment in reducing Tulane virus (TV) population on artificially inoculated strawberry

ClO_2 treatment concentration used (ppm-h/g product)	Initial TV population before treatment (log PFU/g)	Reductions in TV population after treatment (log PFU/g)[a]
0.63 (1 mg/L)	5.32 ± 1.01	2.98 ± 1.14 A
1.25 (1 mg/L)	6.01 ± 0.21	4.22 ± 0.90 B
2.35 (1 mg/L)	5.51 ± 0.11	3.75 ± 1.11 AB
3.01 (1 mg/L)	4.87 ± 0.80	3.66 ± 1.09 AB
3.03 (2 mg/L)	5.69 ± 0.00	4.72 ± 0.21 B
4.40 (2 mg/L)	5.15 ± 0.64	4.00 ± 1.03 AB

Samples were inoculated with TV and allowed to dry for 2 h prior to treatment. Sample were then treated under a constant 1 mg ClO_2/L air for the appropriate exposure time to achieve the required final concentration of ppm-h/g product

[a]Samples with similar letters within a column are not significantly different ($p \leq 0.05$)

Table 2 Efficacy of chlorine dioxide gas (ClO_2) treatment in reducing Tulane virus (TV) population on artificially inoculated raspberry

ClO_2 treatment concentration used (ppm-h/g product)	Initial TV population before treatment (log PFU/g)	Reductions in TV population after treatment (log PFU/g)[a]
0.63 (1 mg/L)	5.21 ± 0.33	3.40 ± 0.70 AB
1.25 (1 mg/L)	6.00 ± 0.08	4.77 ± 0.76 C
2.35 (1 mg/L)	5.58 ± 1.07	2.93 ± 0.44 B
3.01 (1 mg/L)	5.05 ± 1.05	3.71 ± 1.45 AC
3.03 (2 mg/L)	5.79 ± 0.00	3.68 ± 0.00 AC
4.40 (2 mg/L)	5.97 ± 0.29	4.70 ± 0.56 C

Samples were inoculated with TV and allowed to dry for 2 h prior to treatment. Sample were then treated under a constant 1 mg ClO_2/L air for the appropriate exposure time to achieve the required final concentration of ppm-h/g product

[a]Samples with similar letters within a column are not significantly different ($p \leq 0.05$)

Table 3 Efficacy of chlorine dioxide gas (ClO_2) treatment in reducing Tulane virus (TV) population on artificially inoculated blueberry

ClO_2 treatment concentration used (ppm-h/g product)	Initial TV population before treatment (log PFU/g)	Reductions in TV population after treatment (log PFU/g)[a]
0.63 (1 mg/L)	5.03 ± 0.83	3.82 ± 0.45 A
1.25 (1 mg/L)	5.92 ± 0.23	4.09 ± 1.07 A
2.35 (1 mg/L)	5.47 ± 0.45	4.64 ± 0.36 A
3.01 (1 mg/L)	4.19 ± 0.83	3.00 ± 1.10 A
3.03 (2 mg/L)	5.59 ± 0.21	4.62 ± 0.21 A
4.40 (2 mg/L)	5.63 ± 0.23	3.62 ± 0.77 A

Samples were inoculated with TV and allowed to dry for 2 h prior to treatment. Sample were then treated under a constant 1 mg ClO_2/L air for the appropriate exposure time to achieve the required final concentration of ppm-h/g product

[a]Samples with similar letters within a column are not significantly different ($p \leq 0.05$)

effective treatment for inactivation of foodborne viruses on berries.

Assuming that TV responds to ClO_2 in a manner similar to human norovirus, it would appear that a concentration of a minimum of 1 ppm-h/g treated berries should be generally sufficient for ensuring sanitization of berries. Human norovirus and other enteric viruses do not replicate within foods, and detection of these viruses is challenging because these viruses are ordinarily present at very low levels. Thus a 3-log reduction generated by ClO_2 should be capable of inactivating of all but the most grossly contaminated berry fruits.

We demonstrated here that treatment with constant low levels of ClO_2 can inactivate TV, a human norovirus surrogate, on the surfaces of strawberries, blueberries, blackberries and raspberries. Use of direct constant level ClO_2 as opposed to bolus type sachet treatments circumvent issues regarding oxidative burden of different produce products permitting equal treatments regardless of the lot size being treated. This work should serve as a guideline for the industry regarding treatment of berries with chlorine dioxide gas.

Given that optimal concentrations have now been determined, future work will characterize the sensory qualities of

Table 4 Efficacy of chlorine dioxide gas (ClO_2) treatment in reducing Tulane virus (TV) population on artificially inoculated blackberry

ClO_2 treatment concentration used (ppm-h/g product)	Initial TV population before treatment (log PFU/g)	Reductions in TV population after treatment (log PFU/g)[a]
0.63 (1 mg/L)	5.00 ± 0.00	4.17 ± 0.00 A
1.25 (1 mg/L)	6.02 ± 0.25	4.44 ± 0.93 A
2.35 (1 mg/L)	6.18 ± 0.00	3.82 ± 2.17 A
3.01 (1 mg/L)	4.65 ± 1.11	3.63 ± 1.13 A
3.03 (2 mg/L)	5.43 ± 0.23	4.60 ± 0.00 A
4.40 (2 mg/L)	5.69 ± 0.40	4.21 ± 0.00 A

Samples were inoculated with TV and allowed to dry for 2 h prior to treatment. Sample were then treated under a constant 1 mg ClO_2/L air for the appropriate exposure time to achieve the required final concentration of ppm-h/g product

[a]Samples with similar letters within a column are not significantly different ($p \leq 0.05$)

ClO_2 treated berries directly after treatment, after extended cold storage, and after freezing since noroviruses are also a major problem for the frozen berry industry. Currently, we are validating this technology for its efficacy in inactivating the human hepatitis A virus.

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