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Chloroxyanion Residue on Seeds and Sprouts after Chlorine Dioxide Sanitation of Alfalfa Seed

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ABSTRACT: The effects of a 6-h chlorine dioxide sanitation of alfalfa seed (0, 50, 100, and 200 mg/kg seed) on total coliform bacteria, seed germination, and the presence of chlorate and perchlorate residues in seed rinse, seed soak, and alfalfa sprouts was determined. Chlorate residues in 20,000 mg/L calcium hypochlorite, commonly used to disinfect seed, were quantified. Chlorine dioxide treatment reduced ($P < 0.05$) total coliforms on seeds with no effect ($P > 0.05$) on germination. Dose-dependent sodium chlorate residues were present in seed rinse (4.1 to 31.2 μg/g seed) and soak (0.7 to 8.3 μg/g seed) waters, whereas chlorate residues were absent (LOQ 5 ng/g) in sprouts, except for 2 of 5 replicates from the high chlorine dioxide treatment. Copious chlorate residues were present (168 to 1260 mg/L) in freshly prepared 20,000 mg/L calcium hypochlorite solution, and storage at room temperature increased chlorate residues significantly ($P < 0.01$).

KEYWORDS: *calcium hypochlorite, chlorate, chlorine dioxide, perchlorate, residue, sprouts*

INTRODUCTION

Edible sprouts have been implicated in repeated outbreaks of human food poisoning in the US, Europe, and Asia[1−4] during the last two decades. Sprout contamination with *Salmonella* spp, *Escherichia coli*, and less frequently *Listeria monocytogenes* has been associated with outbreak events.[1−4] While relatively infrequent, sprout-associated outbreaks involving mortality have occurred, as exemplified by a 2014 *Listeria* outbreak that killed 2 of 5 individuals infected[5] and an *E. coli* 0157:H7 outbreak in Sakai City, Japan, that killed 9 individuals.[6]

Among vegetables consumed raw, sprouts are particularly susceptible to contamination by pathogens because of the moist warm conditions encountered during presoaking and germination of seeds. Such conditions provide excellent environments for the propagation of commensal[7] and pathogenic[8] bacteria. In instances when pathogens are present on dry seed, germinating seed, and (or) sprouts, no single sanitation measure is entirely effective for pathogen removal. In 1999 the National Advisory Committee on Microbial Criteria for Foods[8] recommended that just prior to sprouting, seeds should be treated with "one or more" treatments able to "reduce or eliminate" pathogens and 20,000 mg/L (2%) calcium hypochlorite was suggested as an intervention.[8] The recommendation was based on the relatively few available data on seed intervention technologies at the time and on data showing partial effectiveness of calcium hypochlorite on reducing pathogen numbers on inoculated seeds.[9,10] Seed treatment with calcium hypochlorite was recognized, however, to be imperfect because pathogens were incompletely removed from seeds; ineffectiveness was hypothesized to be caused, in part, by cracks, abrasions, and crevices that might harbor and protect bacteria from direct action of chemical.[10] Empirical evidence also suggests that while calcium hypochlorite treatment reduces pathogen burdens on treated seed, risk of human infection is not eliminated by calcium hypochlorite treatment. For example, Dechet et al.[1] indicated that at least 3 of 18 disease outbreaks associated with the consumption of sprouts from 1998 to 2010 occurred in seeds produced by sprouting facilities that practiced calcium hypochlorite seed treatment.

Subsequent to the promulgation of the 1999 recommendations from the National Advisory Committee on Microbial Criteria for Foods,[8] a substantial amount of research has been conducted on alternative, chemically based seed sanitizing treatments including organic acids (acetic, lactic, malic, peroxyacetic, fatty, phytic, and oxalic), acidified sodium chlorite, stabilized oxychloro complexes, electrolyzed waters (acidic and alkaline and slightly electrolyzed), and ozone.[11] No chemically based treatment is entirely satisfactory,[11] partly because of limitations of treatments (usually aqueous based) reaching pathogens on and within seed crevices and imperfections or negative effects on germination or product quality. Because alternative treatments have not been suggested by regulatory authorities, 20,000 mg/L calcium hypochlorite remains the industry standard for pathogen reduction on seeds.

Whereas the use of 20,000 mg/L calcium hypochlorite as a seed treatment has been evaluated for its effect on bacteria and pathogens on seeds and sprouts, its contributions to chemical residues in sprouts are unknown. Yang and Her[12] demonstrated that 91% of surveyed market soybean sprouts contained perchlorate residues and that soybean sprouts accumulate perchlorate during germination of seed. Yang and Her[12] did not mention if market sprouts were treated with calcium hypochlorite as a disinfecting agent prior to germination. Similarly, Kettlitz et al.[13] measured a high detection rate (48 of 50 samples) and high average concentrations (1.05 mg/kg; range <0.01 to 5.1 mg/kg) of chlorate residues in frozen bean sprouts, but again, conditions under which sprouts were

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produced were not reported. Treatment with 20,000 mg/L calcium hypochlorite is a plausible source of both chlorate and perchlorate residues because granular calcium hypochlorite may contain chlorate at g/L concentrations[14,15] and perchlorate at mg/L concentrations.[15] Thus, hypochlorite itself is a possible source of chlorate and(or) perchlorate residues in treated sprouts.

Chlorine dioxide gas has been tested for use as a possible seed disinfectant treatment in the repertoire of techniques available to treat seeds used for sprouting. Efficacy across a number of gas concentrations, exposure durations, temperatures, and humilities has been documented[16−18] against pathogen and nonpathogen bacteria. In contrast to aqueous solutions, chlorine dioxide gas has favorable characteristics with respect to its ability to diffuse into porous surfaces or otherwise inaccessible exterior seed imperfections. The gas is highly active against a number pathogens and rot organisms that affect vegetables and produce[19] and against a large number of human viral[20] and bacterial pathogens.[21] If chlorine dioxide gas is protected from light during sanitation, chlorate and perchlorate residue formation on vegetables[22,23] or meat products[24] is mitigated. If, however, sanitation occurs in chambers not protected from light, chlorate and perchlorate residues may form.[25] The purpose of the present study was to determine the effect of the chlorine dioxide sanitation of germination, total coliforms, and residue formation on alfalfa seeds and on sprouts germinated subsequent to chlorine dioxide treatment.

MATERIALS AND METHODS

Chemicals. Chlorine dioxide gas was generated from Fruitgard media provided by ICA Trinova (Newnan, GA). Potassium iodide and sodium chlorite were obtained from Fluka Chemcial; St. Louis, MO, USA. Sodium bicarbonate, sodium carbonate, and sodium perchlorate, HPLC-grade water, and 40% methylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chlorate (>99%) was a gift from EKA Chemicals (Magog, Quebec). Sodium chlorate internal standard, labeled with heavy oxygen ($NaCl^{18}O_3$), was synthesized in-house and sodium perchlorate internal standard ($NaCl^{18}O_4$) was purchased from Icon Isotopes (Summit, NJ, USA). Water was purified in-house using a Milli-Q purifier (Millipore Sigma, Burlington, MA USA). Glacial acetic acid and HPLC-grade acetonitrile were purchased from EMD Chemicals (Darmstadt, Germany). Calcium hypochlorite pellets, beads, or powders were purchased from Acros Organics (71.4% available chlorine; Geel, Belgium), Robelle Industries (65.0% available chlorine; Braintree, MA), US Water Systems (powder and pellets, both 70% available chlorine; Indianapolis, IN), and Sigma-Aldrich (66% available chlorine; St. Louis, MO).

Study Design. The effects of four chlorine dioxide treatment levels (0, 50, 100, and 200 mg ClO_2 per kg seed) on alfalfa seed germination, total coliforms, and on chlorate and perchlorate residues in seed rinses, seed soak water, and sprouted seeds were determined. Each treatment was replicated across day in 5 separate trials (n = 5 for each treatment) as shown in Figure 1. During each trial, two sets of control tanks were run; tanks without seed, but with chlorine dioxide gas (100 mg/kg seed equivalent) to measure gas production and consumption by the experimental apparatus, and tanks with seed, but no chlorine dioxide gas.

Seed Treatments. Aliquots (0.5 kg) of Vernal alfalfa (*Medicago sativa*) seed (Agassiz Seed Supply; West Fargo, ND) were weighed into 6-L rotating drum marinate tanks (Chef's Elite model STX-1000-CE; Lincoln, NE). The marinate tank lids were modified to accommodate 13 mm butyl stoppers (Kimble Kontes) through which gases could be sampled. The transparent marinate tanks were enveloped with duct tape to ensure the exclusion of laboratory lighting. Chlorine dioxide is light sensitive,[26] and protection from light will prevent, or reduce, the formation of perchlorate and chlorate.[25] A 10

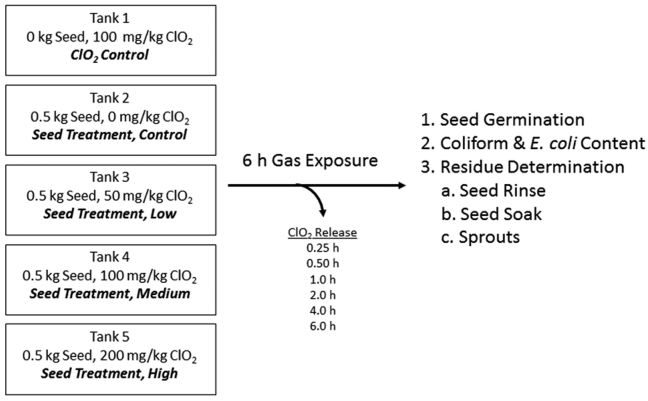

Figure 1. Design of experiment showing treatment tanks, samples taken during gas exposures, and end points measured after the end of the gas exposure period. The complete experiment was replicated on each of five separate days.

mL aliquot of purified water was added immediately prior to the start of each trial to seed within each marinate tank. Equal masses of Fruitgard media A and B (ICA TriNova; Newnan, GA) were then added to Tyvek sachets with zip lock closures and mixed briefly by hand before being added to marinate tanks. The dry media was formulated to release 8.9 mg of ClO_2 per gram of each media over a 6-h period. Marinate drums were sealed with lids, drums were placed on the rotor base, and rotation was initiated. After the completion of 6 h treatment periods, rotation was stopped, lids were detached, and the sachets were removed for discard. Separate aliquots of seed were removed for germination tests (~10 g) and for determination of total coliform (~400 g), and the remainder of the treated seed was stored in labeled containers at −20 °C until rinse and sprouting experiments could be conducted.

Chlorine Dioxide Gas Measurement. Gas samples (1 mL) were removed at 0, 0.25, 0.5, 1, 2, 4, and 6 h from each treatment using gastight syringes (SGE Analytical Science; Melbourne, Australia) equipped with 22 gauge syringe needles (Becton Dickinson; Franklin Lakes, NJ). At collection, gas was bubbled through 8 mL of 0.02% KI in buffer composed of 1.5 mM $NaHCO_3$ and 1.5 mM NaH_2CO_3 to trap ClO_2 as chlorite ion (ClO_2^-). Chlorite was quantified the day of collection by injecting 100 μL aliquots of KI trapping buffer onto a Dionex AS2100 ion chromatograph equipped with Dionex AG9 and AS9 guard and analytical columns, respectively. Chlorite was eluted using an isocratic mobile phase of 9 mM potassium carbonate originating from an in-line eluent generator. Chlorite was detected using suppressed conductivity detection and quantified using least-squares regression of peak area against a standard curve with points at 0, 75, 225, 750, 1500, 3000, and 5000 ng/mL of freshly prepared sodium chlorite dissolved in KI buffer. Chromeleon version 7.1.1 software was used to control the chromatograph (ThermoFisher; City State) and to analyze and summarize data sets.

Seed Germination. Quintuplicate 5.7-L plastic bins were lined with paper towels and sufficient tap water was added to completely saturate the towels. Within replicate and treatment, germination tests were conducted in quintuplicate so that each 5.7-L bin contained germination tests from each treatment. Germination was measured by lining polycarbonate Petri dishes (15 × 100 mm) with blotter seed germination paper (8.57 cm), adding 8 mL of purified water, and adding 25 to 30 treated alfalfa seeds. Lids were placed on Petri dishes, dishes were added to bins, and bins placed into a germination chamber set to 25 °C, with a 16/8 h light/dark cycle. Germination was enumerated after a 48-h incubation period and the amount of germination was expressed as a percentage of the total seed added to a plate.

Total Coliforms and *E. coli*. Within 24 h of chlorine dioxide gas treatment, control (25 g) and treated (100 g) alfalfa seeds were added to respective 1-L Erlenmeyer flasks and 500 mL of 0.9% NaCl (w/vol) were added. Seeds were rinsed by manual swirling for 5 min and seeds

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were allowed to settle. Triplicate aliquots (100 mL) of seed rinse waters were transferred through cheesecloth into 100 mL sterile bottles containing Colisure media (Idexx; Westbrook, ME). Antifoaming agent (5 drops) was added to each bottle and total Coliforms and *E. coli* rinsed from seeds were enumerated using the Colisure assay (Idexx; Westbrooke, ME) as described in the product literature. Sample trays were incubated at 35 °C for 24 h and *E. coli* and total coliforms quantified as per product literature. Triplicate negative control assays consisting of 100 mL aliquots of purified water were run simultaneously with treated seed assays for each replicate.

Sprouting Experiment. Within trial and treatment, duplicate aliquots of seed (20 ± 0.2 g) were added to 0.94 L glass jars (Ball; Fishers, IN) and 100 mL of purified water was added. Seeds were rinsed by had swirling for approximately 5 min and the rinsewater was decanted into 50 mL polypropylene tubes (Sarstedt; Newton, NC). A subsample (10 mL) from each seed rinse was filtered (0.45 μM PTFE, 17 mm) into an autosampler vial for ion chromatographic analysis. To rinsed seeds, 250 mL of purified water were added; seeds were allowed to soak for 16−22 h at room temperature. Soak water was decanted and 50 mL aliquots were retained for chemical analysis. Seeds were sprouted by placing jars, capped with rings and a cheesecloth cover, on their sides in a germination chamber set to 25 °C, with a 16/8 h light/dark cycle. Seeds were sprouted for a minimum of 4 days; twice daily seeds were rinsed with 250 mL of purified water and the jars rotated/shaken gently to distribute the seeds/sprouts. At harvest, sprouts were transferred to a labeled container and sufficient water was added to cover the sprouts. Empty hulls and ungerminated seeds were removed by gravity and gentle stirring. Sprouts were removed from the water, placed onto absorbent paper, and the weight was recorded. Prior to storing at −20 °C, sprouts were placed in blending jars, diluted with 30 mL of water (to facilitate puree formation), and homogenized. Puree aliquots (50 g each) were transferred to 50 mL polypropylene tubes for storage.

Residue Analysis of Seed Rinses. Filtered seed rinsewater from the sprouting experiment was analyzed for chlorate using ion chromatography. Chlorate residues were quantified using identical chromatographic conditions (instrument, column, injection volume, mobile phase, flow rate) as described for the chlorite determination for chlorine dioxide analysis. Quantitative results were derived from a matrix-matched standard curve consisting of points of 100, 250, 500, 750, 1000, 2500, and 5000 ng/mL. The LOQ for the chlorate analysis of seed rinses was set to the lowest analytical standard (100 ng/mL; 10 ng on-column) used for the analysis which was equivalent to a method LOQ of 0.5 μg/g for seed rinse. In some instances, the presence of false-positive, background level, chlorate peaks in negative control samples were verified by LC-MS/MS using chromatographic procedures described below for chlorate analysis of soak water. The presence of chlorate in these negative control samples could not be verified using LC-MS/MS.

Perchlorate residue was assayed using a modification of the Krynisky method.[27] Briefly, 1 g of treated seed was diluted in 9.85 mL of 1% acetic acid and subsequently fortified with 150 μL of a 2000 ng/mL $NaCl^{18}O_4$ internal standard solution. Recovery samples containing untreated seed were fortified with 300 ng/g of perchlorate. Tubes were capped and mixed for 5 min on a rotary mixer while solid phase extraction tubes (ENVI-Carb, 0.5 g, 6 cc; Sigma-Aldrich, St. Louis) were conditioned with 6 mL of acetonitrile followed by 6 mL of 1% acetic acid. Samples (10 mL) were loaded onto SPE tubes and the nonretained aqueous fractions were collected into 15 mL tubes. Tubes were vortex-mixed and 3−5 mL of each sample was filtered (17 mm; 0.45 μm; Thermo Scientific; Rockwood, TN) into respective 1 mL LC-MS/MS and 2 mL storage vials (for archival purposes). Samples were stored at −20 °C until LC-MS/MS analysis. Matrix-matched calibration standards were prepared at 0, 0.5, 1.0, 5, 25, 125, and 250 ng/mL of sodium perchlorate and 30 ng/mL of internal standard. Quantitative data were collected on a Waters Aquity triple quadrupole mass spectrometer. The chromatographic component consisted of Dionex AG21 and AS21 (2 × 250 mm; 10 μm) guard and analytical columns, respectively, a column flow rate of 0.350 mL/min, and an injection volume of 24 μL. The mobile phase consisted of solvents A (180 mM methylamine) and B (400 mM methylamine) in a gradient having the following composition: Time 0, 100% A; 4.0 min, 100% A; 4.5 min, 100% B; 6.5 min, 100% B; 6.6 min, 100% A; 11 min 100% A. Data were acquired, processed, and quantified using MassLynx 4.1 with QuanLynx software. Ion chromatograms were summed for the ^{35}Cl transition m/z 99 → 83 and ^{37}Cl transition m/z 101 → 85 for native perchlorate and the ^{35}Cl transition m/z 107 → 89 and the ^{37}Cl transition m/z 109 → 91 for ^{18}O-perchlorate. $^{37}Cl/^{35}Cl$ Isotope ratios of native and labeled perchlorate were used for confirmation.

Residue Analysis of Seed Soak Water. Samples from water used to soak seeds prior to germination were thawed overnight at approximately 5 °C; approximately 6 mL of each sample was filtered (17 mm; 0.45 μm; Thermo Scientific; Rockwood, TN) into a labeled 15 mL tube. In-house synthesized ^{18}O-labeled sodium chlorate[28] and perchlorate internal standards (150 ng each; 27 and 75 μL additions, respectively) were each added to a 5 mL volumetric flask designated for individual soak-water samples. Volumetric flasks were diluted to the mark with filtered soak water samples, capped, and mixed by inversion a minimum of 10 times. Approximate 1 mL volumes were added to LC-MS/MS vials prior to analyses. Chlorate and perchlorate were analyzed simultaneously using the previously described LC-MS/MS system and mobile phase. For quantitative determinations the ^{35}Cl transition m/z 83 → 67 and the ^{37}Cl transition m/z 85 → 69 were summed for native chlorate and as were the ^{35}Cl transition m/z 89 → 71 and the ^{37}Cl transition m/z 91 → 73 for ^{18}O-labeled chlorate.

Residue Analyses of Sprouts. In preparation for chlorate analysis, sprout purees were thawed, weighed (1.0 ± 0.1 g) into 15 mL polypropylene tubes, and subsequently diluted with 9.65 to 9.95 mL of 1% acetic acid, depending on the nature of the sample. Recovery samples were fortified with 30 ng (300 μL) of sodium chlorate and all samples were fortified with 30 ng (52 μL) of ^{18}O-labeled internal standard. Blanks were diluted in 10 mL of 1% acetic acid with no chlorate or internal standard fortification. Vials were capped and mixed for 5 min on an rotary shaker and subsequently centrifuged at 2800 x *g* for 5 min. Extracts were subjected to SPE, filtered, and aliquoted into LC-MS/MS vials as previously described. Samples for perchlorate analysis were prepared in the same manner except that 30 ng of fortification and internal standards were added in respective volumes of 300 and 150 μL, respectively. Chlorate and perchlorate residues were quantified by LC-MS/MS as described for soak water.

Residue Analysis of Calcium Hypochlorite. Calcium hypochlorites from five commercial sources were each dissolved in 1 L of purified water to make 20,000 mg/L (2%) solutions after adjusting for available chlorine content of the source material. Immediately prior to analysis, 0.250 mL of each calcium hypochlorite solution was transferred to a 500 mL volumetric flask partly filled with purified water and diluted to the mark. Flasks were mixed by inversion a minimum of 15 times and aliquots removed for ion chromatographic analyses. Ion chromatography was performed as described for chlorate under "Chlorine Dioxide Gas Measurement" except a standard curve containing sodium chlorate, dissolved in water, at 0.05, 0.1, 0.25, 0.5, 1, 2, 5, and 10 μg/mL was used.

Statistical Analyses. The effects of chlorine dioxide on total coliform bacteria were determined using a one-way analysis of variance after square-root transformation to ensure equal variance. Differences in seed germination rates were determined using Student-*t* tests. Main effects of time on chlorate concentrations of calcium hypochlorite source solutions was determined using simple 2-way ANOVA with main effects of time of measurement and calcium hypochlorite source. Within calcium hypochlorite source, differences in chlorate concentration across time were determined using a *t* test. Statistical procedures were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA.

RESULTS AND DISCUSSION

The release of chlorine dioxide in control and treatment tanks is shown in Figure 2. The control tank (containing no seed) was charged with sufficient dry media to mimic the 100 mg/kg seed treatment and verified that targeted amounts of chlorine

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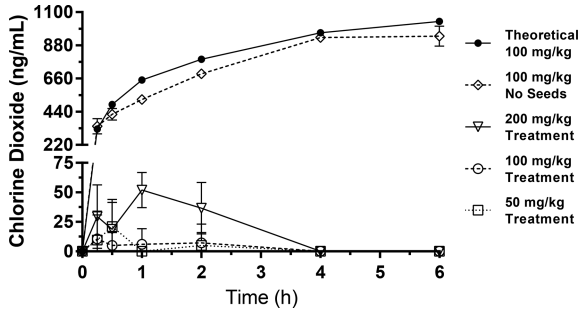

Figure 2. Chlorine dioxide release from controlled release media equivalent to the 100 mg/kg seed treatment in control tanks (open diamond, dashed line), and in 50 (open square, dotted line), 100 (open circle, dashed line), and 200 (open triangle, solid line) mg/kg treatment tanks. Data represent means and standard deviations of 5 replicates per treatment. Solid circles connected by a solid line represent the theoretical release of chlorine dioxide from the controlled release media, equivalent to the 100 mg/kg seed treatment.

dioxide were produced. In the absence of seed, chlorine dioxide release was consistent with, but slightly lower than, theoretical levels of gas release. Slightly lower gas production may have resulted from minor leaks and, perhaps more importantly, the consumption of gas by the plastic drum and seal of the modified marinate tank. Nevertheless, the data clearly indicate that in the absence of seed, chlorine dioxide was released from the media at levels that closely approached theoretical levels and that chlorine dioxide accumulated within sanitation drums during the 6-h treatment period. Data presented in Figure 2 also clearly indicate that when seeds were present in the test system, chlorine dioxide did not accumulate to an appreciable degree in the 50 and 100 mg/kg treatments and accumulated on a transitory basis in the 200 mg/kg treatment. The data are consistent with previous studies indicating that chlorine dioxide gas does not accumulate during treatment of produce[23] or ready-to-eat meat products[26] when controlled release media is used to generate chlorine dioxide. Gasses do accumulate, however, under other scenarios. For example, when chlorine dioxide is rapidly generated by the mineral acid-catalyzed oxidation of sodium chlorite, gas is so rapidly produced that it exceeds the capacity of eggs,[29] fruit[22,23] or meat[24] to adsorb the gas. In continuous flow systems, the degree of chlorine dioxide gas adsorption by fruits is variable depending upon gas concentrations and exposure times,[30] but under continuous flow, chlorine dioxide gas is generally present in excess concentrations.[31]

Smith et al.[24,25] and Kaur et al.[22] demonstrated that adsorption of chlorine dioxide on produce and ready-to-eat meat seems to be primarily a surface phenomenon. That is on melons, no chlorine-dioxide-related residues were present within edible flesh fractions and quantitative recovery of melon associated radioactivity occurred in rind fractions. Residues remaining on produce and ready-to-eat meat products were comprised primarily of chloride ion with chlorate and perchlorate being present in variable amounts depending upon the amount of light to which experiments were exposed.[22,24,25] In the current study chlorate and perchlorate were measured as potential end points of chlorine dioxide exposure; measurements of chlorite were not attempted because of chlorite's relative instability in aqueous matrices containing biological reductants (discussed by Smith et al.[23]) and the unlikelihood of this residue surviving the sprouting process. Therefore, both chlorate and perchlorate were measured along the major steps of sprout production, the initial seed rinse and soak waters and in germinated sprouts (Table 1).

Table 1. Concentrations of Chlorate and Perchlorate in Seed Rinses, in Seed Soak Waters, and in Sprouts Generated from Treated Alfalfa Seeds (Means and Standard Deviations of 5 Replicates Per Treatment)[a]

	Chlorine Dioxide Treatment (mg/kg)			
Fraction	0	50	100	200
	Sodium chlorate (μg/g)[b]			
Seed rinse	ND	4.06 ± 0.38	12.81 ± 1.13	31.17 ± 3.32
Seed soak	ND	0.72 ± 0.14	3.01 ± 0.45	8.25 ± 1.54
Sprouts	ND	ND	<LOQ	0.029 ± 0.003[c]
	Sodium perchlorate (ng/g)[d]			
Seed rinse	ND	ND	ND	ND
Seed soak	ND	ND	ND	ND
Sprouts	ND	ND	ND	ND

[a]Data are expressed as sodium chlorate and perchlorate equivalents. [b]Method LOQ for chlorate in seed rinse was 0.5 μg/g seed; was 6.25 ng/g seed for seed soak; and was 10 ng/g for sprouts. Method LOD was 5 ng/g for sprouts. [c]Two replicates had quantifiable residues; mean and standard deviation were calculated from only those samples with quantifiable residues. [d]Method LOQ for perchlorate in seed rinse was 5 ng/g seed; seed soak was 6.25 ng/g seed; and in sprouts, 5 ng/g. LOD was 1 ng/g.

Perchlorate residues were not present in seed rinse, soak, or sprout fractions. Previous studies have indicated that perchlorate residues may form from chlorine dioxide, especially when chlorine dioxide gas is exposed to light.[25,26] In chlorine dioxide sanitation of produce and ready-to-eat meat, the formation of perchlorate could be essentially eliminated if sanitations were protected from light,[23,24] thus the protection from light in this study. We did not run additional tests under laboratory illumination to determine if perchlorate residues would have formed under such conditions. Given the reactivity of chlorine dioxide and the known byproducts formed during the photodegradation of aqueous or gaseous chlorine dioxide[32−34] we did not believe such testing was necessary.

In contrast to perchlorate, chlorate residues were formed in a dose-dependent manner and in appreciable quantities during chlorine dioxide sanitation (Table 1) with 4 to 31 μg/g removed from seeds during a water rinse and 0.72 to 8.3 μg/g removed from seed during the 24-h soak prior to germination. Chlorate formation was independent of exposure to light in these experiments and is presumably due to disproportionation reactions that might have been favored by the addition of water (10 mL) at the beginning of the experiments.[35] Sodium chlorate residues in sprouts were not detectable (50 and 100 mg/kg seed treatments) or were just above the method limit of quantitation (LOQ = 10 ng/g) in the 200 mg/kg seed treatment group. The data suggest that although chlorate residues were formed during chlorine dioxide sanitation of alfalfa seed, the residues were quantitatively removed from seeds during rinsing and soaking. Alternatively, chlorate residues that possibly remained on seeds after soaking might have been transformed by bacterial action during the sprouting process as chlorate residues are prone to biodegradation in biotic environments conducive to bacterial growth.[36−38]

The current recommendation for sanitizing seeds for sprout production is to incorporate "one or more treatments that can

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effectively reduce or eliminate pathogenic bacteria".[8] A commonly used resource is a 2,000 to 20,000 mg/L calcium hypochlorite[8,39] seed or sprout rinse. Previous work, however, has established that calcium hypochlorite rinses up to 20,000 mg/L are not completely effective at eliminating pathogens from seeds or sprouts.[8,40,41] In the present study, chlorine dioxide treatment was associated with a dose-dependent reduction in removable total coliforms (Figure 3), consistent

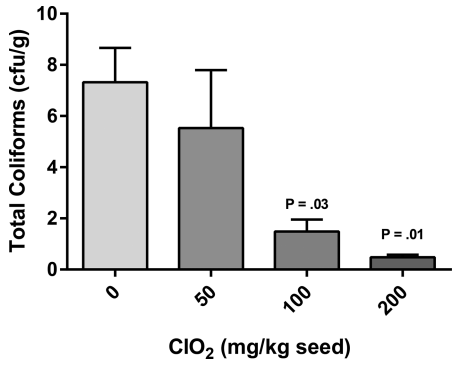

Figure 3. Effect of chlorine dioxide gas on total coliforms (mean ± std dev) rinsed from treated alfalfa seeds. Relative to controls (0 mg ClO_2 gas), total coliforms were reduced by 100 (P = 0.03) and 200 (P = 0.01) mg of ClO_2 gas per kg of seed (n = 5 replicates per treatment level).

preparation and use in food disinfection applications.[51] Calcium hypochlorite granules, pellets, or powders from 5 sources were diluted to 20,000 mg/L active chlorine and assayed for chlorate content three times within 1 week (days 2−7) of preparation and again just prior to 3 weeks of preparation (days 16−21) to mimic what might occur in stock solutions or in unused, but prepared, calcium hypochlorite solution. As demonstrated in Figure 4, chlorate concentrations in solutions less than 1 week

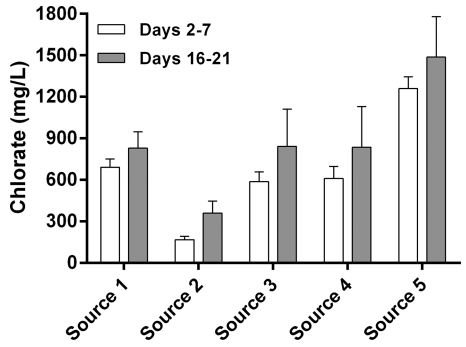

Figure 4. Chlorate content (mg/L) of commercial calcium hypochlorite sources of laboratory, technical, and food-grade qualities. Specific commercial sources are described in the Chemicals section of the Materials and Methods. Data are means ± std deviations of triplicate measures taken between 2 and 7 days of preparation and from 16 to 21 days of preparation.

with previous studies showing the reduction of bacterial contamination of seeds using aqueous[42,43] or gaseous chlorine dioxide.[16−18] Total coliforms in this study were not used as a metric of pathogenicity, but rather as a measure of the general effectiveness of the treatment to reduce organisms typically associated with fecal contamination.

In the present study, significant quantities of chlorate residues were formed from chlorine dioxide treatment of seeds, but this residue was removed during rinsing and soaking steps of the sprouting process. Using fairly sensitive LC-MS/MS methods (LOQ 10 ng/g), no chlorate residue was present in sprouted alfalfa from the lowest two chlorine dioxide treatments and was sporadically quantified (2 of 5 replicates) at the 200 mg/kg seed treatment level. Market surveys of sprouts in Europe[13] and South Korea[12] have measured chloroxyanions (chlorate and/or perchlorate) in >90% of sprout samples assayed. Bean sprout samples (n = 50) assayed by Kettlitz et al.[13] contained an average chlorate concentration of 1,000 ng/g of sprout (1 mg/kg). Entry points of chlorate and/or perchlorate on produce could be contamination of chlorine-based disinfection solutions (discussed by Kettlitz et al.[13]), the use of hypochlorite disinfectants in hydroponic production systems,[44] and perhaps the use of 20,000 mg/L calcium hypochlorite as a seed disinfectant. The relative instability of sodium hypochlorite solution and contamination with chlorate and perchlorate have been well characterized.[15,45−47] Although less well characterized in the literature, commercial calcium hypochlorite also contains chlorate residue,[14,48] sometimes at appreciable (percentage) levels[49] which may form at appreciable rates[50] in solution. Unlike sodium hypochlorite, calcium hypochlorite is usually handled as a dry reagent until dissolution in water for use. We characterized chlorate levels within a variety of laboratory and commercial calcium hypochlorite sources; some of the sources were specifically labeled for potable water and (or) recommended for

of age ranged from 168 ± 23 to 1260 ± 84 mg/L. Across all sources, chlorate concentrations increased (P = 0.003) from the initial measurements (days 2−7) to the final analysis (days 16−21), consistent with the notion that aqueous hypochlorite solutions—whether as sodium or calcium hypochlorite—are not stable at room temperature.[45,46,48,49] In the present experiment, the degree of chlorate contamination exceeded 0.5 parts per thousand within 7 days in 4 of the 5 calcium hydroxide sources; in three sources, the average chlorate concentration exceeded 0.8 parts per thousand after 16−21 days in solution, with source 5 always containing >1 part per thousand (>1g/L) of chlorate. Source 2 (Figure 4) was a laboratory supply company; although the chlorate content of this source increased (P = 0.02) over time, its chlorate content remained below the initial concentrations of the other sources, even after a 2 week period. Based on the high concentrations of chlorate measured in relatively fresh food and potable water grade calcium and sodium hypochlorite solutions, sanitation of produce handling facilities and produce could be an important source of chloroxyanions in vegetable foods.[12,13]

Chlorine dioxide gas would not be useful as a sanitizing agent for sprout production if it was to impair germination in any way. In our experiments, there were no differences (P > 0.05) in germination between untreated or chlorine dioxide treated seeds at any level (Figure 5), consistent with previous studies using gaseous[17,18] or aqueous[42,43] ClO_2.

Previous studies have clearly demonstrated the possible utility of using chlorine dioxide gas for seed or sprout sanitation. While the efficacy of chlorine dioxide treatment against a number of viral and bacterial pathogens and (or) rot organisms is not generally questioned, regulatory authorities have not given approval to chlorine dioxide gas use on edible foods because of concerns with chemical residues. Our data suggest that with respect to sanitation of sprouting seeds, the impacts of chlorate and/or perchlorate residues subsequent to

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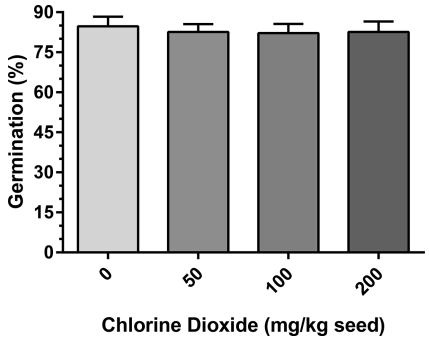

Figure 5. Alfalfa seed germination (% ± standard deviation) of control and chlorine dioxide treated alfalfa seeds (n = 4 per treatment).

chlorine dioxide sanitation of seeds could be prevented or minimized during the commercial production of sprouts. That is, seed rinse and seed soak steps are generally conducted to remove soluble contaminants, dusts, and particulates from seeds and to soften the seed coat prior to germination.[8] Our results suggest that chlorate residues formed during chlorine dioxide sanitation are effectively removed during seed rinsing and soaking steps prior to seed germination.

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Notes

The authors declare no competing financial interest.

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