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Formation of Oleic Acid Chlorohydrins in Vegetables during Postharvest Chlorine Disinfection

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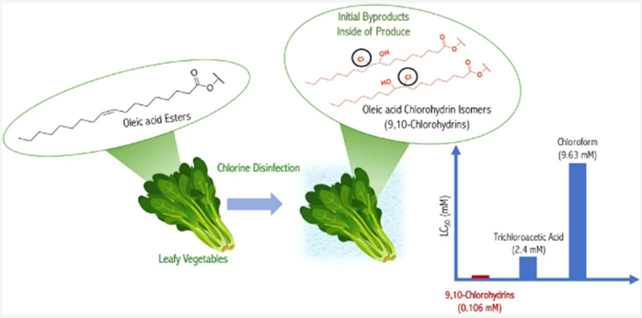

ABSTRACT: High chlorine doses (50−200 mg/L) are used in postharvest washing facilities to control foodborne pathogen outbreaks. However, chlorine can react with biopolymers (e.g., lipids) within the produce to form chlorinated byproducts that remain in the food. During chlorination of micelles of oleic acid, an 18-carbon alkene fatty acid, chlorine added rapidly across the double bond to form the two 9,10-chlorohydrin isomers at a 100% yield. The molar conversion of lipid-bound oleic acid to 9,10-chlorohydrins in chlorine-treated glyceryl trioleate and produce was much lower, reflecting the restricted access of chlorine to lipids. Yields from spinach treated with 100 mg/L chlorine at 7.5 °C for 2 min increased from 0.05% (0.9 nmol/g-spinach) for whole leaf spinach to 0.11% (2 nmol/g) when shredding increased chlorine access. Increasing temperature (21 °C) and chlorine contact time (15 min) increased yields from shredded spinach to 0.83% (22 nmol/g) at 100 mg/L chlorine and to 1.8% (53 nmol/g) for 200 mg/L chlorine. Oleic acid 9,10-chlorohydrin concentrations were 2.4−2.7 nmol/g for chlorine-treated (100 mg/L chlorine at 7.5 °C for 2 min) broccoli, carrots, and butterhead lettuce, but 0.5−1 nmol/g for cabbage, kale, and red leaf lettuce. Protein-bound chlorotyrosine formation was higher in the same vegetables (5−32 nmol/g). The Chinese hamster ovary cell chronic cytotoxicity LC_{50} value for oleic acid 9,10-chlorohydrins was 0.106 mM. The cytotoxicity associated with the chlorohydrins and chlorotyrosines in low masses (9−52 g) of chlorine-washed vegetables would be comparable to that associated with trihalomethanes and haloacetic acids at levels of regulatory concern in drinking water.

KEYWORDS: *chlorine, postharvest washing, fatty acid, chlorohydrin*

■ INTRODUCTION

Foodborne pathogenic outbreaks are estimated to cause 600 million illnesses and 420 000 deaths per year worldwide.[1] Concerns over the nearly 6 million illnesses per year in the United States[2] culminated in the Food Safety Modernization Act (FSMA) in 2011.[3] The FSMA may lead to the development of guidelines for the application of chemical sanitizers to control pathogens in sprays, flumes, and dump tanks within postharvest food washing facilities. Among chemical sanitizers, chlorine remains the most widespread due to low operational costs.[4,5] For ready-to-eat produce, free chlorine doses between 50 and 200 mg/L as Cl_2 are applied to produce for contact times of ≤5 min.[4−12] However, particularly for shredded or chopped produce, the high chemical oxygen demand (COD up to 1000 mg/L) in washwaters can exert high chlorine demand, such that chlorine must be continually added to maintain chlorine residuals.[5]

Relative to the low chlorine (~5 mg/L as Cl_2) and precursor concentrations (~2 mg C/L dissolved organic carbon (DOC)) in drinking water, the high chlorine doses and precursor concentrations (both dissolved COD and the solid produce) in postharvest washing facilities are expected to form high levels of disinfection byproducts (DBPs).[5] Just as in drinking water, postharvest food washing facilities ultimately will need to balance the acute risk posed by foodborne pathogens against the chronic risk associated with exposure to food-based DBPs (F-DBPs).

Current F-DBP research has focused on the same low molecular weight (1−2 carbons), soluble DBPs of interest in drinking water, including regulated classes (trihalomethanes (THMs), haloacetic acids (HAAs), and chlorate) and unregulated classes (e.g., haloacetonitriles and haloacetamides).[8,13−17] DBP formation depends on the produce, but formation typically correlates with COD and can be substantial.[18] For example, Shen et al.[16] found up to 860 μg/L THMs and 2100 μg/L HAAs when homogenized lettuce (maximum COD 1130 mg/L) was treated with 80 mg/L as Cl_2

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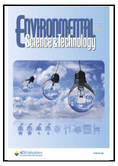

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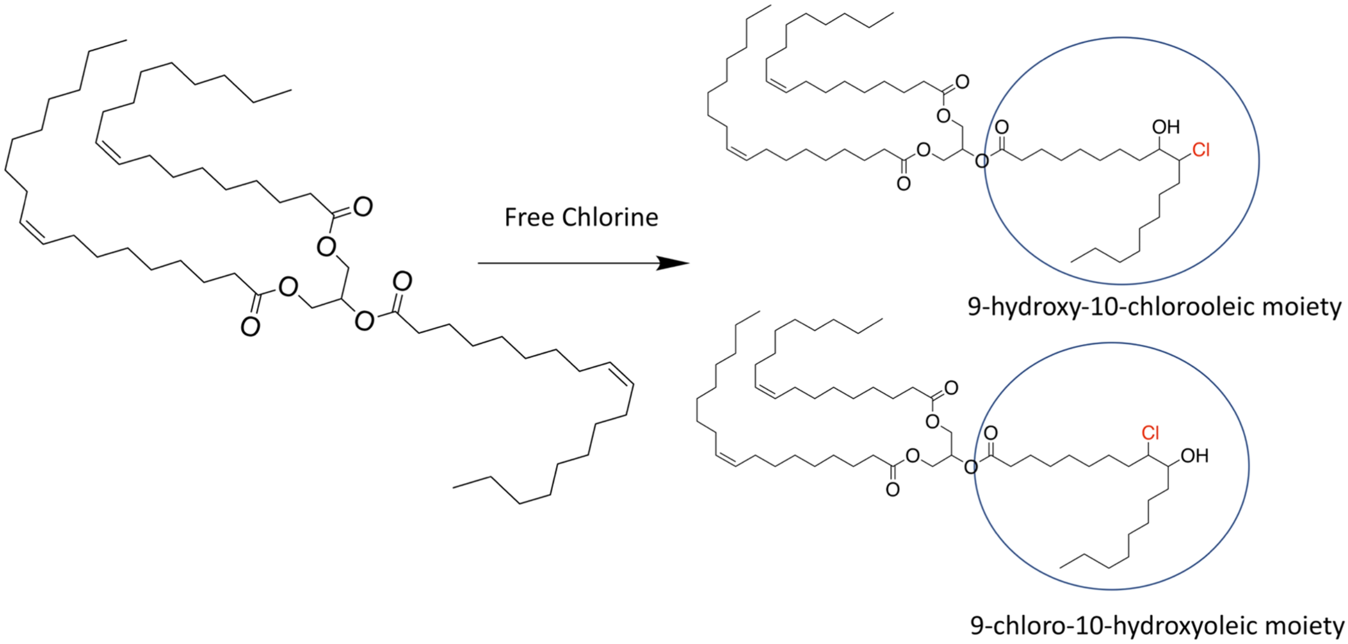
Scheme 1. Chlorination of Glyceryl Trioleate, a Model Triacylglyceride, Forms Two Monochlorohydrin Products (i.e., 9-Hydroxy-10-chlorooleic and 9-Chloro-10-hydroxyoleic Moieties)[a]



[a]The monochlorohydrin can be formed on any unsaturated bond on glyceryl trioleate.

of chlorine. Despite these high absolute concentrations, low molecular weight DBPs form at low yields (<10%) from chlorine reactions, with the biopolymers (e.g., proteins, lipids, and carbohydrates) serving as precursors in produce.[19,20] The low yields are attributable to the long series of elementary steps, including carbon−carbon bond cleavage, needed to liberate these 1−2 carbon molecules from biopolymers. Moreover, previous research has indicated that these low molecular weight DBPs partition to the washwater and so are more of a concern for washwater disposal than for consumption of the produce.[21]

The initial products of chlorine reactions with biopolymers in produce include chlorine addition to the monomers constituting biopolymers. The short chlorine contact times in postharvest washing facilities inhibit further reactions leading to the liberation of low molecular weight DBPs, such that the initial products of chlorine addition to monomers should form at higher yields and remain bound to the biopolymers within the produce. A subset of the monomers constituting biopolymers are reactive with chlorine, including certain amino acids within proteins and unsaturated fatty acids within lipids.[22] Regarding amino acids, previous research has identified the high-yield, initial transformation products for cysteine (cysteic acid), methionine (methionine sulfoxide), tyrosine (3-chlorotyrosine and 3,5-dichlorotyrosine), and lysine (lysine nitrile).[23−26] Using *N*-acetyl-tryptophan to mimic peptide-bound tryptophan, recent research demonstrated the formation of seven novel, tryptophan-derived intermediate DBPs, including two chlorinated DBPs.[19] Together, these byproducts accounted for a 55−100% yield, while the sum of all low molecular weight DBPs accounted for ≤20% yield.[19] Similarly, the chlorination of *N*-acetyl-histidine formed β-cyanoalanine at an ~50% yield, while the sum of all low molecular weight DBPs accounted for ≤7% yield.[20] Importantly, these products retained the *N*-acetyl group, indicating that they would remain bound within proteins in the produce. Previous research demonstrated the conversion of protein-bound tyrosines to chlorotyrosines during chlorination of lettuce, spinach, and gilthead seabream fish.[7,21,27] During chlorination of lettuce and spinach, protein-bound chlorotyrosines formed at comparable or greater concentrations than the sum of all of the soluble low molecular weight DBPs, even though low molecular weight DBPs could form from multiple precursors, while chlorotyrosines would only form from tyrosine.[21] Protein-bound chlorotyrosines are expected to be liberated during digestion, enabling uptake within the intestines.

Less research has evaluated lipids. Lipids are important components of cellular membranes and the waxy cuticles of plants.[28−30] Biomedical research has indicated that chlorine addition across the double bonds in unsaturated fatty acids forms the two chlorohydrin isomers as stable products.[31−35] Oleic acid is an 18-carbon fatty acid featuring one alkene (i.e., an 18:1 fatty acid) between the 9 and 10 positions. Chlorination of free oleic acid vesicles formed both isomers of 9,10-chlorohydrins (i.e., 9-chloro-10-hydroxyoleic acid and 9-hydroxy-10-chlorooleic acid; Scheme 1).[32] Recent research identified a series of novel, brominated or iodinated byproducts that formed when rice or wheat flours were heated in chlorinated or chloraminated tap waters to simulate cooking waters; byproducts included soluble free (i.e., not lipid-bound) brominated fatty acids.[36,37] However, the halogenated free fatty acids were not quantified and the analysis did not include lipid-bound fatty acid byproducts.

The goal of this study was to evaluate the formation of lipid-bound oleic acid chlorohydrins within produce treated with chlorine under conditions relevant to postharvest washing facilities as an exemplar for chlorine reactions with lipid-bound, alkene-containing fatty acids. Fatty acids with multiple alkenes are more prevalent than oleic acid in produce. For example, linolenic acid, an 18-carbon fatty acid with three alkenes (18:3), constitutes 69% of total fatty acids in spinach compared to only 1.1% for oleic acid (18:1).[28] However, the occurrence of only one alkene in oleic acid facilitated the synthesis and purification of chlorohydrin byproducts and thus the development of an analytical method to quantify the concentrations of lipid-bound oleic acid chlorohydrins in produce. The first objective was to compare the yields of chlorohydrins during chlorination between free and triacylglyceride-bound oleic acid vesicles to evaluate the extent to which the alkene functional groups are available to react with chlorine when sequestered within lipid bilayers and waxy cuticles. After developing a method to liberate oleic acid 9,10-chlorohydrins from lipids, the second objective was to apply this method to measure oleic acid chlorohydrin formation in spinach and lettuce over chlorine exposure conditions (e.g., temperature, time, chlorine concentration, shredded vs. whole leaf) relevant to postharvest washing conditions. The third objective was to compare the concentrations of lipid-bound oleic acid chlorohydrins and protein-bound chlorotyrosines within a range of produce treated with chlorine under postharvest-relevant conditions. The mammalian cell cytotoxicity of the chlorohydrins was measured to facilitate an evaluation of the importance of the chlorohydrin and chlorotyrosine concentrations for consumer exposure.

■ MATERIALS AND METHODS

Text S1 provides reagent sources and purities. The synthesis of the 9,10-chlorohydrins of oleic acid is provided in Text S2. Chlorine addition across the alkene in free or lipid-bound oleic acid produces two 9,10-chlorohydrin isomers: 9-hydroxy-10-chlorooleic acid and 9-chloro-10-hydroxyoleic acid (Scheme 1). These isomers were not separated during synthesis or during analysis by liquid or gas chromatography mass spectrometry (GC/MS or LC/MS) and so were quantified together. Sodium hypochlorite stocks were standardized by UV absorbance at 292 nm (365 M^{-1} cm^{-1}).[38]

Chlorination of Oleate and Glyceryl Trioleate. To evaluate the stoichiometry of free chlorine reactions with free oleate, potassium oleate (1.5 mM) in deionized water buffered at pH 7 with 10 mM phosphate buffer was treated with 0−10 molar equiv of free chlorine in 60 mL borosilicate glass vials in triplicate. The 1.5 mM oleate concentration just exceeds the 1 mM critical micelle concentration for oleate.[39] Samples were vortexed and left to react for 1 min at room temperature. Aliquots (100 μL) were added to 1000 μL of methanol containing 150 μM 12-hydroxystearic acid as the internal standard for analysis by LC/MS (Text S3); since this experiment evaluated stoichiometry and not kinetics, LC chromatographic separation was used to halt the reaction rather than the addition of chlorine quenching agents.

To determine the pseudo-first-order loss rate constant of free oleate within micelles during reaction with free chlorine, 1.9 mM potassium oleate was treated at 8 °C with 30 mM free chlorine in deionized water buffered with 20 mM sodium phosphate at pH 7 in borosilicate glass vials in triplicate. Since initial experiments indicated rapid kinetics, the 8 °C temperature, representative of chilled produce washing lines, was selected to evaluate conservative (i.e., slow) kinetics. The reaction solutions were stirred with a magnetic stir bar. Aliquots (100 μL) were withdrawn from the vials at 15 s intervals for up to 105 s. To quench the chlorine residual, the aliquots were placed into 2 mL amber glass vials prefilled with 50 μL of an L-ascorbic acid solution at a concentration sufficient to achieve a 20% molar excess relative to the initial free chlorine concentration. The aliquots were then diluted with 900 μL methanol containing 150 μM 12-hydroxystearic acid as an internal standard and analyzed via LC/MS (Text S3).

To characterize the reactivity of chlorine with lipid-bound oleic acid, chlorine was applied to glyceryl trioleate, a triacylglyceride containing three oleic acid substituents in 50 mL solutions. Because the glyceryl trioleate formed a separate phase, the solutions were vortexed during the reaction. After quenching the chlorine residual with a 20% molar excess of thiosulfate, certain samples of the glyceryl trioleate phase were analyzed directly by LC/MS (Text S3). Other samples were extracted using methyl *tert*-butyl ether (MtBE) and then treated with acidic methanol to liberate oleic acid 9,10-chlorohydrins from the triacylglyceride and enable quantification of their methyl esters by GC/MS (Text S3).

Vegetable Chlorination Experiments. Heads of lettuce and cabbage, bunches of spinach and kale, broccoli florets, and whole carrots were purchased from local stores in California, apart from one bunch of butterhead lettuce that was grown at the O'Donohue Family Stanford Educational Farm. The butterhead lettuce sample from the farm had not been exposed to chlorinated water. While the history of prior exposure of the store-bought vegetable samples to chlorinated washwaters was unknown, these types of produce typically are rinsed with 15−40 mg/L as Cl_2 to remove dirt after harvest.[40] This lower level of sanitization is in contrast to the higher chlorine exposures encountered in postharvest washing facilities for ready-to-eat produce (e.g., bagged salads). Samples were stored at 4 °C, and washing experiments were conducted within 72 h of sample collection. Prior to washing experiments, vegetable samples were hand-selected to be without observable damage or discoloration, were rinsed with deionized water, and were dewatered using a salad spinner. The skin of carrots was removed using a vegetable peeler. For most experiments, vegetable samples were cut to mimic "shredded" conditions. For example, all leaf samples (i.e., lettuce, cabbage, kale, and spinach) were cut into 0.5 cm × 1 cm strips, except where otherwise noted. Broccoli florets were cut into 1 cm × 1 cm × 1 cm cubes, while carrots were cut into 0.5 cm × 0.5 cm × 0.5 cm cubes. Additional experiments with spinach involved "whole leaf" conditions in which whole leaves were used without cutting. The wet weight of samples was individually recorded.

Samples were shaken with 0−200 mg/L as Cl_2 chlorine in deionized water buffered at pH 7 with 10 mM phosphate buffer in glass vials for 15 min at room temperature (21 °C) or for up to 2 min at 7.5 °C in a temperature-controlled room. The occurrence of bromide in source waters used in authentic washing facilities may promote the formation of oleic acid 9,10-bromohydrins. Since we only had purified standards for oleic acid 9,10-chlorohydrins, the use of a deionized water matrix enabled the quantification of chlorohydrin yields. Most experiments involved ∼1 g samples in 50 mL of chlorinated water, although ∼10 g samples within 500 mL of chlorinated water were used for certain samples featuring lower concentrations of oleic acid and its chlorohydrins. After measuring the chlorine residual with the DPD colorimetric method,[41] the chlorine residual was quenched with a 20% molar excess of L-ascorbic acid and the washwater was decanted. The vials containing the ∼1 g samples were placed in a −20 °C freezer overnight in preparation for freeze-drying; when a standard of oleic acid 9,10-chlorohydrins was frozen in

10 mM phosphate buffer at pH 7 (Text S4), no loss of chlorohydrins was observed. The frozen samples were then placed on a freeze drier for a minimum of 24 h. The ∼10 g samples were transferred to aluminum foil before being placed in the −20 °C freezer and then the freeze drier. Experiments were either conducted in duplicate over 3 separate days ($n = 6$) or in triplicate on the same day.

Freeze-dried samples were weighed and then pulverized on a BioSpec Mini-BeadBeater at a speed of 4.5 m/s for 20 s in 2 mL twist cap tubes containing one 3.2 mm stainless steel bead. Certain pulverized samples were split for the analysis of lipid-bound oleic acid and its chlorohydrins or for the analysis of protein-bound tyrosines and chlorotyrosines. The fatty acid protocol was adapted from a previous method;[42] the detailed method is provided in Text S3. Briefly, the pulverized aliquots were transferred to preweighed 15 mL glass centrifuge tubes and then weighed again to determine the sample mass after the transfer from the pulverization step. Lipids were extracted into methyl *tert*-butyl ether (MtBE). The MtBE extracts were treated with acidic methanol to liberate fatty acids from lipids and methylate the resulting free fatty acids. The samples were then treated with a silylating agent to convert the hydroxyl group in the oleic acid chlorohydrins to their trimethylsilyl derivatives for analysis using electron impact GC/MS. The oleic acid 9,10-chlorohydrin method-reporting limit was 0.53 nmol/g wet weight of vegetable for the 1 g sample size; the two 9,10-chlorohydrin isomers were quantified together since they were not separated by GC/MS.

The tyrosine protocol was adapted from our previous study of chlorotyrosines in spinach and lettuce (Text S5).[21] Briefly, the freeze-dried and pulverized samples were treated with methanesulfonic acid at 110 °C in an anaerobic glovebox to liberate tyrosines from proteins. After neutralizing the samples, the samples were supplemented with methanol and then extracted with chloroform to remove lipids. The aqueous/methanol phase was evaporated to near dryness, reconstituted in pH 5 buffer, and then free tyrosines were extracted by passing over Sep-Pak C18 cartridges. The eluents were treated with 6-aminoquinoline-*N*-hydroxy-succinimidyl ester (AQC) to derivatize the *N*-terminus of the free amino acids. Derivatized amino acids were quantified using LC/MS using electrospray ionization in the negative ion mode. The method-reporting limits were 1.7 nmol/g for 3-chlorotyrosine and 1.9 nmol/g for 3,5-dichlorotyrosine.

Chinese Hamster Ovary (CHO) Cell Chronic Cytotoxicity Analyses. The CHO cell chronic cytotoxicity assay captures adverse biological impacts that cause a reduction in cell density after exposure to a test agent for 72 h, a chronic exposure for these cells. Cytotoxicity was selected as an end point because (1) it provides a broad and sensitive metric of toxicity in that multiple toxicity pathways could result in a reduction in cell growth and (2) the existing, extensive library of CHO cytotoxicity evaluations for DBPs[43] permits a quantitative comparison of the toxic potency of oleic acid chlorohydrins to other DBPs. For the in vitro chronic cytotoxicity analyses of oleic acid chlorohydrin, CHO-K1 cell line (AS52, clone 11−4−8) was employed. CHO cells are genomically stable, adherent, have normal morphology, express cell contact inhibition, and grow as a monolayer without the expression of neoplastic foci.[44,45] A description of the growth conditions for the CHO cells, the cytotoxicity assay, and the statistical analyses was previously published (Text S6).[43] This assay was calibrated, and there is a direct relationship between the absorbance of the crystal violet dye associated with the cell membranes and the number of viable cells.[46,47] Oleic acid chlorohydrins were diluted in cell culture medium (F12 + 5% FBS). A cytotoxicity concentration−response curve for the oleic acid chlorohydrins was generated from summary data that included a range-finding experiment plus two repeated experiments with 8−24 independent replicates per concentration (Figure S3). Regression analysis was applied to the concentration−response curve to calculate the LC_{50} value. LC_{50} is the calculated molar concentration of oleic acid chlorohydrins that reduced the cell density to 50% of the negative control. An ANOVA test was conducted to determine the concentration that induced a statistically significant level of cytotoxicity as compared to their concurrent negative control ($P \leq 0.05$). The power of the ANOVA test was maintained at >0.8 at $\alpha = 0.05$. A Bootstrap statistic with multiple regression analyses was used to generate a mean and a standard error of the mean for the LC_{50} value sample (Table S1).

RESULTS AND DISCUSSION

Conversion of Free Oleic Acid and Glyceryl Trioleate to 9,10-Chlorohydrins. Initial experiments attempted to characterize the pseudo-first-order conversion rate of oleic acid to 9,10-chlorohydrins. Oleate (1.9 mM; just above the 1 mM critical micelle concentration[37]) was treated with 30 mM free chlorine at pH 7 in 20 mM phosphate buffer and 8 °C. Conversion to 9,10-chlorohydrins was complete within 15 s, indicating a pseudo-first-order conversion rate > 0.09 s^{-1} (Figure S4).

To evaluate the reaction stoichiometry, 1.5 mM oleate was treated for 5 min with up to 15 mM free chlorine at room temperature (∼21 °C) at pH 7 (Figure 1). The results indicated a 1:1 stoichiometry. At 0.75 mM free chlorine (0.5 molar equiv), the conversion to 9,10-chlorohydrins was ∼50%, while ∼100% conversion was observed for free chlorine concentrations ≥ 1.5 mM (≥1 molar equiv).

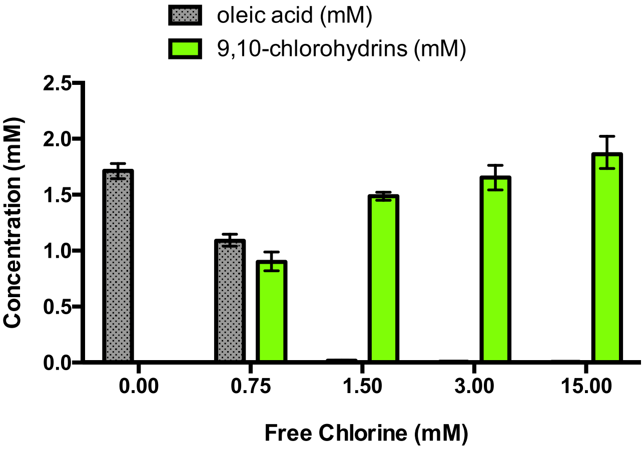


Figure 1. Stoichiometric conversion of potassium oleate (1.5 mM) micelles to form 9,10-chlorohydrins upon exposure for 5 min to 0, 0.5, 1.0, 2.0, and 10.0 molar equiv of free chlorine in deionized water at pH 7 with 10 mM sodium phosphate. Error bars represent the range of experimental replicates ($n = 2−3$).

Due to the low solubility of lipids, lipid-bound fatty acid alkenes tend to form a separate phase, restricting the access of aqueous chlorine to reactions with the alkenes. To characterize the importance of this restriction in access, chlorine was

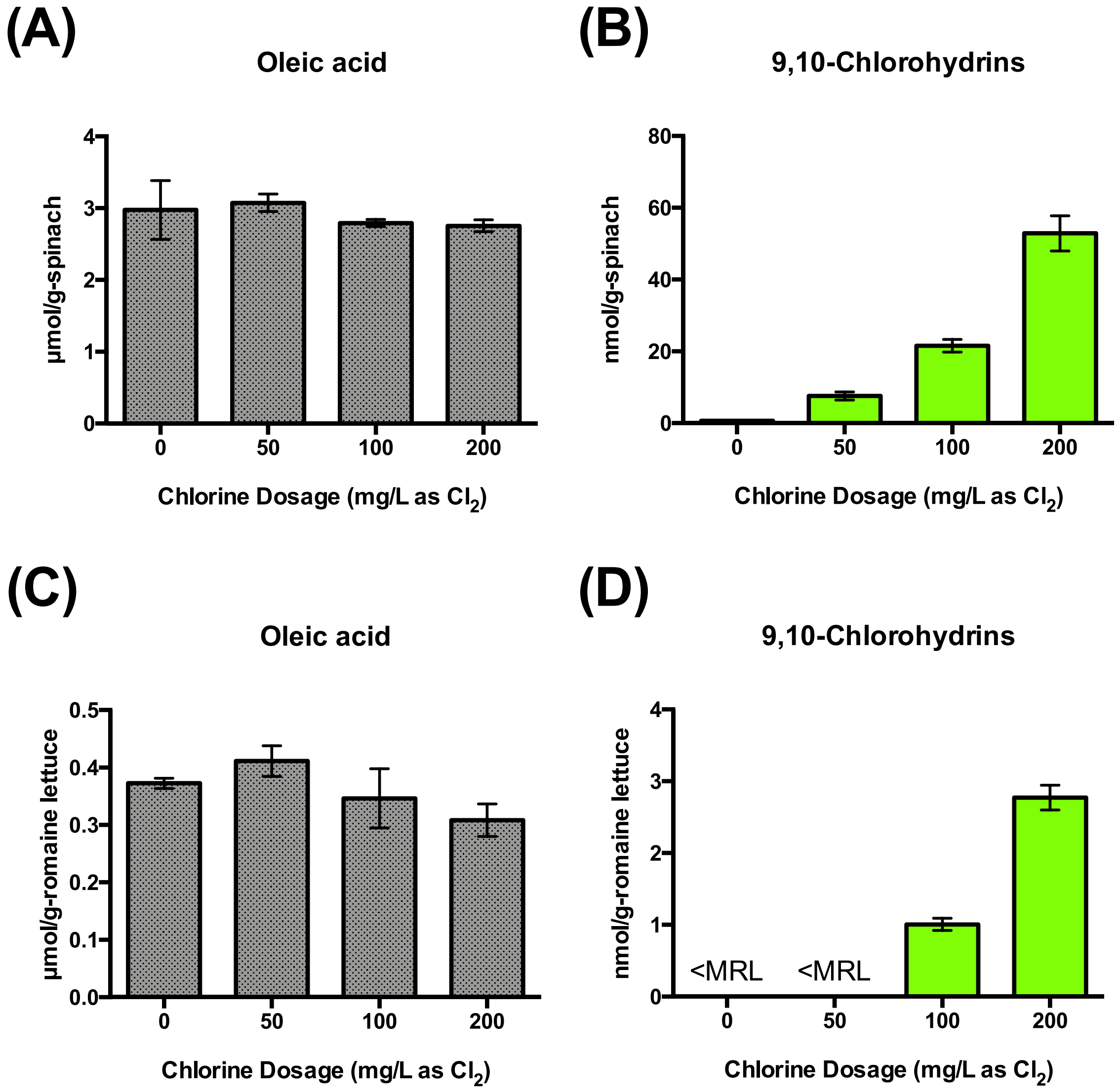


Figure 2. Concentrations of oleic acid and its 9,10-chlorohydrins after chlorination (0−200 mg/L as Cl_2) for 15 min of 1 g shredded (A, B) spinach (Cal-Organic Farms) or (C, D) romaine lettuce (Tanimura & Antle, sample A) in deionized water at pH 7 with 10 mM phosphate at 21 °C. Leaves were cut to 0.5 cm × 1 cm. Compounds were detected in the leaf phase and were normalized by the leaf wet weight. Error bars represent the standard error of experimental replicates ($n = 6$). MRL = minimum reporting limit (0.53 nmol/g).

applied to glyceryl trioleate, a triacylglyceride containing three oleic acid substituents. Because the glyceryl trioleate formed a separate phase, the solutions were vortexed during the reaction. Two conditions were evaluated: (1) 9 mM chlorine with 340 μM glyceryl trioleate (9-fold molar excess of chlorine relative to oleate) for 2 min at 21 °C buffered at pH 7 (10 mM phosphate) and (2) 612 mM chlorine with 17 mM glyceryl trioleate (12-fold molar excess relative to oleate) for 8 min at 21 °C unbuffered; the second condition represented extreme conditions to facilitate the detection of glyceryl trioleate chlorohydrins by LC/MS because of their poor ionization efficiency. LC/MS analysis did indicate the formation of glyceryl trioleate 9,10-monochlorohydrins, 9,10-dichlorohydrins, and 9,10-trichlorohydrins (Figures S5 and S6). No free oleic acid 9,10-chlorohydrins were detected by LC/MS, demonstrating that chlorination does not liberate oleic acid from triacylglycerides. After liberation of 9,10-chlorohydrins from the lipids, analysis by GC/MS indicated that the molar conversion of glyceryl-bound oleate to glyceryl-bound 9,10-chlorohydrins was 0.03% (±0.003%; $n = 3$) for 9 mM chlorine with 340 mM glyceryl trioleate and 4.1% (±0.01%; $n = 3$) for 612 mM chlorine and 17 mM glyceryl trioleate. Note that triacylglycerides represent an extreme case due to their high hydrophobicity; in phospholipids, which are prevalent in cell membranes, a polar phosphate group replaces one of the fatty acids attached to the glycerol, which would reduce the hydrophobicity and facilitate chlorine access to the fatty acid alkenes. Overall, these results indicate that chlorine addition to alkenes to form chlorohydrins is highly favorable but that the hydrophobicity of lipids, including their tendency to form a separate phase, can restrict access to reactions with chlorine.

Effect of Washing Conditions on Formation of Oleic Acid 9,10-Chlorohydrins. A series of experiments were conducted to characterize the effect of washing conditions on lipid-bound oleic acid 9,10-chlorohydrin formation, including chlorine dose, temperature, contact time, and whether the produce was shredded prior to washing. To evaluate the effect of chlorine dose, spinach (Cal-Organic Farms) and romaine lettuce (Tanimura & Antle, sample A) leaves were cut to 0.5 cm × 1 cm dimensions to mimic shredded vegetables and were

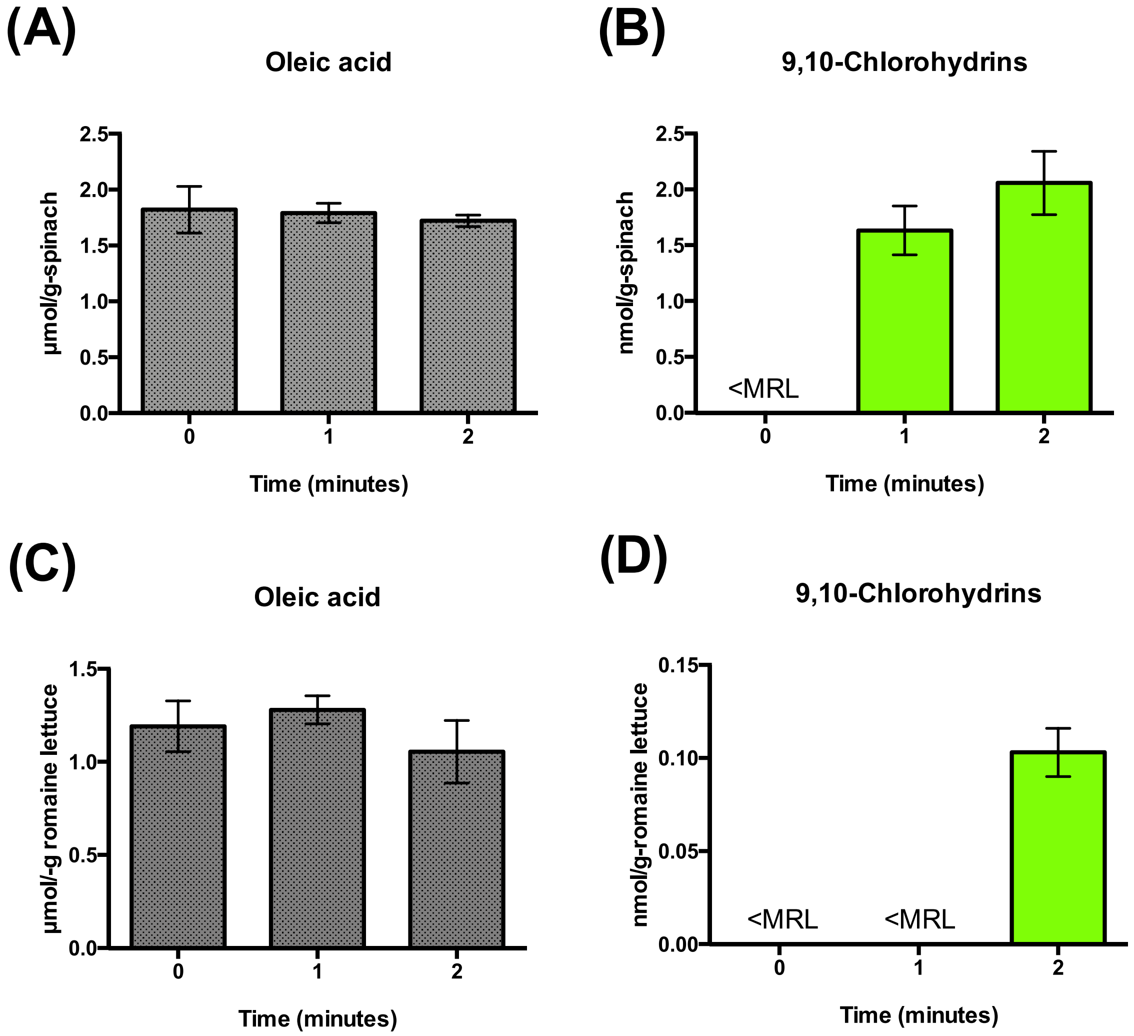


Figure 3. Concentrations of oleic acid and its 9,10-chlorohydrins after chlorination with 100 mg/L as Cl_2 for 0–2 min of 1 g shredded (A, B) baby spinach (Josie's Organics) or 10 g shredded (C, D) romaine lettuce (Tanimura & Antle, sample B) in deionized water at pH 7 with 10 mM phosphate at 7.5 °C. Leaves were cut to 0.5 cm × 1 cm. Compounds were detected in the leaf phase and were normalized by the leaf wet weight. Error bars represent the standard error of experimental replicates (n = 3–6). MRL = minimum reporting limit (0.53 nmol/g-spinach for 1 g-spinach samples and 0.05 nmol/g-romaine lettuce for 10 g lettuce samples).

treated for 15 min at pH 7 and 21 °C with 0–200 mg/L as Cl_2 free chlorine (Figure 2). The oleic acid concentrations in the spinach (3.0 μmol/g) were nearly an order of magnitude higher than in the romaine lettuce (0.38 μmol/g). For shredded spinach, the decrease in chlorine concentration was 66% for 50 mg/L as Cl_2 chlorine and 39% for 100 and 200 mg/L as Cl_2 (Table S2); a chlorine residual was always measurable. For shredded lettuce, the decrease in chlorine concentration was always <20%. Even after a free chlorine dose of 200 mg/L as Cl_2, the loss of oleic acid was <10%. For both shredded spinach and lettuce, the yield of 9,10-chlorohydrins increased with chlorine dose, reaching 53 nmol/g for spinach (1.8% molar yield) and 2.8 nmol/g for lettuce (0.74% molar yield) for the 200 mg/L as Cl_2 chlorine dose. The higher absolute concentrations of 9,10-chlorohydrins found in spinach concur with the higher oleic acid concentrations. These molar yields are much lower than the 100% yields observed for free oleic acid micelles. The maintenance of free chlorine residuals over the experimental time scales indicates that the reduced yields were not due to a limitation on chlorine exposure resulting from scavenging of the chlorine by reaction with other molecules in the produce. Rather, the results suggest that the low yields result from limited access of the chlorine to lipid-bound oleic acids within the interior of the leaves; experiments comparing chlorohydrin yields between whole leaf and shredded spinach further evaluate this hypothesis (see below).

Washing of certain leafy vegetables in postharvest facilities can be conducted under colder conditions with shorter chlorine contact times. To evaluate the impact of lower temperature and shorter contact times, shredded baby spinach (Josie's Organics) and romaine lettuce leaves (Tanimura & Antle, sample B) were treated with 100 mg/L as Cl_2 free chlorine for 0–2 min at pH 7 and 7.5 °C (Figure 3). These experiments involved different batches of spinach and romaine lettuce than the experiments represented in Figure 2 and hence different starting concentrations of oleic acid. However, the oleic acid concentrations were still higher in the spinach (1.8 μmol/g) than in romaine lettuce (1.2 μmol/g). The decrease in chlorine residual was <10% (Table S2). Concentrations of the 9,10-chlorohydrins increased over 2 min to reach 2 nmol/g (0.11% molar yield) in spinach, much lower than the 22 nmol/

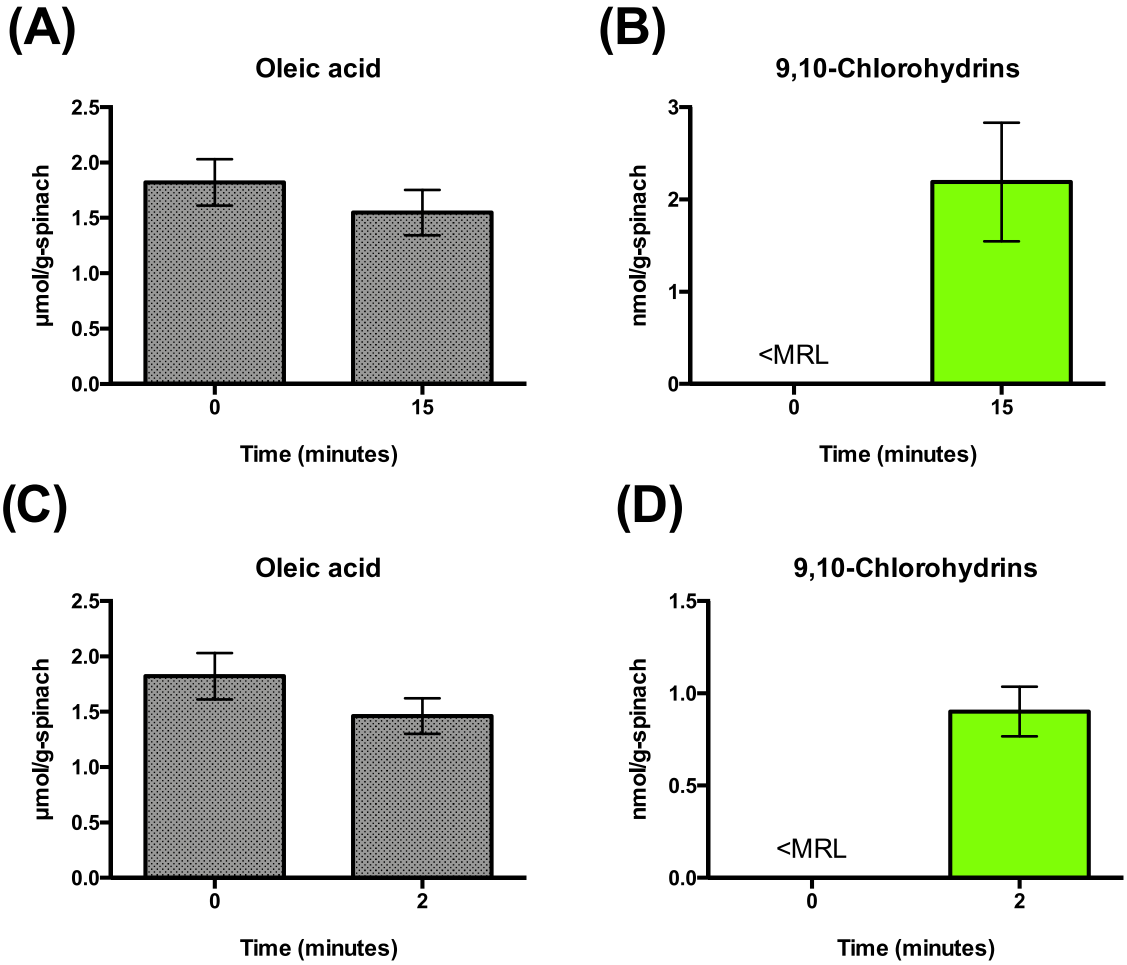


Figure 4. Concentrations of oleic acid and its 9,10-chlorohydrins after chlorination with 100 mg/L as Cl_2 of 1 g whole baby spinach (Josie's Organics) in deionized water at pH 7 with 10 mM phosphate at 21 °C for 15 min (A, B) and 7.5 °C for 2 min (C, D). Compounds were detected in the leaf phase and were normalized by the leaf wet weight. Error bars represent the standard error of experimental replicates (n = 3−6). MRL = minimum reporting limit (0.53 nmol/g-spinach).

g (0.83% molar yield) observed for the same 100 mg/L as Cl_2 chlorine dose but over 15 min at room temperature (Figure 2). Similarly, the oleic acid 9,10-chlorohydrin concentrations increased over 2 min at 7.5 °C to 0.10 nmol/g (0.0083% molar yield) in shredded lettuce (Figure 3), much lower than the 1 nmol/g (0.26% molar yield) observed over 15 min at room temperature (Figure 2).

As indicated previously, the low conversion of total oleic acid to 9,10-chlorohydrins observed in shredded spinach and lettuce relative to the 100% molar conversion observed in free oleic acid micelles may be attributable to the limited access of chlorine to lipid-bound oleic acid within the interior of leaves. To evaluate this possibility, oleic acid 9,10-chlorohydrin yields were compared between shredded and whole leaf spinach, where shredding was expected to increase the access of chlorine to oleic acid within the leaves. Chlorination of ~1 g whole baby spinach leaves (Josie's Organics) with 100 mg/L as Cl_2 chlorine at pH 7 and 21 °C for 15 min produced 2.2 nmol/g-spinach of 9,10-chlorohydrins from 1.8 µmol/g-spinach of oleic acid (0.12% molar yield; Figure 4A,B). Chlorination of ~1 g of the same batch of whole baby spinach leaves with the same chlorine dose at a lower temperature (7.5 °C) for a shorter time (2 min) generated only 0.90 nmol/g-spinach of 9,10-chlorohydrins (0.05% molar yield; Figure 4C,D). The decrease in chlorine residual was <10% (Table S2). Molar yields of 9,10-chlorohydrins were higher when the spinach was shredded before chlorination with 100 mg/L as Cl_2 at both 21 °C for 15 min (0.83%; Figure 2) and 7.5 °C for 2 min (0.11%; Figure 3). These results indicate that shredding increases the access of chlorine to oleic acid within the interior of the leaves. Similarly, we had previously observed an increased molar yield of chlorotyrosines from tyrosines for chlorination of shredded spinach leaves relative to whole leaves.[21]

Oleic Acid 9,10-Chlorohydrins and Chlorotyrosines across Vegetables. Oleic acid 9,10-chlorohydrins and chlorotyrosines (i.e., 3-chlorotyrosine and 3,5-dichlorotyrosine) were measured in two varieties of shredded lettuce (butterhead and red leaf) and four other shredded or cubed vegetables (broccoli, cabbage, carrot, and kale) after chlorination with 100 mg/L as Cl_2 chlorine for 2 min at pH 7 and 7.5 °C (Figure 5). Oleic acid concentrations were highest in broccoli (2.0 µmol/g) and butterhead lettuce (1.34 µmol/g) and lowest in kale. Tyrosine concentrations were highest in kale (7.6 µmol/g) and broccoli (6.0 µmol/g) and lowest in cabbage. Tyrosine concentrations were higher than oleic acid concentrations in all vegetables except cabbage, where they were similar. The decrease in chlorine residual was <10% (Table S2).

The concentrations of 9,10-chlorohydrins were highest for cubed or shredded broccoli, carrot, and butterhead lettuce

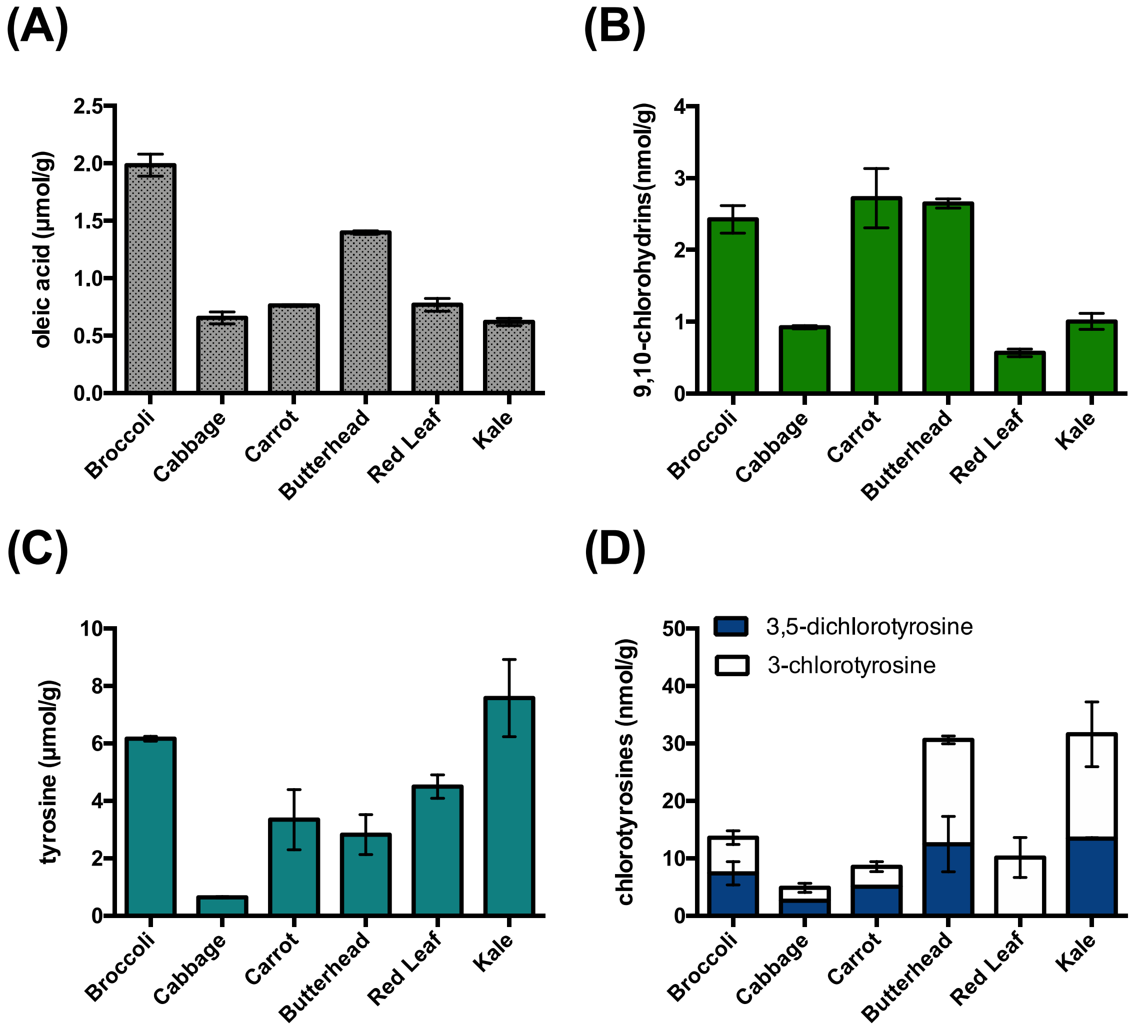


Figure 5. Concentrations of (A) oleic acid, (B) oleic acid 9,10-chlorohydrins, (C) tyrosine, and (D) chlorotyrosines after chlorination of shredded vegetables with 100 mg/L as Cl_2 for 2 min in deionized water at 7.5 °C and pH 7 with 10 mM phosphate. Shredded vegetables included broccoli (1 cm × 1 cm × 1 cm), cabbage, carrot (0.5 cm × 0.5 cm × 0.5 cm), butterhead, and red leaf lettuces and kale. If not otherwise stated, all samples were cut into 0.5 cm × 1 cm pieces. 9,10-Chlorohydrins, 3-chlorotyrosine, and 3,5-dichlorotyrosine were detected in the solid sample phase and were normalized by the wet weight of vegetable precursor material. All vegetables were washed at 1 g wet weight in 50 mL wash solution, except for cabbage, carrot, and butterhead lettuce, which were washed at 10 g wet weight in 500 mL wash solution. Error bars represent the standard error of experimental replicates (n = 2−3). MRL = minimum reporting limit (0.53 nmol/g for 1 g samples and 0.053 nmol/g for 10 g samples).

(2.4−2.7 nmol/g; Figure 5B) and somewhat higher than the 2.0 nmol/g observed in shredded spinach under the same conditions (Figure 3). Shredded cabbage, kale, and red leaf lettuce formed lower levels of 9,10-chlorohydrins, comparable to the 0.9 nmol/g observed during chlorination of whole baby spinach leaves under the same conditions (Figure 4D). 9,10-Chlorohydrin formation typically tracked with oleic acid concentrations.

Total chlorotyrosine concentrations were always higher than the concentrations of oleic acid 9,10-chlorohydrins (Figure 5D). Total chlorotyrosine concentrations were highest in kale (31 nmol/g) and butterhead lettuce (27 nmol/g), with concentrations ranging from 3.1 to 13 nmol/g in the other vegetables. Previously, we measured 60 nmol/g total chlorotyrosines when shredded spinach was treated with 100 mg/L as Cl_2 chlorine over a longer contact time (5 min at 7 °C).[21] 3,5-Dichlorotyrosine was the dominant chlorotyrosine in all vegetables except red and butterhead lettuces and kale. While no 3,5-dichlorotyrosine was detected in red leaf lettuce during these experiments, we detected it previously in a different batch of shredded red leaf lettuce under different conditions (20 mg/L as Cl_2 chlorine for 15 min at 21 °C).[21]

Cytotoxicity and Implications. The CHO cell chronic cytotoxicity concentration−response curve for 9,10-chlorohydrins of oleic acid is shown in Figure S3. The concentration of 9,10-chlorohydrins associated with a 50% decrease in CHO cell growth relative to untreated controls (i.e., the LC_{50} value) was 0.106 mM. This value is significantly lower than the LC_{50} values for 3-chlorotyrosine (3.17 mM) and 3,5-dichlorotyrosine (0.71 mM),[21] indicating that the oleic acid 9,10-chlorohydrins are more cytotoxic by ∼30-fold and ∼7-fold, respectively.

To provide an initial estimate of the importance of exposure to DBPs in chlorine-disinfected vegetables relative to chlorinated drinking water, we compared the exposure associated with lipid-bound oleic acid chlorohydrins and protein-bound chlorotyrosines in vegetables to the consumption of a drinking water containing 80 μg/L chloroform and 60 μg/L trichloroacetic acid, levels of regulatory concern for trihalomethanes and haloacetic acids in tap water.[48] The CHO cell cytotoxicity LC_{50} values for chloroform and trichloroacetic acid are 9.63 and 2.4 mM, respectively,[43] indicating that they are less cytotoxic than oleic acid 9,10-chlorohydrins and 3,5-dichlorotyrosine. Even though brominated trihalomethanes and haloacetic acids are more cytotoxic than their chlorinated analogues,[43] we considered only chlorinated analogues of the drinking water DBPs since we evaluated only chlorotyrosines and 9,10-chlorohydrins in the vegetables. Consumption of 2 L of water per day would involve exposure to 1.34 μmol chloroform and 0.74 μmol trichloroacetic acid. These masses were normalized by their respective LC_{50} values, providing cytotoxic potency-weighted exposures of 1.4×10^{-4} for chloroform and 3.07×10^{-4} for trichloroacetic acid. In accordance with our previous research demonstrating that mammalian cell cytotoxicity of these DBPs in mixtures is additive,[49] we summed these cytotoxic potency-weighted exposures to provide a total daily exposure of 4.47×10^{-4} from the water.

Normalizing the concentrations of oleic acid 9,10-chlorohydrins, 3-chlorotyrosine and 3,5-dichlorotyrosine measured in shredded or cubed vegetables (Figure 5) by their respective LC_{50} values (Figure S7) indicated that oleic acid 9,10-chlorohydrins were more significant contributors to the cytotoxicity than the chlorotyrosines for four of the six vegetables. We calculated how many grams of each vegetable (on a wet weight basis) would provide a total cytotoxic exposure equivalent to the 2 L of drinking water. The results indicated that between 9 and 52 g of shredded or cubed vegetables washed with 100 mg/L as Cl_2 chlorine for 2 min at pH 7 and 7.5 °C provided an equivalent cytotoxic exposure (Figure S8). Only 9−13 g of butterhead lettuce, broccoli, carrot, and kale were needed, which are low relative to the 85 g lettuce (~1.5 cups) and 148 g broccoli (~1 cup) recommended daily intakes;[50] for perspective, 10 g broccoli is approximately one fork full. These results suggest that the consumption of chlorine-disinfected produce could be an important pathway for DBP exposure. However, the high chlorine exposures associated with these levels of oleic acid chlorohydrin and chlorotyrosine formation are typically applied to ready-to-eat produce within postharvest washing facilities, while other produce (e.g., lettuce heads) experience lower chlorine doses (<40 mg/L as Cl_2) to rinse off dirt after harvest. DBP exposure can also decrease when produce is cooked. Heating 200 nM oleic acid 9,10-chlorohydrins to boiling at pH 7 (10 mM phosphate buffer) resulted in a 62% (±2%; $n = 3$) decrease in chlorohydrin concentrations (Text S7). However, ready-to-eat produce typically is consumed raw.

Just as tap water contains many other DBPs beyond THMs and HAAs, disinfected produce likely contains DBPs beyond oleic acid chlorohydrins and chlorotyrosines. Noteworthy is that oleic acid is one of many biomolecules featuring alkene functional groups, including other fatty acids and pigments (e.g., carotene in carrots). For instance, oleic acid (18:1) constitutes only 1.1% of the total fatty acids in spinach, relative to the 3.5% accounted for by palmitoleic acid (16:1), 4.6% by linoleic acid (18:2), and 69% by linolenic acid (18:3).[28] Since all of these alkenes are expected to form their respective chlorohydrins, the total cytotoxicity exerted by chlorohydrins within chlorine-disinfected vegetables may be far higher than calculated here. However, analysis of other chlorohydrins can be complex, particularly due to the multiple chlorohydrin isomers that can arise from chlorination of polyunsaturated fatty acids, such as linolenic acid.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c04362.

> Material sources; synthesis procedures; analytical method details; CHO cell cytotoxicity results; chlorohydrin and chlorotyrosine concentrations in disinfected vegetables; and masses of vegetables exhibiting comparable cytotoxicity to chlorine-disinfected drinking water (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) WHO. *WHO Estimates of the Global Burden of Foodborne Diseases Short*; WHO, 2015.

(2) CDC. National Outbreak Reporting System (NORS). https://wwwn.cdc.gov/norsdashboard/ (accessed April 15, 2020).

(3) United States 111th Congress. 124 Stat. 3885. Food Safety Modernization Act; United States of America, 2011; pp 3885−3973.

(4) Francis, G. A.; Thomas, C.; O'Beirne, D. The Microbiological Safety of Minimally Processed Vegetables. *Int. J. Food Sci. Technol.* **1999**, *34*, 1−22.

(5) Fukayama, M. Y.; Tan, H.; Wheeler, W. B.; Wei, C. I. Reactions of Aqueous Chlorine and Chlorine Dioxide with Model Food Compounds. *Environ. Health Perspect.* **1986**, *69*, 267−274.

(6) Simpson, A. M.-A.; Mitch, W. A. Chlorine and Ozone Disinfection and Disinfection Byproducts in Postharvest Food Processing Facilities: A Review. *Crit. Rev. Environ. Sci. Technol.* **2020**, 1−43.

(7) Bao Loan, H. N.; Jacxsens, L.; Kurshed, A. A. M.; De Meulenaer, B. 3-Chlorotyrosine Formation in Ready-to-Eat Vegetables Due to Hypochlorite Treatment and Its Dietary Exposure and Risk Assessment. *Food Res. Int.* **2016**, *90*, 186−193.

(8) Cardador, M. J.; Gallego, M. Effect of the Chlorinated Washing of Minimally Processed Vegetables on the Generation of Haloacetic Acids. *J. Agric. Food Chem.* **2012**, *60*, 7326−7332.

(9) Huang, A.-T.; Batterman, S. Formation of Trihalomethanes in Foods and Beverages. *Food Addit. Contam., Part A* **2009**, *26*, 947−957.

(10) Klaiber, R. G.; Baur, S.; Wolf, G.; Hammes, W. P.; Carle, R. Quality of Minimally Processed Carrots as Affected by Warm Water Washing and Chlorination. *Innovative Food Sci. Emerging Technol.* **2005**, *6*, 351−362.

(11) Suslow, T. *Postharvest Chlorination: Basic Properties & Key Points for Effective Distribution*; University of California, Agriculture and Natural Resources, 1997.

(12) Suslow, T. Chlorination in the Production and Postharvest Handling of Fresh Fruits and Vegetables. https://www.siphidaho.org/env/pdf/Chlorination_of_fruits_and_veggies.PDF (retrieved November 1, 2020).

(13) Gómez-López, V. M.; Marín, A.; Medina-Martínez, M. S.; Gil, M. I.; Allende, A. Generation of Trihalomethanes with Chlorine-Based Sanitizers and Impact on Microbial, Nutritional and Sensory Quality of Baby Spinach. *Postharvest Biol. Technol.* **2013**, *85*, 210−217.

(14) Coroneo, V.; Carraro, V.; Marras, B.; Marucci, A.; Succa, S.; Meloni, B.; Pinna, A.; Alberto, A.; Adriana, S.; Schintu, M. Presence of Trihalomethanes in Ready-to-Eat Vegetables Disinfected with Chlorine. *Food Addit. Contam., Part A* **2017**, *34*, 2111−2117.

(15) López-Gálvez, F.; Tudela, J. A.; Allende, A.; Gil, M. I. Microbial and Chemical Characterization of Commercial Washing Lines of Fresh Produce Highlights the Need for Process Water Control. *Innovative Food Sci. Emerging Technol.* **2019**, *51*, 211−219.

(16) Shen, C.; Norris, P.; Williams, O.; Hagan, S.; Li, K. W. Generation of Chlorine By-Products in Simulated Wash Water. *Food Chem.* **2016**, *190*, 97−102.

(17) Van Haute, S.; Sampers, I.; Holvoet, K.; Uyttendaelea, M. Physicochemical Quality and Chemical Safety of Chlorine as a Reconditioning Agent and Wash Water Disinfectant for Fresh-Cut Lettuce Washing. *Appl. Environ. Microbiol.* **2013**, *79*, 2850−2861.

(18) Tudela, J. A.; López-Gálvez, F.; Allende, A.; Hernández, N.; Andújar, S.; Marín, A.; Garrido, Y.; Gil, M. I. Operational Limits of Sodium Hypochlorite for Different Fresh Produce Wash Water Based on Microbial Inactivation and Disinfection By-Products (DBPs). *Food Control* **2019**, *104*, 300−307.

(19) Hua, L.; Kim, E.; McCurry, D. L.; Huang, C.; Mitch, W. A. Novel Chlorination Byproducts of Tryptophan: Initial High-Yield Transformation Products versus Small Molecule Disinfection By-products. *Environ. Sci. Technol. Lett.* **2020**, *7*, 149−155.

(20) Choe, J. K.; Hua, L.-C.; Komaki, Y.; Simpson, A. M.-A.; McCurry, D. L.; Mitch, W. A. Evaluation of Histidine Reactivity and Byproduct Formation during Peptide Chlorination. *Environ. Sci. Technol.* **2021**, *55*, 1790−1799.

(21) Komaki, Y.; Simpson, A. M.-A.; Choe, J. K.; Plewa, M. J.; Mitch, W. A. Chlorotyrosines versus Volatile Byproducts from Chlorine Disinfection during Washing of Spinach and Lettuce. *Environ. Sci. Technol.* **2018**, *52*, 9361−9369.

(22) Pattison, D. I.; Hawkins, C. L.; Davies, M. J. Hypochlorous Acid-Mediated Protein Oxidation: How Important Are Chloramine Transfer Reactions and Protein Tertiary Structure? *Biochemistry* **2007**, *46*, 9853−9864.

(23) Sivey, J. D.; Howell, S. C.; Bean, D. J.; McCurry, D. L.; Mitch, W. A.; Wilson, C. J. Role of Lysine during Protein Modification by HOCl and HOBr: Halogen-Transfer Agent or Sacrificial Antioxidant? *Biochemistry* **2013**, *52*, 1260−1271.

(24) Walse, S. S.; Plewa, M. J.; Mitch, W. A. Exploring Amino Acid Side Chain Decomposition Using Enzymatic Digestion and HPLC-MS: Combined Lysine Transformations in Chlorinated Waters. *Anal. Chem.* **2009**, *81*, 7650−7659.

(25) Hawkins, C. L.; Pattison, D. I.; Davies, M. J. Hypochlorite-Induced Oxidation of Amino Acids, Peptides and Proteins. *Amino Acids* **2003**, *25*, 259−274.

(26) Huang, H.; Wu, Q. Y.; Hu, H. Y.; Mitch, W. A. Dichloroacetonitrile and Dichloroacetamide Can Form Independently during Chlorination and Chloramination of Drinking Waters, Model Organic Matters, and Wastewater Effluents. *Environ. Sci. Technol.* **2012**, *46*, 10624−10631.

(27) Bao Loan, H. N.; Devlieghere, F.; Van Hoeke, C.; De Meulenaer, B. 3-Chlorotyrosine Formation in Gilthead Seabream (Sparus Aurata) and European Plaice (Pleuronectes Platessa) Fillets Treated with Sodium Hypochlorite. *Food Res. Int.* **2015**, *69*, 164−169.

(28) Wolf, F. T.; Coniglio, J. G.; Davis, J. T. Fatty Acids of Spinach Chloroplasts. *Plant Physiol.* **1962**, *37*, 83−85.

(29) Speer, J. H.; Wise, E. C.; Hart, M. C. The Composition of Spinach Fat. *J. Biol. Chem.* **1929**, *82*, 105−110.

(30) Holloway, P. J. Intracuticular Lipids of Spinach Leaves. *Phytochemistry* **1974**, *13*, 2201−2207.

(31) Carr, A. C.; Vissers, M. C. M.; Domigan, N. M.; Winterbourn, C. C. Modification of Red Cell Membrane Lipids by Hypochlorous Acid and Haemolysis by Preformed Lipid Chlorohydrins. *Redox Rep.* **1997**, *3*, 263−271.

(32) Winterbourn, C. C.; van den Berg, J. J. M.; Roitman, E.; Kuypers, F. A. Chlorohydrin Formation from Unsaturated Reacted with Hypochlorous Acid Fatty Acids. *Arch. Biochem. Biophys.* **1992**, *296*, 547−555.

(33) Winterbourn, C. C.; Kettle, A. J. Biomarkers of Myeloperoxidase-Derived Hypochlorous Acid. *Free Radical Biol. Med.* **2000**, *29*, 403−409.

(34) Carr, A. C.; Winterbourn, C. C.; Van Den Berg, J. J. M. Peroxidase-Mediated Bromination of Unsaturated Fatty Acids to Form Bromohydrins. *Arch. Biochem. Biophys.* **1996**, *327*, 227−233.

(35) Carr, A. C.; Winterbourn, C. C.; Blunt, J. W.; Phillips, A. J.; Abell, A. D. Nuclear Magnetic Resonance Characterization of 6α-Chloro-5β-Cholestane-3β,5-Diol Formed from the Reaction of Hypochlorous Acid with Cholesterol. *Lipids* **1997**, *32*, 363−367.

(36) Pan, Y.; Zhang, X.; Li, Y. Identification, toxicity and control of iodinated disinfection byproducts in cooking with simulated chlor(am)inated tap water and iodized table salt. *Water Res.* **2016**, *88*, 60−68.

(37) Zhang, D.; Wu, Y.; Zhang, X.; Li, W.; Li, Y.; Li, A.; Yang, P. Identification, formation and control of polar brominated disinfection byproducts during cooking with edible salt, organic matter and simulated tap water. *Water Res.* **2020**, *172*, No. 115526.

(38) Feng, Y.; Smith, D. W.; Bolton, J. R. Photolysis of Aqueous Free Chlorine Species (HOCl and OCl^-) with 254 nm Ultraviolet Light. *J. Environ. Eng. Sci.* **2007**, *6*, 277−284.

(39) National Standard Reference Data System. In *Critical Micelle Concentrations of Aqueous Surfactant Systems*; Mukerjee, P.; Mysels, K. J., Eds.; National Bureau of Standards: Washington, DC, 1971.

(40) Suslow, T. V. Extension Research Specialist, University of California at Davis, *Personal Communication* (accessed October 18, 2021).

(41) Rice, E. W.; Baird, R. B.; Eaton, A. D. *Standard Methods for the Examination of Water & Wastewater*, 23rd ed.; American Public Health Association, American Water Works Association and the Water Environment Association, 2017.

(42) Matyash, V.; Liebisch, G.; Kurzchalia, T. V.; Shevchenko, A.; Schwudke, D. Lipid Extraction by Methyl-Tert-Butyl Ether for High-Throughput Lipidomics. *J. Lipid Res.* **2008**, *49*, 1137−1146.

(43) Wagner, E. D.; Plewa, M. J. CHO Cell Cytotoxicity and Genotoxicity Analyses of Disinfection By-Products: An Updated Review. *J. Environ. Sci.* **2017**, *58*, 64−76.

(44) Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J. Analysis of Mutagens with Single Cell Gel Electrophoresis, Flow Cytometry, and Forward Mutation Assays in an Isolated Clone of

Chinese Hamster Ovary Cells. *Environ. Mol. Mutagen.* **1998**, *32*, 360−368.

(45) Xu, X.; Nagarajan, H.; Lewis, N. E.; Pan, S.; Cai, Z.; Liu, X.; Chen, W.; Xie, M.; Wang, W.; Hammond, S.; et al. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat. Biotechnol.* **2011**, *29*, 735−741.

(46) Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D. Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2002**, *40*, 134−142.

(47) Plewa, M. J.; Wagner, E. D. *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*; Water Research Foundation: Denver, CO, 2009, p 134.

(48) EPA. National Primary Drinking Water Regulations. https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations (accessed June 22, 2021).

(49) Lau, S. S.; Wei, X.; Bokenkamp, K.; Wagner, E. D.; Plewa, M. J.; Mitch, W. A. Assessing Additivity of Cytotoxicity Associated with Disinfection Byproducts in Potable Reuse and Conventional Drinking Waters. *Environ. Sci. Technol.* **2020**, *54*, 5729−5736.

(50) FDA. Nutrition Information for Raw Vegetables. https://www.fda.gov/food/food-labeling-nutrition/nutrition-information-raw-vegetables (accessed June 14, 2021).