Ozonated Water Extends the Shelf Life of Fresh-Cut Lettuce

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The use of ozonated water as a sanitizer to extend the shelf life of fresh-cut lettuce and the effect on the antioxidant constituents (polyphenols and vitamin C) were investigated. Fresh-cut iceberg lettuce (*Lactuca sativa* L.) was washed at 4 °C using three different ozonated water dips [10, 20, and 10 activated by ultraviolet C (UV-C) light mg L^{-1} min total ozone dose], and the dips were compared with water and chlorine rinses. Treated lettuce was packaged in air or active modified atmosphere packaging (MAP) (4 kPa of O_2 + 12 kPa of CO_2 balanced with N_2) and stored for 13 days at 4 °C. Despite its strong oxidizing activity, ozonated water did not stimulate the respiratory activity of fresh-cut lettuce. Moreover, ozonated water maintained the initial visual appearance of fresh-cut lettuce and controlled browning during storage in air. Initially, ozonated water and chlorine reduced the total mesophilic population by 1.6 and 2.1 log, respectively, when compared with water. Active MAP was effective in controlling total microbial growth, achieving 2.0 log reduction in relation to samples stored in air at the end of storage. On the other hand, active MAP caused a 2.0−3.5 reduction of coliforms on sanitized samples compared with water-washed samples. The most efficient treatments were ozone 20 and ozone 10 activated by UV-C, which were as effective as chlorine. Changes in individual phenolic compounds were independent of the washing treatments. In air, chlorogenic and isochlorogenic acid contents increased noticeably after 13 days while monocaffeoyltartaric and dicaffeoyltartaric acids remained unchanged. MAP effectively suppressed accumulation of caffeoylquinic derivatives, whereas caffeoyltartaric derivatives decreased during MAP storage to reach similar levels. The content of vitamin C (ascorbic acid and dehydroascorbic acid) decreased during storage, particularly under MAP. Ozonated water could be an alternative sanitizer to chlorine for fresh-cut lettuce due to good retention of sensorial quality and browning control with no detrimental reduction in the antioxidant constituents.

KEYWORDS: Lactuca sativa L.; browning; chlorine; microbial quality; minimal processing; modified atmosphere packaging; polyphenols; sanitizers; sensory quality; vitamin C

INTRODUCTION

In the past 10 years, there has been an increasing demand of fresh-cut fruits and vegetables, mainly because of their convenience as ready to eat products as well as the health benefits associated with their consumption (*1*, *2*). Fresh-cut lettuce has been one of the commodities with higher request by salad bars and fast food services over the past 10 years, and it may represent more than 80% of the total production of fresh-cut produce (*3*). The nutrient content of lettuce is important because of the large quantities consumed, and it includes several important constituents such as phenolic antioxidants as caffeic acid derivatives (*4*, *5*) and flavonoids (*6*), and vitamins A and C, calcium, and iron (*7*). The phenolic compounds may serve as natural substrates for oxidative enzymes such as polyphenol oxidase (PPO) to yield *o*-quinones that polymerize in non-enzyme-catalyzed reactions, resulting in brown pigments in the presence of oxygen (O_2) (*8*). Low O_2 levels are favorable to prevent cut edge browning, and therefore, active atmosphere modification (gas flushing) is routinely employed (*9*). In general, fresh-cut iceberg lettuce is gas flushed to quickly attain atmospheres of less than 1 kPa of O_2 to slow browning caused by PPO (*10*, *11*). In addition, fresh-cut lettuce is often packaged in an atmosphere of ≥10 kPa of carbon dioxide (CO_2) to inhibit biosynthesis of enzymatic browning reaction substrates (*12*).

Apart from the packaging, the washing or sanitizing step is one of the most important stages in the processing of fresh-cut products to remove dirt, pesticide residues, and microorganisms responsible for quality loss and decay (*13*). This step is also used to precool cut produce and remove cell exudates that adhere to product cut surfaces and may support microbial growth and browning. Chlorine-based sanitizers have been used for sanitation purposes in food processing for several decades and are perhaps the most widely used sanitizers in the food industry (*14*−*16*). Conventional fresh-cut lettuce production uses rinse water, usually chlorinated at 100 mg/L. Safety concerns about

the reaction of chlorine with organic residues in the formation of potentially mutagenic or carcinogenic reaction products, such as trihalomethanes (*17*, *18*), and their impact on human and environmental safety have been raised in recent years. For this reason, its use for washing fresh-cut products is banned in several European countries, including Germany, The Netherlands, Switzerland, and Belgium. This is a cause of concern because some restrictions in the use of chlorine might be implemented in other countries, and therefore, other alternatives to chlorine must be investigated.

Ozone as an aqueous disinfectant was declared to be generally recognized as safe (GRAS) for food contact applications in 1997 (*19*, *20*). In some studies ozonated water has been shown to reduce the microbial population and extend the shelf life of fresh-cut fruits and vegetables (*13*, *21*). Furthermore, it has been used for disinfection of lettuce (*22*), fresh-cut cilantro (*23*), fresh-cut celery (*24*), and trimmed lettuce heads although it was less effective as a prewashing step than chlorine (*25*). Moreover, the decrease of pathogens including *Salmonella typhimurium*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 has also been described (*26*, *27*).

However, due to its strong oxidizing activity, ozone may also cause physiological injury (*28*) and loss of antioxidant constituents. The effect of ozone gas on the appearance of fresh lettuce showed an adverse impact on appearance and physiological injury on both photosynthetic and vascular tissues (*29*).

The disagreement of most studies on the effectiveness of ozonated water may be attributed to the ozone generators, which are usually developed for laboratory scale, and are not always consistent in their concentrations because of the difficulties in the solubilization of ozone. However, in terms of process optimization and knowledge of shelf life application, an ozonated system including an ozone analyzer, an ambient sensor, a probe, a temperature controller, and an effective system for dissolving ozone gas in the water is essential. Additionally, a recirculation system that supplements new ozonated water is also required.

The main objective of the present work was to investigate the use of ozonated water, preserving the microbial and sensory quality of fresh-cut lettuce stored under different packaging conditions (air and modified atmosphere packaging (MAP)). The effect of ozone on the antioxidant constituents (polyphenols and vitamin C) and enzymatic browning substrates was also studied.

MATERIALS AND METHODS

Plant Material. Iceberg lettuce (*Lactuca sativa* L.) grown under commercial conditions was harvested in fields located in Campo de Cartagena (Murcia, Spain) between January and May. Lettuce heads were transported to the laboratory (40 km) under refrigerated conditions and kept at 4 °C and 70% relative humidity (RH) in darkness until processing the next day. Wrapper leaves were hand-removed, and heads were then shredded in 3 cm pieces using a sharp stainless steel knife. To obtain a homogeneous sample, shredded lettuce was well mixed and divided into five batches of 2 kg, one for each washing treatment. All the process was conducted at 8 °C under sanitary conditions. Three repetitions separately in time were carried out from January to May. Results from one of the experiments were used in this assay, although similar tendencies were observed in all cases.

Wash Solutions. Shredded lettuces were washed at 4 °C with five different solutions: (A) sterile deionized water (pH 5.81), (B) 80 mg L^{-1} total chlorine prepared from sodium hypochlorite (10%, w/v) (Panreac, Montcada i Reixac, Barcelona, Spain) adjusted at pH 6.50 with citric acid (the total chlorine concentration was determined following the method reported by the American Society for Testing and Materials (*30*)), (C) 10 mg L^{-1} min total ozone dose (ozone 10), (D) 20 mg L^{-1} min total ozone dose (ozone 20), and (E) 10 mg L^{-1} min total dose of ozone activated by ultraviolet C (UV-C) (ozone 10 + UV). The ozonated water had pH 7.50. The washing time was 3 min, except for ozonated water, for which the time for treatment was the time that was sufficient to achieve the reported doses (always shorter than 5 min). Unwashed samples were not investigated because the wash is a prerequisite step after lettuce shredding for shelf life extension as has been suggested in recent studies (*25*, *31*), and therefore, they were not considered of commercial interest.

Ozonated Water. Extradry compressed air (0.7 Pa) was passed through a water-cooled corona discharge generator (model 1A, Steriline, Ozono Electronica Iberica, Granada, Spain) to produce ozone. Gaseous ozone production (3 g h^{-1}) was measured with an ozone gas analyzer (model H1-SPT, IN USA Inc., Needham, MA). A flow of ozone of 0.12 nm^3 h^{-1} was dissolved in deionized water by an inverse mixer in a 100 L dissolution tank. The excess of gas was neutralized by a thermal destroyer (model DOT 1.1, Ozono Electronica Iberica) at 550 °C. Ozonated water was impelled out by a pump at a flow rate of 1 m^3 h^{-1}, and conduced through a stainless steel plate heat exchanger (model UFX 6-11, Barriquand, Roanne Cedex, France), coupled to a water-cooling apparatus with a capacity of 1.98 kW (model TAE 015 PO, MTA Srl, Conselve, Italy). Finally, the ozonated water was delivered to the 50 L treatment tank and returned to the dissolution tank by a second pump, closing the circuit. An amperometric selective probe equipped with a temperature compensation sensor was used to monitor dissolved ozone and connected to a dissolved ozone analyzer (B&C electronics Srl, Carnate, Milano, Italy), which measures the ozone concentrations in two ranges (0−2 and 0−20 mg L^{-1}). The indigo trisulfonate spectrophotometric method was used to calibrate the analyzer, and also to check the ozone concentration applied (*32*, *33*). The decrease in absorbance was measured at 25 ± 0.1 °C in a spectrophotometer (UV-1603, Shimadzu, Tokyo, Japan) equipped with a temperature controller (CPS 240, Shimadzu). The ozone dose applied was controlled with an integration system of concentration by temperature implemented in a programmable automaton (model Siemens S7 + TD200, Ingeniera y Control Remoto S.L., Granada, Spain). This part is essential to control the applied treatments since the concentration applied must be sufficient to reach the desired ozone dose. This is because immediately after the product is dipped in the ozonated water the concentration of ozone decreases drastically in the tank due to product demand as a consequence of organic residues, cell exudates, etc., and therefore, the ozone cannot exert its sanitizer action if the concentration is not monitored and adjusted. For that reason, the term dose or CT value is used. Thus, the inactivation or destruction of microorganisms is related to the concentration of disinfectant (*C*, mg L^{-1}) and the contact time (*t*, min). The product of these two parameters is called the CT value or dose, and it is expressed in units of mg L^{-1} min (*34*). For the advanced oxidation treatment a UV-C tubular reactor in line was used (Ecotronic MX1, Montagna srl Ultraviolet System, Lacchiarella, Milano, Italy) at an irradiation of 30 mW/cm^2 and a wavelength of 257 nm. The flow rate was the same as that of the ozonated water treatment. All the experiments with ozone were made in the pilot plant of CEBAS-CSIC (Spain) following strict safety and protection rules.

Packaging. Shredded lettuce was packaged in polypropylene trays (165 × 120 × 57 mm) using two different atmospheres: air and active MAP. A gas exchange device with a vacuum packaging machine (Zermat, Carburos Metlicos S.A., Madrid, Spain) and a mixing station (Witt-Gasetechnik, model KM 100-3 M, Carburos Metlicos S.A.) were used. Active MAP was carried out by flushing a gas mixture of 4 kPa of O_2 and 12 kPa of CO_2 (N_2 as the balanced gas) into the trays. The batches of fresh-cut lettuce undergoing MAP were sealed with polyethylene terephthalate (PET)−polypropylene (PP) multilayer films (Tecknopack S.L., Barcelona, Spain) with the following film characteristics: 92 μm thickness, specific mass of 1.40 and 0.9 g m^{-3} for PET and PP, respectively, and permeance at 25 °C and 90% RH of 4.2 × 10^{-13} mol s^{-1} m^{-2} Pa^{-1} for O_2. For air, samples of shredded lettuce were placed in trays sealed with a perforated plastic film (PET−PP multilayer films, 1255 perforations m^{-2}, 0.6 mm diameter, Tecknopack S.L.). The film had the same characteristics as the one used for active MAP. Packaging was carried out with a gas exchange device for trays (Reetray 25, Reepack SRL, Milano, Italy). All samples were stored

for up to 13 days at 4 °C and evaluated on day 0 and after 5, 9, and 13 days. Three replicates of 100 g of fresh-cut lettuce were used for each treatment and sampling date. All sanitizer solutions were combined with the storage under air and MAP.

Respiration Rate and Headspace Analysis. The rate of carbon dioxide (CO_2) production was measured using an open system (*35*). Three replicates of 100 g of fresh-cut iceberg lettuce per washing treatment were placed in 1500 mL glass jars at 4 °C for 13 days. A flow of 10 mL min^{-1} of humidified air was pumped into the jars to avoid dehydration and excessive CO_2 accumulation. The increase in CO_2 content in the headspace was determined using a gas chromatograph (Shimadzu GC-14, Kyoto, Japan) equipped with a thermal conductivity detector (TCD). Samples were analyzed in triplicate and monitored for 13 days.

Changes in CO_2 and O_2 concentrations in the headspace of sealed trays were monitored using a gas chromatograph (Shimadzu GC-14) equipped with a TCD detector. A sample of 0.5 mL of headspace gas was taken from each tray with a calibrated syringe, and three replicates per treatment were used.

Sensory Evaluation. The organoleptic characteristics including visual quality, browning, texture, and aroma of fresh-cut lettuce were evaluated on day 0 and after 5, 9, and 13 days of storage by a four-membered expert panel. Lettuce visual quality was evaluated including the appearance features of gloss, freshness, and color uniformity and intensity and scored on a 9−1 scale, where 9 = excellent, 5 = acceptable (limit of marketability), and 1 = poor, inedible. Leaf edge browning and leaf surface browning were evaluated as browning on a 5−1 scale, where 5 = severe, 3 = moderate, and 1 = no browning. Texture was scored on a scale of 5−1, where 5 = very firm and turgid, 3 = moderately firm, and 1 = very soft. Aroma was determined on a scale of 5−1, where 5 = full typical aroma or flavor, 3 = moderate, and 1 = none.

Microbial Analysis. The growth of mesophilic and psycrotrophic bacteria and coliforms in fresh-cut lettuce was followed during the experiment. A 25 g sample of fresh-cut lettuce was homogenized with a 1:10 dilution of sterile 1% peptone-buffered water (AES Laboratoire, Combourg, France) in sterile 400 Lab Stomacher bags (Seeward Medical, London, U.K.) by using a Stomacher (IUL Instrument, Barcelona, Spain) for 90 s. All culture media used in this study were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Total aerobic mesophilic bacteria were enumerated by the standard plate count method using plate count agar (PCA) at 30 ± 1 °C for 48 h. Total psycrotrophic bacteria were enumerated by the standard plate count method using PCA at 4 ± 1 °C for 7 days. Coliforms were isolated using Endo agar at 37 ± 0.5 °C for 24 h. Microbial analyses were achieved on day 0 and after 5, 9, and 13 days of storage. All samples were analyzed in duplicate, and each microbial count is the mean of three samples from three packages. Microbial counts were expressed as log CFU g^{-1} of tissue.

Extraction and Analysis of Phenolic Compounds. For each treatment, the content of 50 g per tray was rapidly frozen by immersion in liquid nitrogen and then freeze-drying. Lyophilized lettuce (3 g) was homogenized with an Ultra Turrax (Ika, Staufen, Germany) for 1 min on ice with 30 mL of extraction solution (methanol/water, 7:3 v/v, containing 4 mM NaF to inactivate polyphenol oxidases and prevent phenolic degradation due to browning). Homogenates were centrifuged at 10500*g* in an Eppendorf centrifuge (model Sigma 1-13, Braun Biotech International, Osterode, Germany) for 5 min at 2−5 °C. The supernatant was recovered, filtered through a 0.45 μm Osmonics/MSI cameo nylon filter (Fisher Scientific, Los Angeles, CA), and directly analyzed by HPLC.

Samples of 50 μL of extracts were analyzed using an HPLC system (Merck Hitachi, Tokyo, Japan) equipped with a model L-7100 pump and a model L-7455 photodiode array UV/vis detector. The samples were injected with a model L-7200 autosampler. The separations were achieved on a LiChrocart C_{18} column (250 mm × 4 mm i.d., 5 μm, Merck, Darmstadt, Germany) using water/formic acid (95:5 v/v) (A) and methanol (B) as the mobile phases. The linear gradient started with 3% B in A to reach 25% B in A at 6 min, 35% B at 25 min, and 90% B at 35 min. The flow rate was 1 mL min^{-1}, and chromatograms were recorded at 330 nm.

Phenolic compounds were identified as previously described (*36*). Individual phenolic acids were quantified by comparison with an external standard of 5′-caffeoylquinic acid (Sigma, St. Louis, MO) and flavonols such as quercetin 3-*O*-rutinoside (Merck). The results are expressed as mg/100 g of fresh weight.

Extraction and Analysis of Vitamin C. Ascorbic acid and dehydroascorbic acid contents were determined as described by Zapata and Dufour (*37*) with some modifications (*38*). A 10 g sample of fresh-cut iceberg lettuce was added to 10 mL of extraction medium (0.1 M citric acid, 0.05% w/v EDTA disodium salt, 5% v/v methanol, and 4 mM NaF). The mixture was directly homogenized for 30 s on ice and filtered through cheesecloth. The filtrate was collected and centrifuged at 10500*g* in an Eppendorf centrifuge for 5 min at 2−5 °C. The filtrate was flushed through an activated Sep-Pak C_{18} cartridge (Waters, Milford, MA) and then filtered through a 0.45 μm filter. Then 250 μL of 1,2-phenylenediamine dihydrochloride (OPDA) solution (35 mg/100 mL) was added to 750 μL of extract for dehydroascorbic acid derivatization into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-*b*]-quinoxaline-1-one (DFQ). After 37 min in darkness, the samples were analyzed by HPLC.

Ascorbic acid and dehydroascorbic acid were evaluated using an HPLC system (Merck Hitachi), equipped with an L-6000 pump and coupled to a D-2500 variable-wavelength UV detector. Samples of 20 μL were injected onto a 250 × 4 mm i.d. 5 μm reversed-phased Kromasil 100 C_{18} column (Teknokroma, Barcelona, Spain) with an ODS guard C_{18} precolumn. The flow rate was kept at 0.9 mL min^{-1}. The detector wavelength was initially set at 348 nm, and after elution of DFQ, it was manually shifted to 261 nm for ascorbic acid detection. The vitamin C content was calculated by adding the ascorbic acid and dehydroascorbic acid contents, and the results are expressed as mg/100 g of fresh weight. The coefficient of variation was less than 8%.

Statistical Analysis. There were three repetitions per treatment and evaluation period. All data represent the mean of three replicates. Analysis of variance (ANOVA) followed by Duncan's multiple range test with a significance level of $P \leq 0.05$ was performed using SPSS (Windows 2000, Statistical Analysis).

RESULTS AND DISCUSSION

Effect of Washing Treatments on the Respiration Rate. The respiration rate of fresh-cut iceberg lettuce was measured during storage to determine if the ozonated water rinse had any effect on the physiology, causing damage or decay to the tissue. The high initial respiration rate of fresh-cut lettuce slightly decreased during the first 5 days to reach 5.0−8.6 mL of CO_2 kg^{-1} h^{-1} during the rest of storage (**Figure 1**). No significant differences in the respiration rate were observed among washing treatments throughout the storage period. Therefore, the respiratory activity of fresh-cut lettuce washed with different doses of ozonated water was similar to that of fresh-cut lettuce rinsed with water and chlorine, and it did not induce an additional stress. These results agree with those previously reported for fresh-cut potatoes washed with different solutions including ozonated water, which had similar O_2 and CO_2 levels in the headspace of the MAP bags during storage (*39*). Up to now, the information regarding the influence of ozonated water on the respiration rate had been scarce for fresh-cut products. Recently, it was shown that a low ozonated water dose (0.03 mg L^{-1}) in fresh-cut celery did not affect the respiration rate compared with nontreated samples (*24*)

Evolution of Modified Atmosphere Packaging during Storage. The recommended active MAP for fresh-cut iceberg lettuce is 0.5−3 kPa of O_2 and 10−15 kPa of CO_2 (*9*). To achieve that gaseous atmosphere, initial active MAP (4 kPa of O_2 + 12 kPa of CO_2) was flushed, and it changed during storage by the respiration of the tissue with the consumption of O_2 and the emission of CO_2 to reach 0.5−2 kPa of O_2 and 18−22 kPa of CO_2 at the end of storage (**Figure 2**). The O_2 level reached

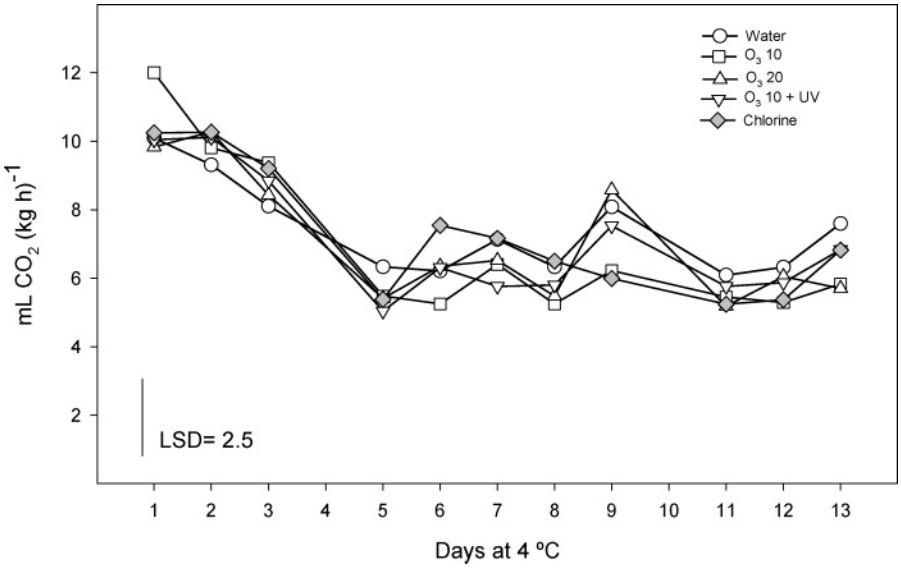

Figure 1. Respiration rate of shredded iceberg lettuce washed with water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}) during 13 days of storage at 4 °C. Each value represents the mean of three 100 g jars, and the vertical bars correspond to the LSD at $P \leq 0.05$.

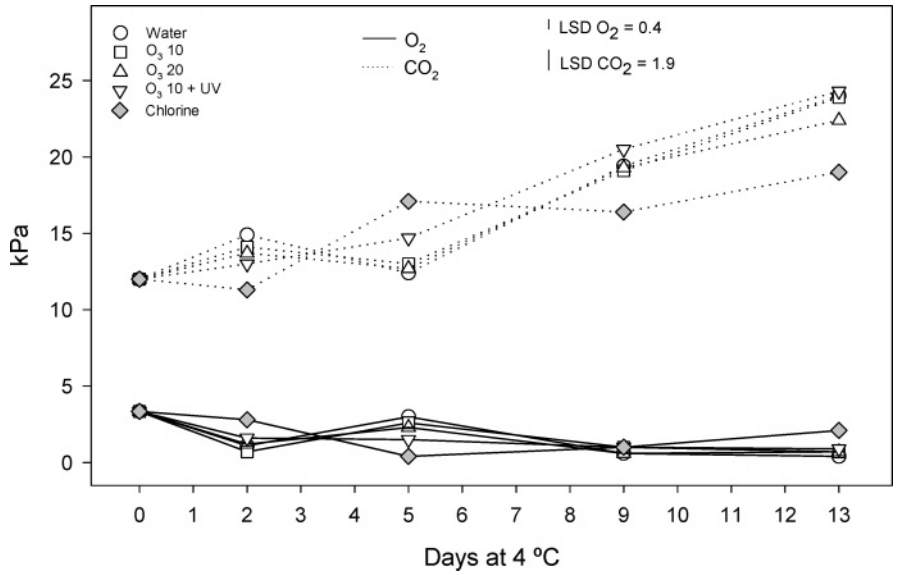

Figure 2. Changes in headspace O_2 (—) and CO_2 (---) levels in active MAP packages over 13 days at 4 °C of shredded iceberg lettuce washed with water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}). Each value represents the mean of three 100 g packages, and the vertical bars correspond to the LSD at $P \leq 0.05$.

in the packages was in the recommended ranges, while the CO_2 level was slightly higher than that suggested.

Effect of Washing Treatments and MAP on Sensory Quality. The visual quality of shredded lettuce was excellent after washing for all treatments, and promotion of browning was not observed for any washing solutions. No significant differences in the initial visual quality were observed among the treatments. After 9 days of storage, fresh-cut lettuce washed with water or chlorine and stored in air showed an important decrease of the visual quality and a pronounced browning (**Figure 3A,B**). However, the highest degree of browning observed in air-stored samples at the end of storage corresponded to those samples washed with water, whereas a chlorine rinse delayed the visual quality deterioration as well as slowed browning. This is in agreement with previous studies where washing fresh-cut lettuce in chlorinated water reduced browning in relation to samples washed with water although it was not enough to control it (*25*). In contrast, samples washed with ozonated water and stored in air maintained an excellent visual quality during storage without significant differences compared to the initial visual quality. Additionally, no browning was observed in these samples for up to 13 days, including the vascular midrib (**Figure 3A,B**). On the other hand, no significant differences were observed in the visual appearance or browning development between the ozonated treatments.

Under active MAP, the visual appearance was preserved for all the washing treatments. However, a moderate decrease of visual quality was observed after 9 days of storage, being more evident in those samples washed with water (**Figure 3C**). Nevertheless, there was no significant difference in the visual appearance among chlorine and ozonated water treatments throughout 13 days of storage at 4 °C. Moreover, no evidence of browning was observed for the different lettuce samples (**Figure 3D**). In addition to browning control achieved with

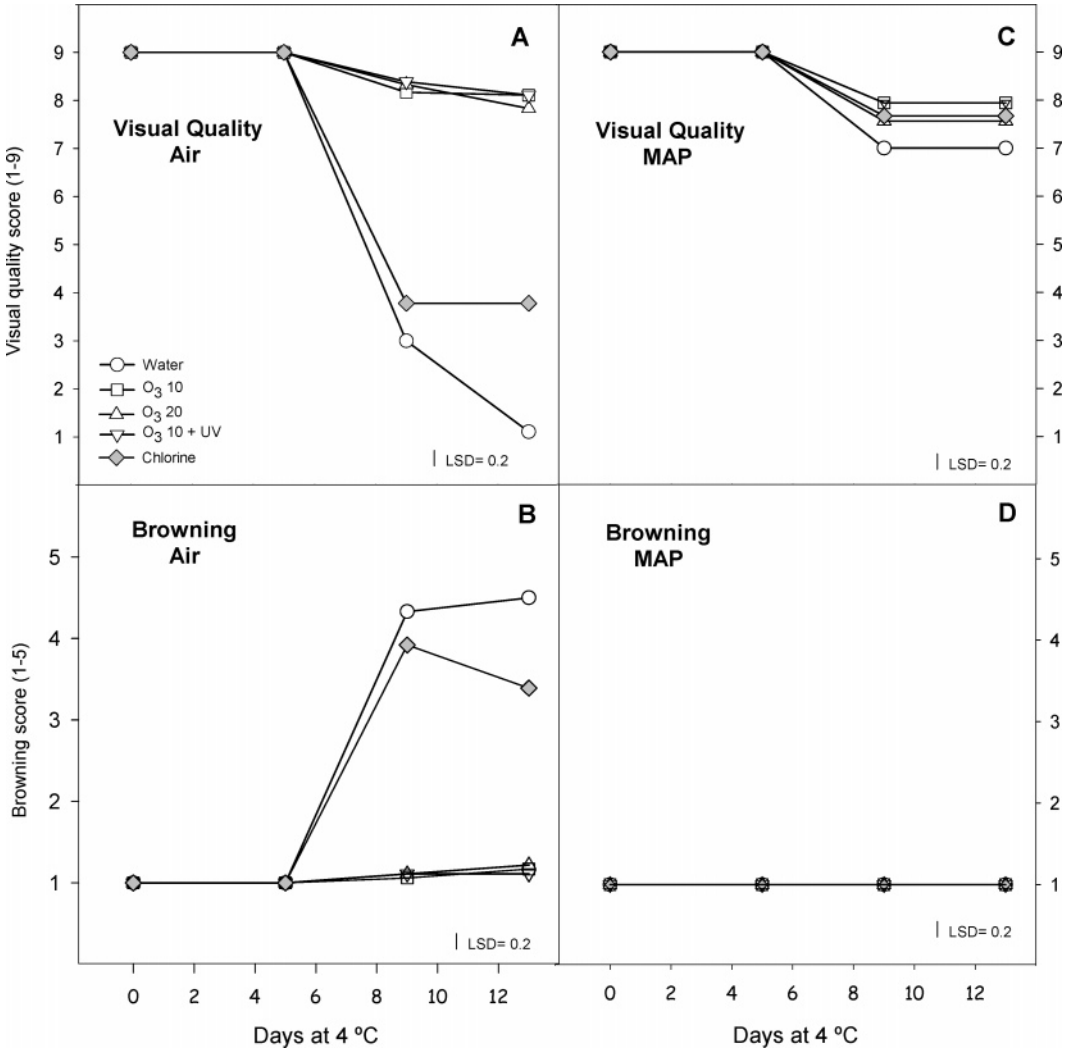

Figure 3. Effect of water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}) washing treatments on the visual quality and browning of shredded iceberg lettuce stored in air or active MAP for 13 days at 4 °C. The values are the mean of four panelists, and the bars represent the LSD at $P \leq 0.05$. Visual quality: 9 = excellent, 5 = acceptable, 1 = poor, inedible. Browning scores: 5 = severe, 3 = moderate, 1 = no browning.

MAP, these samples maintained the full typical aroma, which was not affected by the different washes (data not shown). Immediately after the trays were opened, an off-odor was not detected in samples washed with different solutions at any storage time, without evidence of anaerobic fermentations after 13 days of storage at 4 °C. Sample textures slightly decreased during storage, but no differences were observed between MAP and air-stored samples. By day 13, all samples maintained a moderate crispy texture without significant differences among them (data not shown). Therefore, neither chlorine nor ozone affected the texture of fresh-cut lettuce during storage, in agreement with previous findings (*25*).

Effect of Washing Treatments and MAP on Microbial Quality. The effects of the different washing treatments on the native mesophilic and coliform counts of shredded lettuce stored in air and MAP are shown in **Figure 4**. Initially, the total mesophilic population was reduced by 1.6 and 2.1 log units in those samples washed with ozonated water and chlorine, respectively, when compared with those washed with water. No significant differences were found between the initial numbers of mesophilic bacteria in fresh-cut lettuce washed with different ozone doses (**Figure 4A**). The growth rate of total microorganisms in air was similar for all the washing solutions tested including the water rinse. However, the initial reduction achieved by ozone and chlorine allowed reducing the final counts by 1.8 and 2.7 log, respectively, at the end of the storage. This reduction could be essential to prolong the shelf life of the product as occurs with the Spanish microbial regulation, which allows maximum mesophilic bacteria counts of 10^6 CFU g^{-1} (*40*) as the limit at the consumption date. However, after 13 days of storage in air, water-washed samples exceeded this legal limit while those samples washed with ozone or chlorine were still below the established limit (**Figure 4A**).

For all treated samples, storage under MAP reduced total bacterial counts, achieving 2 log reductions compared with samples stored in air at the end of the storage. This is in agreement with a previous study that showed that CO_2 has the benefit of reducing total microorganisms in fresh-cut produce due to its fungi- and bacteria-static characteristics (*41*). However, some researchers have demonstrated that although 3 kPa of O_2 + 10 kPa of CO_2 levels maintain acceptable visual quality of shredded lettuce, they do not appreciably affect microbial development (*42*). The combination of MAP and washes with chlorine, ozone 20, or ozone 10 activated by UV-C was able to slow microbial growth throughout 13 days of storage in relation to the water rinse (**Figure 4B**). Mesophilic counts resulting from

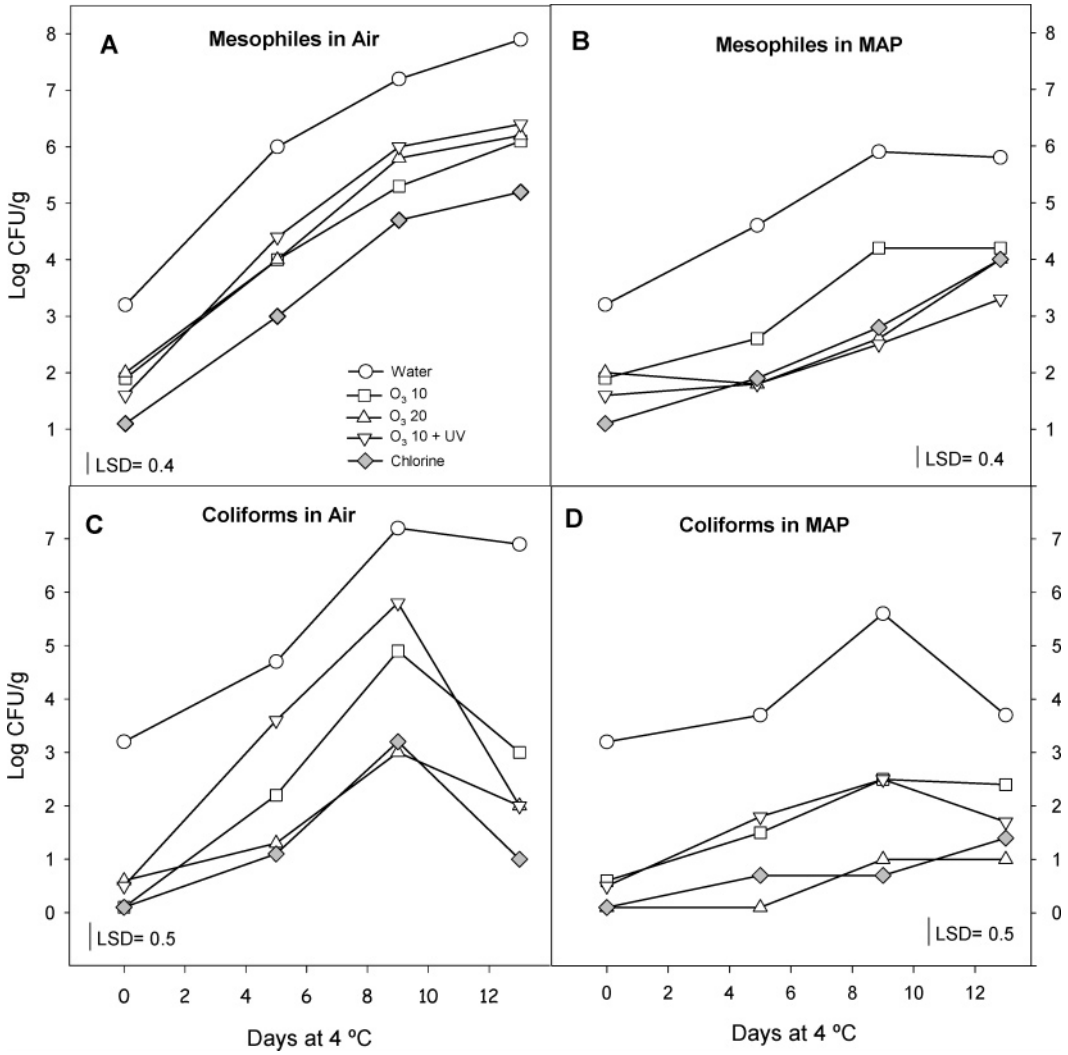

Figure 4. Effect of water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}) on the growth of aerobic mesophilic bacteria and coliforms (log CFU g^{-1}) during 13 days of storage at 4 °C in air and active MAP. The values are the means of three replicates, and the bars represents the LSD at $P \leq 0.05$.

these three treatments (chlorine, ozone 20, or ozone 10 activated by UV-C) were not significantly different. The higher effectiveness of ozone 10 activated by UV-C compared with ozone 10 could be explained considering that ozone absorbs UV radiation and produces hydroxyperoxide, superperoxide, and hydroxyl radicals, which react powerfully with microorganisms (*43*). The effect of MAP controlling mesophilic bacteria was increased when used in combination with sanitizers. Ozone and MAP as well as chlorine and MAP slowed microbial growth in a more effective way than the addition of inhibitory effects of ozone or chlorine and MAP applied individually (**Figure 4B**). Therefore, the atmosphere composition had a decisive effect on bacterial growth when using these sanitizing treatments. Reactive oxygen species produced during the ozone treatment could lead to damage of cell components, thereby reducing cell viability. In addition, MAP storage caused a subsequent inactivation as well as growth inability of cells with sublethal damage. Under MAP, samples treated with ozone 10 had the same microbial growth rate as water-washed samples; however, as the initial microbial reduction was 1.6 log, ozonated samples reached a better microbial quality at the end of the storage. The storage under MAP of samples treated with ozone and chlorine allowed maximum reductions of total mesophilic bacteria counts of 2.5 and 1.8 log, respectively, after 13 days. The best treatment was with ozone 10 activated by UV-C.

Coliforms, which are used as indicator microorganisms of fecal contamination in water and foods, include potentially pathogenic species such as *E. coli*. This microbial group was of special concern in this study due to the high initial level presented in water-washed samples (3.2 log), and it increased markedly at day 9, reaching 7 log, and remained constant for up to 13 days of storage in air (**Figure 4C**). Therefore, it was necessary to investigate the use of an effective treatment (ozone or chlorine) to control this microbial group. Initially, ozone and chlorine dips performed an important reduction of coliforms until 3.2 log units compared to water-washed samples, and no significant differences were found between treatments (**Figure 4C**). In air, the maximum density of coliforms was achieved after 9 days of storage, being significantly lower (4.2 log reductions) in those samples treated with ozone 20 and chlorine. When samples were stored under MAP, growth of coliforms was slowed in all cases when compared to that of samples stored in air. Reductions of 4.9 log units after 9 days confirmed the efficiency of sanitizers (chlorine or ozone 20) and active MAP (**Figure 4D**). To achieve the maximum efficiency of sanitizer treatments on microbial control, it will be necessary to combine them with active MAP and storage at low temperature.

Phenolic Content of Fresh-Cut Iceberg Lettuce. Poor raw material homogeneity has made it very difficult, in the case of shredded iceberg lettuce, to study and evaluate the content of

phytonutrients (polyphenols). Either constitutive or biosynthesized after wound stress, its content differs depending on the cultivar, the tissue type (photosynthetic and midrib tissues), and its location within the lettuce head (inner and outer leaves) (*44*, *45*). Processing conducted at a pilot plant scale under industrial operation conditions has been recommended to reduce problems associated with raw material heterogeneity (*25*). Most of the research on wound-induced phenolic metabolism in iceberg lettuce has been focused on midrib tissues (*4*, *46*−*48*), but because commercial fresh-cut iceberg lettuce contains both photosynthetic and vascular tissue, the evaluation of polyphenols in homogeneous lettuce samples was essential.

Flavonol derivatives in iceberg lettuce are mainly located in the photosynthetic tissue and play a minor role in browning since their content is rather small compared with that of other lettuce cultivars (*35*, *49*, *50*). Apart from flavonoids, caffeic acid derivatives were found to be the most predominant soluble phenolics in iceberg lettuce. The chromatogram recorded at 330 nm confirmed the presence of two classes of polyphenols in shredded iceberg lettuce: caffeic acid derivatives as the main compounds and flavonoids in lower concentration. The major caffeic acid derivatives detected in the extracts of shredded iceberg lettuce were characterized as monocaffeoyltartaric acid (CAFTA), chlorogenic acid (5′-caffeoylquinic acid, CGA), isochlorogenic acid (3,5-dicaffeoylquinic, ICGA), chicoric acid (dicaffeoyltartaric acid, DCAFTA), and two further dicaffeoylquinic derivatives. Chicoric acid was identified as the main caffeic acid derivative and found in large amounts with regard to the total polyphenol content (50−58% depending on the washing treatment), in agreement with previous reports (*48*). The caffeoyltartaric derivatives have been reported to be quite stable after wounding, the caffeoylquinic derivatives, chlorogenic acid first and isochlorogenic acid in a second stage, being those which synthesis increases as a response to wounding (*48*).

Effect of Washing Treatments and MAP on Phenolic Content. The main interest of this polyphenol analysis was to evaluate the possible effect of ozonated water treatments on the antioxidant phenolic content. Therefore, the phenolic analysis was conducted on the sampling dates (initially and after 5, 9, and 13 days) as the study of wound-induced changes in phenolic metabolism was not the purpose of the present work. During the first 3−5 days, wounding of iceberg lettuce produces a signal that migrates through the tissue and induces the synthesis of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) to produce caffeic acid that conjugates with quinic acid to form chlorogenic and isochlorogenic acids (*48*). These phenolic compounds accumulate in shredded iceberg lettuce, and are associated with subsequent tissue browning (*51*). In our case, the storage under reduced O_2 and elevated CO_2 (active MAP) has been used to control lettuce tissue browning. The phenolic content of shredded lettuce was mainly affected by MAP, whereas the influence of different washing treatments was insignificant (**Table 1**). These findings are in agreement with previous studies, obtaining a high accumulation of phenolic compounds when fresh-cut lettuce was stored in air, whereas carbon dioxide levels of >10 kPa led to a reduced phenolic content (*50*). Recently, the influence of ozone and chlorine as prewashing treatments before cutting on the phenolic metabolism of fresh-cut lettuce stored in passive MAP has been reported (*25*). However, the recommended MAP for fresh-cut lettuce in this study was reached after 9 days of storage, and this differs considerably from the real situation in industrial fresh-cut lettuce processing. This is probably why Baur et al. (*52*) observed nearly constant values for caffeic acid derivatives during storage.

Table 1. Effect of Water, Ozonated Water [10 mg L^{-1} min (Ozone 10), 20 mg L^{-1} min Ozone (Ozone 20), 10 mg L^{-1} min Ozone Activated by UV-C (Ozone 10 + UV)], and Chlorine (80 mg L^{-1}) on the Caffeic Acid Derivatives, Flavonoids, and Total Polyphenols of Shredded Iceberg Lettuce Stored in Air and Active MAP for 13 days at 4 °C[a]

washing solution	no. of days	caffeic acid derivatives		flavonoids		total polyphenols	
		air	MAP	air	MAP	air	MAP
water	0	19.9		3.4		23.3	
	5	19.6	20.5	3.7	4.1	23.3	24.6
	9	20.6	12.2	3.6	3.2	24.1	15.4
	13	24.0	10.1	3.9	3.8	27.9	13.9
ozone 10	0	20.5		3.3		23.8	
	5	19.9	21.0	3.8	3.5	23.8	24.5
	9	18.1	12.7	3.5	3.0	21.7	15.6
	13	21.7	6.7	4.0	3.7	25.7	10.3
ozone 20	0	17.6		3.4		20.9	
	5	20.6	15.2	3.3	3.0	23.9	18.2
	9	19.0	9.4	3.3	3.0	22.3	12.4
	13	21.9	8.3	3.9	4.2	25.8	12.5
ozone 10 + UV	0	16.2		3.5		19.8	
	5	17.1	14.4	2.9	2.6	20.0	17.0
	9	18.6	17.0	3.3	3.4	21.6	20.4
	13	21.9	8.3	3.5	3.1	25.4	11.4
chlorine	0	17.7		3.4		21.1	
	5	22.2	23.5	4.8	2.4	26.9	25.9
	9	19.0	8.7	3.3	3.1	22.3	11.8
	13	17.5	8.5	3.6	3.4	21.1	11.8
LSD		3.2		0.7		3.6	

[a] Means (three replicates) in mg/100 g of fresh weight. LSD at $P \leq 0.05$.

The influence of different washing treatments on the individual phenolic compounds was insignificant considering the heterogeneous nature of lettuce tissues affecting the phenolic composition. Chlorogenic acid and isochlorogenic acid, the compounds whose biosynthesis has been associated with wound-induced browning in iceberg lettuce midribs, increased their contents in all washed samples stored in air (**Figure 5A**). Additionally, monocaffeoyltartaric and dicaffeoyltartaric acids remained unchanged in air-stored samples, and their contents were not affected by the washing treatment, except for a slight decrease in chlorine-washed samples during storage (**Figure 5B**). MAP effectively suppressed accumulation of caffeoylquinic derivatives, whereas caffeoyltartaric derivatives decreased during MAP storage, reaching similar levels in all washed samples throughout storage (**Figure 5C,D**). Ozone treatments, which prevent browning when lettuce is stored in air, had no effect in preventing chlorogenic acid biosynthesis, suggesting that the biochemical mechanism of action used by ozone to prevent browning is different from that of MAP. Taking all phenolic compounds as a global content, the different washing treatments, including ozone treatments, had no effect on the final phenolic content of fresh-cut iceberg lettuce, apart from the reported effects of MAP. However, Zhang et al. (*24*) reported an inhibition effect of ozonated water on the PPO activity of fresh-cut celery.

Effect of Washing Treatments and MAP on Vitamin C Content. The vitamin C content of lettuce is variable among cultivars, but in the case of iceberg lettuce, it is low compared with that of other lettuce cultivars (*53*). In addition, the content of vitamin C in lettuce also differs in the tissue types. Despite the greater potential for browning of phenolics in photosynthetic iceberg tissue than vascular tissue, the level of browning was lower due to antioxidant-protecting systems, which include ascorbates (*45*). Also, the processing methods affect the vitamin C in shredded iceberg lettuce where higher levels of ascorbic acid were retained in samples prepared manually compared to machine cut (*54*).

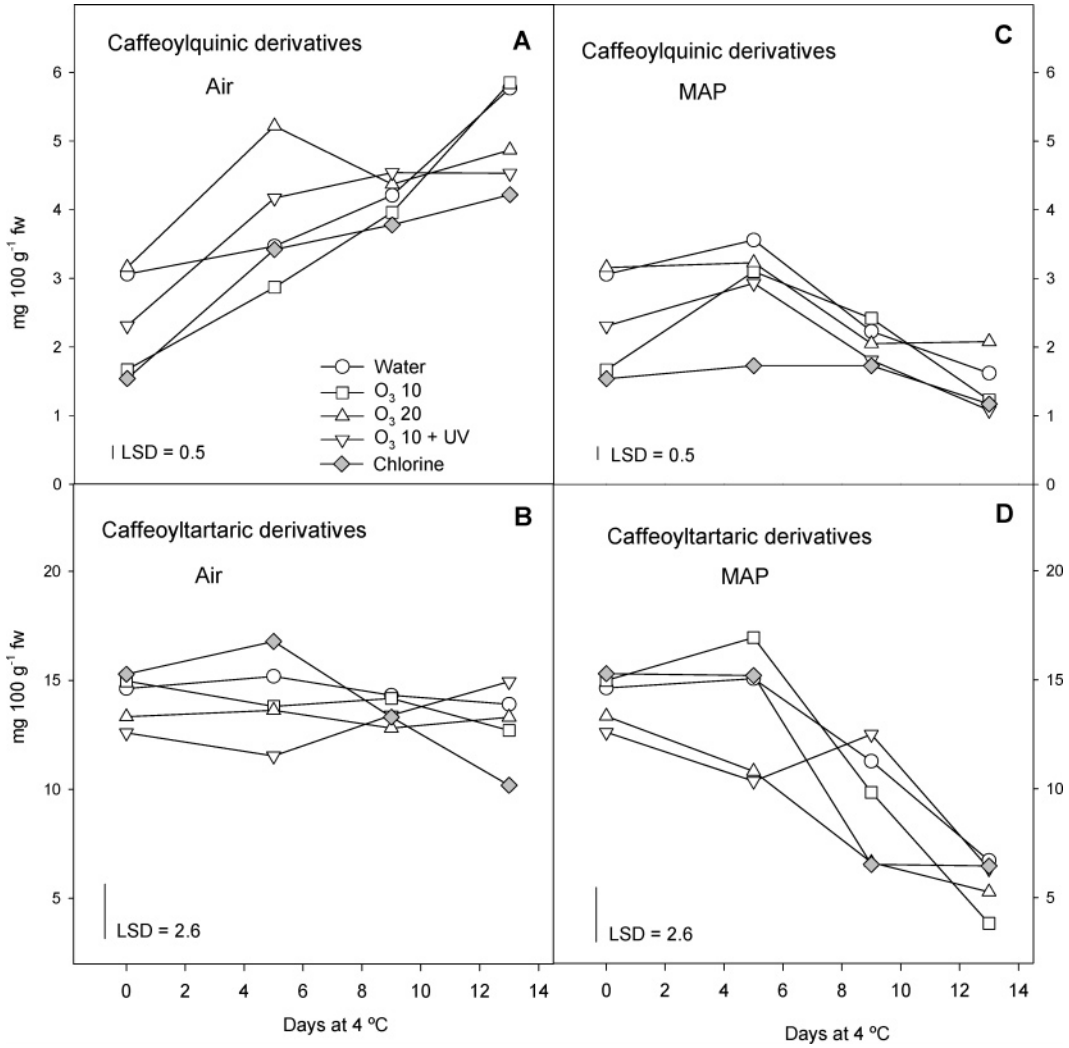

Figure 5. Effect of water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}) on the caffeoylquinic and caffeoyltartaric derivatives of shredded iceberg lettuce stored in air or active MAP for 13 days at 4 °C. The values are the means of three replicates, and the bars represent the LSD at $P \leq 0.05$.

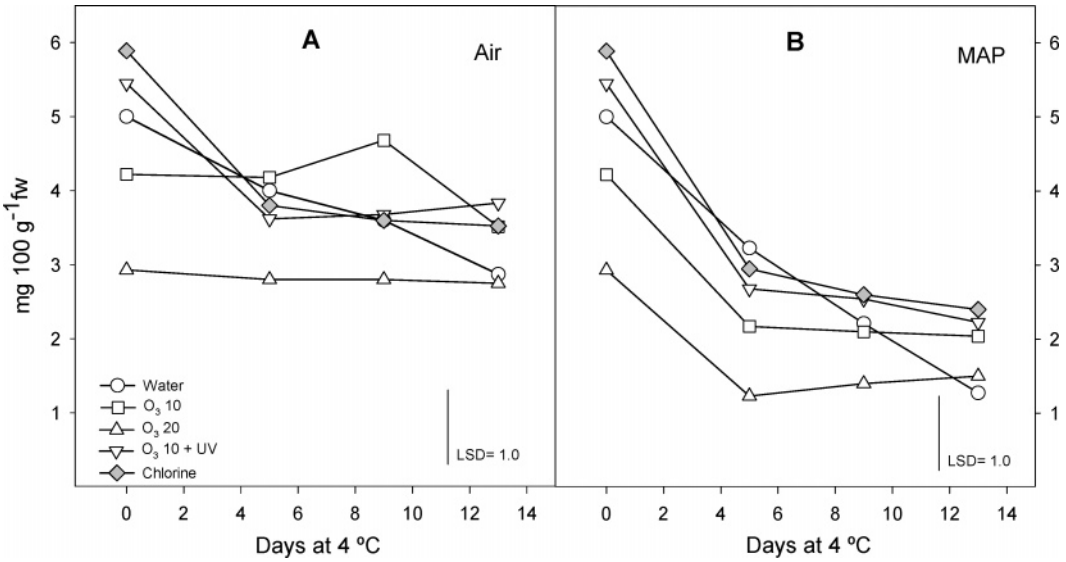

Figure 6. Effect of water, ozonated water [10 mg L^{-1} min (O_3 10), 20 mg L^{-1} min ozone (O_3 20), 10 mg L^{-1} min ozone activated by UV-C (O_3 10 + UV)], and chlorine (80 mg L^{-1}) on the vitamin C content of shredded iceberg lettuce stored in air or active MAP for 13 days at 4 °C. The values are the means of three replicates, and the bars represent the LSD at $P \leq 0.05$.

Under stress conditions, such as chemical exposure, ascorbate oxidase has been described to promote the degradation of ascorbic acid (AA) to dehydroascorbic acid (DHA) (*55*). Since AA can be easily converted into DHA by a potent oxidizer, it

was important to measure both AA and DHA for vitamin C content. Ascorbic acid was the predominant form of vitamin C in fresh-cut iceberg lettuce, representing 55−65% of the total vitamin C content. As described above for phenolic compounds, outer and inner leaves as well as midrib and photosynthetic tissues affect the variability of the vitamin C content. Initially, shredded lettuce washed with 20 mg L^{-1} ozonated water had the lowest content of vitamin C although it was maintained during storage (**Figure 6A**). Shredded lettuce washed with water, chlorine, and ozone 10 activated by UV had a higher vitamin C content, though it was slightly reduced during storage in air, all washed samples reaching similar levels. Wright and Kader (*56*) reported that washing of either sliced or intact strawberries in 100 mg L^{-1} chlorine promoted the oxidation of AA over unwashed fruit. In this study, the trend observed in MAP was similar to that described for air but the effect was significantly enhanced by a modified atmosphere (**Figure 6B**). Reduction in the vitamin C content by MAP has been previously described in potato strips, while it was retained in air-stored samples (*57*). However, in other studies controlled atmospheres of low O_2 and high CO_2 had no significant effect on vitamin C although the predominant form of vitamin C changed during storage, maintaining the same vitamin C content as in the initial samples. That was the case for sliced strawberries and sliced persimmons as well as fresh-cut spinach (*56*, *58*). In contrast, Agar et al. (*59*) observed a reduction in vitamin C content by high CO_2 levels (13−30 kPa) in strawberries. In this case, despite strong oxidizing activity of ozone, vitamin C retention after storage in ozonated water- and chlorine-washed samples was similar. For all washing treatments, samples stored in air experienced a slight decrease of the vitamin C content compared with the initial values (**Figure 6A**). Water-washed samples stored in MAP decreased in vitamin C content to reached a 75% reduction at the end of the storage. Although active MAP was more effective in controlling total microbial growth and sensorial quality, the vitamin C content was not well preserved. This is in agreement with a previous report on Swiss chard where vitamin C was better maintained in air than in MAP (*60*).

In conclusion, it is clear that chlorine replacement sanitizer is urgent to find. Ozonated water could be an excellent alternative for washing shredded lettuce not only by reducing microbial populations on the product but also by keeping the visual quality and controlling browning without any detrimental effect on the antioxidant constituents. The lack of an offensive chemical residue makes this technique an attractive method from a human and environmental safety standpoint. The effect of ozonated water in controlling browning should be investigated further as this may provide additional insight into the endogenous mechanism that controls browning.

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