The effect of pretreatment of ozone and citric acid on quality of fresh-cut lettuce (*Lettuce sativa* L.) preserved into modified atmosphere packaging (MAP)

Farzaneh Najafi*, Safoora Shaban

*Department of Plant Science, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran*

**Key words:** Iceberg lettuce, microbial load, modified atmosphere, ozonated water.


**Abstract**

In this study, the effect of three levels of citric acid (0, 0.5 and 1 g l⁻¹) and three levels of ozonated water (0, 0.6 and 1 ppm) and the interaction of these two treatments on microbial load and qualitative features of fresh-cut iceberg lettuce were studied. After treatment, microbial load and qualitative features of cut lettuce were evaluated in modified atmosphere packaging (5% O₂ + 5% CO₂ + 90% N₂) in four different storage times (3, 6, 9 and 12 days). The effects of these treatments were also compared with percidine (concentration 1 ml to 1200 ml in water). After treatment, fresh-cut lettuce pieces were stored at 4°C with relative humidity 75% for 12 days. The results showed that the total count of microorganisms and the weight loss percentage were increased by increasing the storage time, whereas parameters such as surface color, vitamin C and polyphenol oxidase activity were decreased. In lettuces treated with different concentrations of citric acid, the lowest total count of microorganisms was related to 1 g l⁻¹ citric acid concentration and also treatment with 1 ppm ozonated water concentration. In total, treatments of citric acid and ozonated water, especially in high concentrations and interaction of these treatments resulted in better preservation of qualitative features and reduced microbial load of fresh-cut lettuces during storage time.

*Corresponding Author: Farzaneh Najafi ✉ farzaneh.najafi1@gmail.com*
Introduction
There is a growing use of Modified Atmosphere Packaging (MAP) today aimed to improve the quality of freshly cut produce and to prolong shelf-life. The use of this method for packaging freshly cut fruits and vegetables helps preserve their quality and increase their storage life (Artes et al., 2007). This method reduces the respiration rate, the chemical and biochemical reaction rates and the growth rate of pathogenic microorganisms in freshly cut fruits and vegetables and thus increases their shelf-life (Coles et al., 2009; Regaert et al., 2007).

A big issue presenting itself in the freshly-cut produce industry is finding ways to reduce microbial contamination and to maintain the reduction up to the point when the consumer gets it to their mouth. To reduce the microbial load of these produce, various antimicrobial agents such as ozonated water and organic acids are used. Using ozone is recommended as an alternative to traditional disinfectants due to its efficiency in low concentrations and during short exposure times and due to its way of decomposition into non-toxic compounds. The antimicrobial action of ozone is due to the strong oxidizing properties of ozone molecules or the materials produced from its decomposition, such as hydroxyl radicals, which rapidly react with intracellular enzymes, nucleic materials and their cell constituents, fungal spore coats or viral capsids (Khadre et al., 2001). Organic acids, particularly citrate, lactate, and acetate, have antimicrobial properties (Artes et al., 2007). They are therefore used to deactivate foodborne pathogens in fresh produce. Organic acids act fast and destroy a wide range of bacteria. Citric acid prevents the discoloration of fruits and vegetables through its inhibiting effects on the action of this enzyme; it is therefore extensively used on freshly processed fruits and vegetables (Olmez and Kretzschmar, 2009).

Many scientists have investigated the microbial-load-reducing effect of antimicrobial agents such as ozonated water and organic acids on the quality of produce and found the highest total count of microorganisms to pertain to bacteria, which are often responsible for spoiled vegetables. Ozone is known to change the permeability of bacterial cell membrane and to destroy them. Akbas and Olmez, 2007 demonstrated that immersion treatment with chlorine, citric acid and ozonated water effectively helps reduce microflora in freshly cut iceberg lettuce, and that immersion in organic acids has a greater antimicrobial effect on lettuce microflora and storage life compared to ozone or chlorine treatments (Akbas and Olmez, 2007). In one study, researchers showed that a combined ozone and citric acid treatment has a greater effect on reducing the population of pathogens in iceberg lettuce compared to single treatments (Yuk et al., 2007). It’s found that, when using antimicrobial compounds, ozonated water has great effects on destroying both gram positive and gram negative bacteria in food (Restaino et al.,1995). Immersion of iceberg lettuce in a 0.5% solution of citric or lactic acid for 2 minutes can be as effective in reducing microbial populations in freshly cut iceberg lettuce as treatment with 100 ppm chlorine (Olmez and Akbas, 2009).

Bacteria counts in spinach, lettuce and strawberry treated with 0.05 ppm concentration of ozone and stored at low temperatures for 7 days decreased by 30% to 50% (Kader et al., 1989). Salmonella populations decreased in carrots treated with lemon juice, vinegar and a combination of both (Sengun and Karapinar, 2004). Uyttendaele et al., 2004 reported a significant reduction in Psychrotrophic natural flora in minimally-processed vegetables such as chopped carrots, lettuce and pepper after treatment with a 2% lactic acid for just 1 minute.

Being a salad vegetable, lettuce is consumed by a lot of people. The present study thus investigated the means of preserving the freshness of freshly cut lettuce without sacrificing its nutritional quality and also assessed the effect of proper doses of permitted disinfectants (treatments with ozone and citric acid separately and in combination) on increasing shelf life and reducing microbial load of this produce for supply to consumers.
**Materials and methods**

Iceberg lettuce was provided in the spring of 2011 from farms in Karaj and transferred to the cold storage and stored for 24 hours at 4°C. Damaged and unhealthy lettuce leaves were separated and the remaining leaves were rinsed with water and when the surface moisture dried, they were chopped uniformly with a sharp knife and were kept in a cold storage after treatment.

**Treatments**

In this study, fresh-cut lettuce pieces were immersed in ozonated water treatment in three levels (0, 0.6 and 1 g l\(^{-1}\)) for 10 minutes and in citric acid in three levels (0, 0.5 and 1 g l\(^{-1}\)) for 5 min. Then they were packed using polypropylene package with modified atmosphere of 5% O\(_2\) + 5% CO\(_2\) + 90% N\(_2\) and were kept in the cold storage. In this study, a group of fresh-cut lettuce was treated with percidine (by a ratio of 1 to 1200) and considered sham group. An ozonated water generator (Ozone EUF model MHP\(_{1}\)H) was used to produce ozonated water at needed concentrations.

**Packing**

Two-hundred grams of chopped lettuce was packaged using Henkelman vacuum packaging machine (model 200A) under modified atmosphere (5% O\(_2\) + 5% CO\(_2\) + 90% N\(_2\)). Then lettuce packages were moved to cold storages at temperature of 4°C with relative humidity 75%. The treated lettuce was sampled to assess the factors in four stages, each stage including three days in the cold storage.

**Evaluated characteristics**

**Weight Loss**

In all samplings, the plant fresh weight was measured by a balance with 0.1 g accuracy and was obtained by the following formula and reported as weight loss percent (Saini et al., 2001).

\[
\text{Weight loss percent} = \left(\frac{\text{Secondary weight} - \text{Initial weight}}{\text{Initial weight}}\right) \times 100.
\]

**Discoloration**

The discoloration during the storage time was measured based on parameters L\(^*\), a\(^*\), and b\(^*\) (degree of color transparency) by using a chromameter (Konica Minolta CR-403, Japan) and chroma (purity of color) and hue were calculated from the following formula (Bernalte et al., 2003).

\[
h^0 = \tan^{-1} \frac{b^*}{a^*},
\]

\[
c = \frac{1}{2}(a^* + b^*)
\]

**Vitamin C**

For measuring vitamin C, titration with iodine and potassium iodide and starch solution was used. Five ml filtered lettuce juice was mixed with 20 ml distilled water, and 2 ml starch solution 1% was added to it. The resulting solution was titrated with iodine and potassium iodide and the value of vitamin C was obtained by the following formula (Saini et al., 2001).

\[
C = \left(\frac{0.88 \times V}{5 \times 100}\right).
\]

**Measurement of polyphenol oxidase activity**

One gram of lettuce tissue was pulverized in a mortar with 6 ml sodium phosphate buffer (pH 6.5) and 1 mg polyvinylpyrrolidone and the resulting mixture was centrifuged (12000 rpm, 4°C, 20 min). The reaction mixture contained 0.2 ml catechol in addition to 2.7 ml sodium phosphate buffer (0.1 M) plus 0.1 ml extract. The absorption was read every 10 seconds for 100 seconds at wavelength 420 nm (Huang et al., 2009).

**Evaluation of microbial load**

Microbial load was evaluated during storage time once every three days.

**Sample preparation methods**

The sample was prepared under a sterile hood. First, the surface of the package was cut with a sterile knife and 10 g of the sample was taken with forceps and placed in a sampling container containing 90 ml sterile normal saline and the sampling container was capped. The container was left for 10 minutes so that microbial load of the plant could be transferred into the solution. This container had a dilution of 1/10 with 90 ml of normal saline and 10 g of sample plant. After ensuring homogeneity of the initial dilution of
1/10, serial dilutions were prepared according to standard protocols (Microbiology of Food and Animal Feeding Stuffs. No.8923).

**Sterilization of culture medium and containers and tools needed**

After normal saline was prepared and weighed in glass containers and test tubes, glass containers were capped and test tubes were plugged with cotton, and then tightly wrapped in aluminum foil. Also, when the culture media were prepared, they were covered with cotton and then wrapped tightly in aluminum foil. After normal saline and the culture media were prepared, they were sterilized with other equipment in an autoclave at 121°C for 20 min.

**Instructions for culturing and total count of microorganisms**

First, 1 ml of the dilutions (in this experiment, the dilutions 1/100, 1/1000, 1/10000 were cultured) was poured into the sterile empty plates by a sampler and then 15-20 ml of nutrient agar culture medium sterilized at temperature of 45-50°C was added to sterile plate containing the desired dilution. After solidification of the culture medium, the plates were tightly sealed with parafilm and then the plates were placed in an incubator upside down at 30°C for 72 hours. After 72 hours, the plates were removed from the incubator, and the total colony counts, including bacteria and molds on each plate were counted by using the following formula (Microbiology of Food and Animal Feeding Stuffs. No. 5272).

\[
\text{The number of colonies in one gram or one ml of the sample (cfug}^{-1} \text{ or ml) = The total number of colonies} \\
\text{\times 1/dilution \times 1/volume used.}
\]

**Experimental design and statistical analysis**

A factorial experiment was designed with 3 replicates in a completely random model. Data were analyzed by SPSS software programs and the means were compared with Duncan multiple range test at P≤0.05 level.

**Results and discussion**

**Weight Loss**

Weight loss percentage had an increasing trend with increasing storage time (Fig. 2 and Fig. 3). The results also show that among different concentrations of citric acid, treatment of 0.5 g l⁻¹ acid had the lowest weight loss percentage and among various concentrations of ozone, the lowest weight loss percentage was related to 1 ppm ozone concentration (Fig. 1). Weight loss is very important as it is associated with economic issues and generally weight loss more than 5% can reduce the market value of fruits and vegetables (Brown and Bourne, 2002). In general, evaporation, transpiration and respiration of products after harvest and imbalance of vapor pressure in the product tissues and the air inside the package lead to weight loss and more weight loss over time (Baldwin et al., 1995). In addition to treatments, propylene packaging and also the use of modified atmosphere are the most important factors in preventing weight loss. Less water vapor permeability of the package causes less weight loss percentage (Esti et al., 2002). Weight loss in the used propylene packaging was small due to low permeability to water vapor, no steam escapes and the relative increase of humidity inside the package. The use of modified atmosphere was also effective in preventing weight loss (Serrano et al., 2005). Lettuce stored in propylene packaging at 5°C showed 28% weight loss after 7 days of storage (Allende et al., 2004).

**Hue (h°)**

The amount of hue indicator decreased with increasing storage time (Fig. 5 and Fig. 6). Also treatment with citric acid and ozone led to better
maintenance of hue in storage time and treatment with percidine compared to treatment with ozone and citric acid showed smaller amounts of hue (Fig. 4). The amount of hue represents the actual color parameter which is obtained by calculation a* and b*. Color analysis shows that immersion of fresh-cut iceberg lettuce in chlorine, organic acids and ozonated water does not have adverse effects on lettuce color, however, changes in the color parameters were associated with time as increased value of a*, decreased value of b*, reduced green pigments, decreased value of L* and the darkening of color (Akbas and Olmez, 2007). According to Bolin et al., 1991 reduction of chlorophyll in cells will increase the value of a*. Reduced value of b* can be due to the reduction of β-carotene during the storage time (Bolin et al., 1991). Results show that the reduction of hue indicator during storage time occurred because of discoloration (browning) of cuts due to enzymatic activities.

Fig. 2. The effect of interaction of citric acid and percidine in storage time (day) on weight loss of fresh-cut lettuce.

Fig. 3. The effect of interaction of ozone and percidine in storage time (day) on weight loss of fresh-cut lettuce.

Purity of color decreased over storage time (Fig. 8 and Fig. 9), which was relatively slow. Ozone treatment resulted in better preservation of chroma during storage time, whereas lower concentrations of citric acid showed better color purity during the storage time (Fig. 7).

Fig. 4. The effect of interaction of ozone and citric acid on hue indicator.

Fig. 5. The effect of interaction of citric acid and percidine in storage time (day) on hue indicator.

Brightness of fresh-cut lettuce (L*)
The color brightness indicator decreased because of increased storage time (days) (Fig. 11 and Fig. 12). In treatments used, lower concentrations of citric acid and ozone caused better preservation of the color brilliance during storage time and with increasing concentrations of citric acid and ozone the brightness of fresh-cut lettuce colors was decreased (Fig. 10). Decreased value of L* may indicate the formation of a dark part in the product, which can be attributed to oxidation of phenolic compounds and microbial wastes in the product during storage time (Akbas and Olmez, 2007).

L* indicator in the stored products shows brightness
of the color, and given that $L^*$ is reduced during storage time, it can be concluded that over time the surface color of fresh-cut lettuce darkens. The results of this study are consistent with the results of Jandric et al., 2010. According to this researcher, the browning of cut surface due to enzymatic reactions decreases the color brightness during storage time.

![Fig. 6. The effect of interaction of ozone and percidine in storage time (day) on hue indicator.](image)

![Fig. 7. The effect of interaction of ozone and citric acid on color purity (chroma).](image)

**Vitamin C**

The amount of vitamin C was reduced by increasing storage time (Fig. 14 and Fig. 15) and in comparison of treatments, 0.5 g l$^{-1}$ citric acid concentration and 1 ppm ozone preserved vitamin C better during storage time (Fig. 13). Treatment with percidine was also effective on vitamin C preservation after the other treatments. One reason is that in the early days of storage time, the amount of oxygen in the packaging was higher and oxidation of ascorbic-citric acid (vitamin C) was more than dehydroascorbic acid citric. According to Agar et al. (1997), increased concentration of carbon dioxide by more than 20% can further destroy vitamin C. In this study, actually, with the increase of storage time and product respiration, levels of carbon dioxide are increased and destroy vitamin C during storage time.

![Fig. 8. The effect of interaction of citric acid and percidine in storage time (day) on color purity (chroma).](image)

![Fig. 9. The effect of interaction of ozone and percidine in storage time (day) on color purity (chroma).](image)

It is said that the cut made in fresh-cut products will induce an increase in ethylene production and the ethylene can stimulate other physiological processes such as the degradation of vitamin C etc. (Kader, 1985).

![Fig. 10. The effect of interaction of ozone and citric acid on Brightness ($L^*$).](image)

According to Zhang et al., 2005, increased concentration of ozone leads to better preservation of
vitamin C in fresh-cut celery. The reduced vitamin C during storage time, especially under modified atmosphere conditions was consistent with the results of Beltran et al., 2005 and Zhang et al., 2005.

![Fig. 11. The effect of interaction of citric acid and percidine in storage time (day) on Brightness (L*).](image1)

![Fig. 12. The effect of interaction of ozone and percidine in storage time (day) on Brightness (L*).](image2)

![Fig. 13. The effect of interaction of ozone and citric acid on vitamin C content.](image3)

Polyphenol oxidase activity
As can be seen in Fig. 17 and Fig. 18, the activity of the polyphenol oxidase reduced over time and compared with the applied treatments, citric acid concentrations (0.5 and 1 g l⁻¹) to the concentration of zero had the lowest enzyme activity. However, in terms of the numerical comparison, concentration 0.5 g l⁻¹ showed the lowest activity. In ozone treatment, 1 ppm ozone concentration showed the lowest activity of polyphenol oxidase during storage time (Fig. 16). Enzymatic browning that can cause discoloration in fruits and vegetables is the result of the activity of a group of enzymes especially polyphenol oxidase that has been reported in many plants. The results of Zhang et al., 2005 showed that polyphenol oxidase activity is reduced by treatment with ozonated water, and higher concentrations of ozonated water have more inhibitory effects on the activity of polyphenol oxidase. The results of the present study are consistent with the results of these researchers.

![Fig. 14. The effect of interaction of citric acid and percidine in storage time (day) on vitamin C content.](image4)

![Fig. 15. The effect of interaction of ozone and percidine in storage time (day) on vitamin C content.](image5)

![Fig. 16. The effect of interaction of ozone and percidine in storage time (day) on vitamin C content.](image6)

Total count of microorganisms
As can be seen in Fig. 19, among different concentrations of ozone, 1 ppm ozone concentration,
and among different concentrations of acid, 1 g l⁻¹ acid concentration had the greatest decrease in the total count of microorganisms. Also, among the interaction of different concentrations of citric acid and ozone, the greatest decrease was related to the interaction of 1 ppm ozone concentration in 1 g l⁻¹ citric acid. Total count of microorganisms is increased over time (Fig. 20 and Fig. 21).

The highest total count of microorganisms was related to bacteria that usually spoil vegetables. Most of these bacteria are gram-negative bacteria especially *Erwinia* and *Pseudomonas* that destroy the tissue of these products (Barriga *et al.*, 1991). The results of this study are consistent with the results of Akbas and Olmez, 2007.

Zambre *et al.*, 2010 in their study found that treatment with ozone on tomatoes can reduce the microbial load and increase the shelf life of tomatoes. Ketteringham *et al.*, 2006 also showed the reduction of microbial population treated with ozonated water on cut green pepper.
Francis and O’Beirne (2002) found that the solution of 1% citric acid for 5 minutes can reduce mesophilic bacteria on lettuce about 1.5 log CFU g⁻¹.

**Conclusion**

The results showed that the total count of microorganisms and weight loss percentage were increased by increasing the storage time, while parameters such as surface color, vitamin C and polyphenol oxidase activity were decreased. Among the different concentrations of citric acid, the highest preservation of vitamin C was related to 0.5 g l⁻¹ citric acid concentration. In treatment with different levels of ozonated water, the highest preservation of vitamin C was related to the treatment with 1 ppm ozonated water concentration. In the lettuce treated with different concentrations of citric acid, the lowest total count of microorganisms was related to 1 g l⁻¹ citric acid concentration and also treatment with 1 ppm ozonated water concentration. In all treatments used, the increased concentrations and the interaction of these treatments resulted in better preservation of qualitative features and reduced microbial quality during storage time.

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