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# Efficacy of gaseous ozone against *Salmonella* and microbial population on dried oregano

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ABSTRACT

Interest in potential food applications of ozone has expanded in recent years in response to consumer demands for green technologies. This study was conducted to evaluate the efficacy of gaseous ozone for the microbial reduction and elimination of *Salmonella* on dried oregano. Ozone treatment was performed up to 120 min under continuous stream of two different constant ozone concentrations (2.8 and 5.3 mg/L). Significant ($P < 0.05$) reductions of 2.7 and 1.8 log were observed in aerobic plate counts and yeast and mold counts after ozonation at 2.8 mg/L for 120 min, respectively. Ozonation performed at 5.3 mg/L for 90 min yielded a reduction of over 3.2 log in the aerobic plate counts. Initial population of a cocktail of *Salmonella* serotypes (*S.* Typhimurium, *S.* Newport and *S.* Montevideo) on inoculated oregano determined as 5.8 log CFU/g decreased significantly by 2.8 and 3.7 log after ozonation at 2.8 and 5.3 mg/L for 120 min, respectively. Sensory evaluation results suggested that over the 2 log reduction in the microbial population can be obtained on dried oregano by gaseous ozone treatments with an acceptable taste, flavor and appearance. The results demonstrated that the gaseous ozone treatment is an effective alternative microbial reduction technique for dried oregano.



## 1. Introduction

The commercial products of *Origanum* leaves are known as oregano in the market (Olivier, 1997). The genus *Origanum*, belonging to the family *Lamiaceae*, comprises 43 species (Ietswaart, 1980; Skoula and Harborne, 2002), mainly distributed in the Eastern Mediterranean region. Twenty-four *Origanum* species are endemic to Turkey (Guner et al., 2001). Because of special compositions of essential oil the leaves of *Origanum* plants are widely used as a very popular spice for the flavoring of traditional dishes.

Due to the environments in which they are grown, spices and herbs often contaminated with pathogenic bacteria and fungi. Although a number of microorganisms are killed during the drying of spices and herbs, many bacteria and fungi can survive (ASTA, 2011). Moreover, the traditional method of drying spices and herbs postharvest is to spread them out on the ground to dry under the sun which potentially exposes them to the risk of contamination (Sagoo et al., 2009). Dried spices and herbs may therefore contain high levels of microbial contamination, depending on whether they have received a microbial reduction treatment or not (McKee, 1995). Many spices such as oregano contain inhibitory compounds that will alter conditions required for growth or even inhibit the growth of pathogens. However, the effectiveness of such antimicrobial compounds in preventing survival of foodborne pathogens may be discounted in dry environment (Keller et al., 2013).

*Salmonella* is the most common bacterial pathogen associated with product recalls and outbreaks in spices (ASTA, 2011). In 1993, a nationwide outbreak of salmonellosis occurred in Germany which was traced to contaminated spice with *Salmonella* at very low level (Lehmacher et al., 1995). This outbreak revealed that low numbers of *Salmonella* in spice can cause a large-scale outbreak of salmonellosis. Between 2007 and 2010, three large-scale outbreaks of *Salmonella* due to consumption of *Salmonella*-contaminated spices, were reported in the United States (Van Doren et al., 2012). It is well recognized that *Salmonella* needs moisture to grow but can survive long periods of time in dried foods such as spices (Podolak et al., 2010). Keller et al. (2013) were able to recover the *Salmonella* from artificially contaminated ground black pepper after 8 months of storage at ambient temperature.

In order to provide a greater assurance of spice safety in the absence of cooking, a variety of microbial reduction techniques such as fumigation (ethylene oxide and propylene oxide) and irradiation are employed in the spice industry. These techniques have limitations in quality impact and consumer acceptance. In EU countries the use of ethylene oxide and propylene oxide for microbial reduction in spices is not approved because of the formation of possible toxic residues

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(ASTA, 2011). Irradiation of packaged spices may produce radiolysis products such as 1,3-di-tert-butylbenzene that can migrate into food (Akbas and Ozdemir, 2008). As an alternative to these microbial reduction techniques, gaseous ozone attracts much attention as a green technology. It was recommended for industrial treatment of the spice in several studies (Guzel-Seydim et al., 2004).

Ozone ($O_3$) or triatomic oxygen, a potent oxidant, is suggested to be an effective antimicrobial sanitizer for water, food, and food processing surfaces. A key feature of ozone processing is the lack of residual components on products, as it liberates safe, innocuous oxygen as the major end product (Novak et al., 2008). Gaseous ozone generators are registered with the US Environmental Protection Agency (EPA) as a food contact surface sanitizer. Ozone has the Food and Drug Administration (FDA) acceptance and recognition approval for direct application on food products. Also, gaseous ozone used in food processing is recognized as allowable by organic certification and regulatory bodies (Selma et al., 2008).

There are no published data on the efficiency of the gaseous ozone in the elimination of *Salmonella* from dried oregano. Therefore, the objective of this study was to investigate the inactivation *Salmonella* on dried oregano by gaseous ozone treatment. We also investigated the effects of treatment on microbial and sensory quality of dried oregano.

## 2. Materials and methods

### 2.1. Oregano sample

Dried oregano (*Origanum onites*) leaves was kindly provided by Inan Tarim (Antalya, Turkey). Oregano sample determined as *Salmonella*-negative by the reference method (ISO, 2002) was firstly divided into two sub-samples. One of these sub-samples was artificially contaminated with the mixture of *Salmonella* strains, and it was assayed only for *Salmonella* after ozone treatment.

### 2.2. Salmonella strains and inoculum

A pool of three *Salmonella enterica* serotypes was used for inoculation: *S.* Typhimurium, *S.* Newport and *S.* Montevideo. Lyophilized cultures of *S.* Typhimurium (ATCC 14028) and *S.* Newport (ATCC 6962) were supplied from Microbiologics Inc. (Saint Cloud, USA). Culture of *S.* Montevideo (ATCC 5747) was kindly provided by the National Public Health Agency (Ankara, Turkey). Stock cultures of microorganisms were stored in brain heart infusion broth (Merck, Darmstadt, Germany) supplemented with 20% glycerol at −18 °C.

Each *Salmonella* strain was transferred into tryptic soy broth (TSB, Lab M, Bury, UK) and incubated overnight at 37 °C. Then, equal volumes of cultures grown in TSB were mixed to obtain a pool of the 3 serotypes in the same tube. The mixed culture was centrifuged at 3600 *g* for 10 min at 5 °C (Hettich, Tuttlingen, Germany) and washed three times with phosphate-buffered saline (PBS). The final cell pellet was resuspended in PBS and the cell density of suspension was adjusted to 0.5 McFarland turbidity standard.

### 2.3. Salmonella inoculation

Two hundred grams of oregano sub-sample allocated to the *Salmonella* inoculation was spread on an aluminum foil. Then, about 3 mL of the *Salmonella* suspension, supplemented with 0.3 mL Tween 80 (Merck) in order to help to reduce the surface tension (Nascimento et al., 2012), was sprayed as homogeneously as possible on the oregano sample using a atomizer (DeVilbiss Healthcare, Somerset, PA, USA). Inoculated sample was transferred to a stomacher bag (Gosselin, Hazebrouck cedex, France). After homogenization by hand for 5 min, with the purpose of ensuring a maximum adherence of the inoculum and no change in the initial water activity of the product, the sample was spread again on the aluminum foil and remained for 30 min to dry.

### 2.4. Ozone treatment

Inoculated and non-inoculated sub-samples of dried oregano were divided into portions of 40 g in Petri dishes (150 × 25 mm) and one portion was used for each of the combinations of exposure time–ozone concentration. The effectiveness of ozone on inactivation of microorganisms in foods can be affected by environmental factors such as relative humidity and temperature (Han et al., 2002). In this study, ozone treatment was performed at ambient laboratory conditions for four exposure times (30, 60, 90 and 120 min) under continuous stream of two different constant ozone concentrations in a 4 L gas-tight plexiglas chamber equipped with two ports for the inlet and outlet air flow. Ozone was produced directly from atmospheric oxygen by two ozone generators (Opal, Ankara, Turkey) with different ozone output levels.

The ozone concentrations in the air flow produced by the generators were determined as 2.8 and 5.3 mg/L by the iodometric titration method (IOA, 1996) using a washing bottle equipped with a diffuser. Iodometric method was carried out by bubbling of ozone in 200 mL buffered potassium iodide (KI) solution at a flow rate of 0.7 L/min. When the bubbling was stopped, pH of KI solution was adjusted with sulfuric acid (4.5 M) to pH 2, in order to complete the reaction. Immediately after, the liberated iodine was titrated to a starch endpoint with freshly standardized sodium thiosulfate solution (0.1 M). Ozone concentration was calculated based on ozone/iodine stoichiometry of 1.

Air flow rate in the tube connected to inlet port was adjusted to 0.7 L/min using a flow meter (Dywer Instruments, Michigan City, IN, USA). The times necessary for the ozone concentrations in the treatment chamber to reach asymptotic concentration were calculated by a mass balance equation previously described by Silva et al. (1998):

$$C_0 \times \left(1 - e^{\left(-v \times \frac{t}{V_t}\right)}\right) = C$$

where $V_t$ is the volume of chamber (L), $v$ is the air flow (L/min), $t$ is the time (min), $C_0$ is the concentration of ozone coming from generator (mg/L) and $C$ is the predicted ozone concentration in the chamber for specified time (mg/L).

### 2.5. Microbiological analysis

Microbiological enumerations were performed by plate count technique on plate count agar (PCA, Lab M), dichloran rose bengal chloramphenicol (DRBC) agar (Lab M) and xylose lysine deoxycholate (XLD) agar (Lab M) for aerobic plate count (APC), yeast and mold (YM), and *Salmonella*, respectively. Initial suspensions were prepared with adding 90 mL buffered peptone water (BPW, Lab M) into stomacher bags containing 10 g sample. Totally 2 mL of initial suspension and additional ten-fold dilutions were surface plated on two plates (150 × 15 mm) of enumeration media. Thus, detection limit of <5 CFU/g was achieved. Inoculated PCA and XLD agar plates were incubated at 35 °C for 48 h for APC and *Salmonella*, while DRBC agar plates were incubated at 25 °C for 5 days for YM counts.

After incubation all colonies grown on PCA and DRBC agar plates and characteristic colonies with a black center on XLD agar plates were counted and microorganism counts were calculated as log CFU/g. Occasionally, characteristic *Salmonella* colonies were confirmed using biochemical identification test system (Microgen Bioproducts, Camberley, UK).

### 2.6. Sensory evaluation

The organoleptic characteristics including taste, flavor and appearance of non-inoculated portions were evaluated by a six-member expert panel and scored on a 5–1 scale, where 5 mean "excellent/like very much" and 1 mean "unusable/dislike very much". A sensory panel was done on 24 h after the ozone treatments. Panel samples (5 g) were placed into white plates, labeled with random numbers and given to the panelists in randomized order. Panelists conducted sensory evaluations in individual booths under white illumination (Kader et al., 1982).

### 2.7. Statistical analysis

Three independent trials were conducted. Results were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS Inc., Chicago, USA). Mean values were compared using the Duncan grouping test at $P < 0.05$.

## 3. Results and discussion

The ozone concentrations inside the treatment chamber reached to their maximum levels (2.8 and 5.3 mg/L) within 30 min according to the mass balance equation. It was predicted that the times necessary for the ozone concentrations to reach over half of the asymptotic concentration were less than 5 min.

### 3.1. Reduction of microbial population on dried oregano by ozonation

The effects of ozone treatments on different microflora on dried oregano are shown in Fig. 1. Ozonation performed at 2.8 mg/L for 30 min was not able to significantly reduce initial levels of APC and YM on oregano, determined as 3.9 and 4.0 log CFU/g, respectively. However, ozonation performed at 5.3 mg/L for 30 min was significantly ($P < 0.05$) reduced levels of APC and YM by 0.5 to 0.4 log, respectively. Reductions in the APC and YM counts ranging from 0.8 to 1.8 log were achieved after 60 min of ozonation, depending on the concentrations. Two hours of ozone treatment at the concentration of 5.3 mg/L was

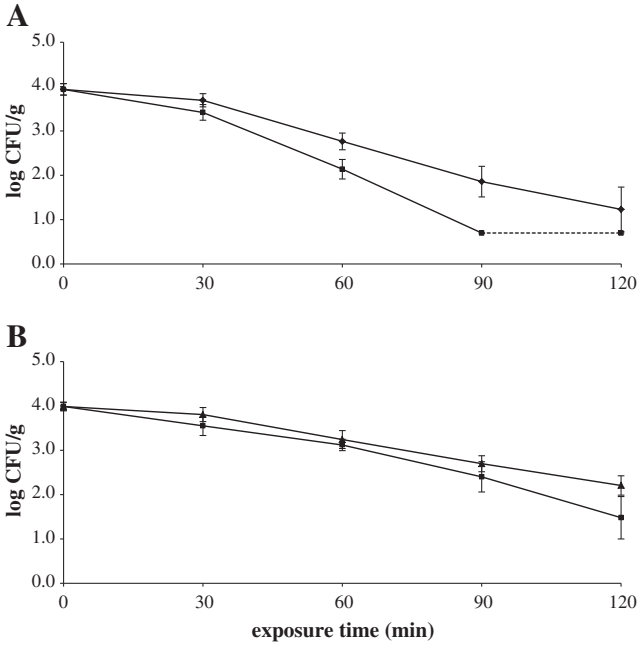


**Fig. 1.** Reduction of APC (A) and YM (B) counts on dried oregano by gaseous ozone treatment at two different concentrations (▲ 2.8 mg/L, ■ 5.3 mg/L) for 120 min. (——) indicates below the detection limit.

reduced the YM counts of oregano from 4.0 to 1.5 log CFU/g, whereas at same concentration the APC counts decreased below the detection limit (<5 CFU/g) with a reduction more than 3.2 log. The reductions observed in the treatments at 2.8 and 5.3 mg/L for 120 min were significantly different ($P < 0.05$).

Our results were in accordance with previous studies that performed on different dried food matrices similar to the dried oregano. Akbas and Ozdemir (2008) achieved 2 log reduction in the counts of *Escherichia coli* on inoculated flaked red pepper by a 360 min exposure to ozone at concentration of 1 mg/L. In another study conducted by Oztekin et al. (2006), a statistically significant ($P < 0.05$) reduction in the APC, coliform and YM counts were obtained on dried figs after ozone treatment for 180 min at 5 and 10 mg/L. Zhao and Cranston (1995) reported that the counts of *E. coli*, *Salmonella* and *Staphylococcus aureus* artificially present on the black pepper was considerably reduced by ozone treatment for 60 min at 6.7 mg/L. They also reported that ozone gas treatment under higher moisture content led to a greater reduction in the microbial load.

Ozone destroys microorganisms by the progressive oxidation of vital cellular components. Antimicrobial action of ozone is mainly attributed to two mechanisms: first mechanism is that ozone oxidizes sulfhydryl groups and amino acids of enzymes, peptides and proteins to shorter peptides (Victorin, 1992). The second mechanism is that ozone reacts with the double bonds of unsaturated lipids in the cell envelope, causing leakage of cell contents and eventually microbial lysis (Scott and Lesher, 1963). In addition to these mechanisms, results of a study performed on *Salmonella* Typhimurium showed that ozone has mutagenic effects on bacteria (Dillon et al., 1992). Disruption or lysis is a faster inactivation mechanism than that of other disinfectants which require the disinfectant agent to permeate through the cell membrane in order to be effective. Moreover, cell lysis mechanism of the ozone action cannot lead to microorganism resistance (Pascual et al., 2007).

The enumeration results at both concentrations for all exposure times except 30 min showed that the reductions in the counts of APC were significantly higher than that of YM. In accordance with our results, gaseous ozone was reported as more effective against bacteria than yeasts and molds (Khadre et al., 2001; Pascual et al., 2007). Hibbe and Stotzky (1969) examined the effects of ozone on fungal spore germination. Compared with bacteria, they found fungi to be less sensitive. Previous studies revealed that sensitivity of bacterial and fungal microflora to ozone is affected primarily by the strains of microorganisms (Zorlugenc et al., 2008). Generally, Gram-negative bacteria are more sensitive to ozone than Gram-positives, spores are more resistant than vegetative cells, and mold species are considerably more resistant compared to yeasts (Moore et al., 2000; Pascual et al., 2007; Restaino et al., 1995).

### 3.2. Survival of Salmonella on dried oregano subjected to ozonation

The efficacy of gaseous ozone in reducing the population of a cocktail of three serotypes of *Salmonella* inoculated on dried oregano is shown in Fig. 2. The initial inoculation level of *Salmonella* determined as 5.8 log CFU/g was decreased by 0.2 and 0.6 log after 30 min of ozone treatments at 2.8 and 5.3 mg/L, respectively. Reduction in the counts of *Salmonella* was 2.5 log for 90 min of treatment at 5.3 mg/L, while same treatment time at 2.8 mg/L reduced *Salmonella* counts by 1.88 log. At the end of the 120 min of ozonation at 2.8 and 5.3 mg/L, reductions in the counts of *Salmonella* on oregano were determined as 2.8 and 3.7 log CFU/g, respectively. Enumeration results indicated that reductions in the levels of *Salmonella* at 5.3 mg/L after 90 and 120 min were significantly ($P < 0.05$) higher than those at 2.8 mg/L. It was reported that nonthermal microbial reduction methods may cause sublethal injury in *Salmonella* (Wuytack et al., 2003). Therefore, it should be noted that selective media such as XLD agar allow for differentiation and enumeration of the specific target microorganisms, but

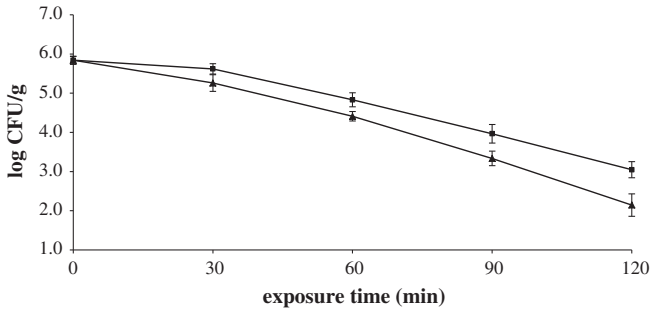


**Fig. 2.** Reduction of *Salmonella* on dried oregano by gaseous ozone treatment at two different concentrations (▲ 2.8 mg/L, ■ 5.3 mg/L) for 120 min.

the media also contain agents which may inhibit repair of sublethally injured cells (Yuste et al., 2004).

Das et al. (2006) achieved over the 3 log reduction in the counts of *Salmonella* on cherry tomatoes within only 20 min by ozone treatment performed at 5 mg/L. This difference in effectiveness of the ozonation on contaminated tomatoes and dried oregano can be explained by the surface area. Previously, surface area was reported as an important factor for microbial decontamination of foods during the ozonation. The smaller particles, thus higher surface area, required a higher concentration of ozone and longer treatment time to achieve the same degree of microbial decontamination (Zagon et al., 1992).

### 3.3. Effects of ozonation on sensory properties of dried oregano

The sensory evaluation of dried oregano treated with gaseous ozone is shown in Table 1. According to the evaluation scores, taste, flavor and appearance of oregano were not affected significantly ($P > 0.05$) by the ozonation performed at 2.8 mg/L for up to 120 min. Taste and flavor of oregano portions treated at concentration of 5.3 mg/L for up to 120 min were graded as similar ($P > 0.05$). However, significant change ($P < 0.05$) was observed in the appearance of oregano ozonated at 5.3 mg/L for 120 min. Zagon et al. (1992) found that prolonged treatments of ozone (>6 h) at relatively high concentrations (>9 mg/L) caused color degradation on spices. Akbas and Ozdemir (2008) reported that ozonation performed at up to 5 mg/L for 360 min did not yielded significant changes in organoleptic properties of flaked red pepper, whereas concentration of ozone over the 5 mg/L resulted significant changes in color of samples. Color degradation effect of ozone on spices can be explained by the oxidative cleavage of chromophores (Nebel, 1975) due to the breakdown of conjugated double bonds (Sarasa et al., 1993).

**Table 1**
Sensory evaluation of dried oregano treated with gaseous ozone for up to 120 min.

| Ozone concentration | Time (min) | Taste | Flavor | Appearance |
|---|---|---|---|---|
| 2.8 mg/L | 0 | 4.7[a] | 4.5[a] | 4.8[a] |
| | 30 | 4.7[a] | 4.5[a] | 4.8[a] |
| | 60 | 4.5[a] | 4.3[a] | 4.7[a] |
| | 90 | 4.5[a] | 4.2[a] | 4.7[a] |
| | 120 | 4.3[a] | 4.2[a] | 4.5[a] |
| 5.3 mg/L | 0 | 4.7[a] | 4.5[a] | 4.8[a] |
| | 30 | 4.5[a] | 4.3[a] | 4.7[ab] |
| | 60 | 4.3[a] | 4.2[a] | 4.7[ab] |
| | 90 | 4.2[a] | 4.0[a] | 4.3[ab] |
| | 120 | 4.2[a] | 4.0[a] | 4.0[b] |

Data within the same column followed by the same letter are not significantly different ($P > 0.05$).

### 3.4. Conclusions

An antimicrobial agent is defined in food industry as an agent that can provide minimum 2 log in microbial reduction (Tiwari and Rice, 2012). Microbiological counts obtained suggested that over the 2 log reduction in the levels of microbial population and *Salmonella* can be obtained on dried oregano by gaseous ozone treatment without significant sensory changes. This result shows that ozone treatment is a promising alternative technique for microbial reduction on dried oregano. A 5 log reduction of *Salmonella* is considered appropriate for risk management by FDA for several foods (ASTA, 2011). Therefore, future validation of the gaseous ozone treatment is still needed to determine the critical limits (e.g., ozone concentration and time parameters) required to achieve 5 log reduction of *Salmonella* on dried oregano.

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