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Article

Ozone Treatment for the Removal of Residual Chlorothalonil and Effects on the Quality of Table Grapes

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Due to uncontrolled use of pesticides and disregard for harvest intervals, some pesticide residue is present in various foods. Treatments using ozone as an alternative for food decontamination have been studied in recent years due to ozone's high oxidation potential even at low concentrations. The present work aimed to evaluate chlorothalonil removal from table grapes by the immersion of fruit in distilled water continuously bubbled with ozone gas. This strategy allowed the removal of 60% of chlorothalonil from table grapes (pulp and skin), regardless of ozone concentration. Ozone treatment of table grapes at a gas concentration of 3 mg L^{-1} changed most of the quality parameters evaluated. Treatment at 2 mg L^{-1} maintained the fruit quality for a longer storage period compared to the untreated control table grapes.

Keywords: ozone, *Vitis vinifera* L., pesticide residue, degradation, fruit quality

Introduction

Many fungicides, such as chlorothalonil (tetrachloroisophthalonitrile, Figure 1), are widely used to control grapevine diseases. The correct use of these fungicides causes no impact on the environment; however, when the recommended application doses and harvest intervals are not followed, residue from these pesticides may remain on postharvest table grapes (*Vitis vinifera* L.). Such contamination poses a risk to consumer health and therefore requires the development of methods for the removal of such pesticide residues, thereby reducing the risks to human health and the environment.

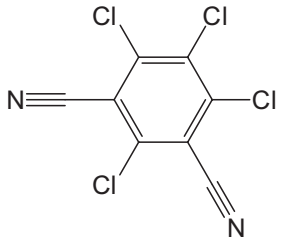

Figure 1. Chemical structure of chlorothalonil.

Among the procedures employed for food decontamination, the use of ozone (O_3) has been investigated as a promising alternative,[1-11] and its effectiveness depends on the combination pesticide/food.

Ozone is generated when molecular diatomic oxygen (O_2) receives an electrical discharge. Because O_3 is unstable, it rapidly degrades back to O_2, allowing the released free oxygen atom ($O^{\bullet}$) to combine either with another $O^{\bullet}$ to form O_2 or with other chemical moieties to cause oxidation. Upon release of the third oxygen atom, ozone acts as a strong oxidizing agent.[12] After reaction, O_3 decomposes back into O_2, leaving no residue on food products.

The adequacy of the treatment of grapes with O_3 gas technology becomes relevant due to the high use of pesticides to control pests in this product. Food quality aspects, such as color, pH, acidity, soluble solids and weight loss, should be evaluated after treatment. It is important to consider alternatives for the removal of these residues that maintain the quality characteristics that make the product attractive to consumers.

For table grapes, the use of ozone bubbles in water becomes relevant due to the presence of postharvest pesticide residues and fruit fragility. This study aimed to evaluate the removal of chlorothalonil from spiked table grapes treated with ozone gas in water. The impact on the physicochemical proprieties of the table grapes was investigated.

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Experimental

Chemicals and reagents

Analytical grade ethyl acetate (99.5% v/v) and HPLC grade acetonitrile (99.5% v/v) were purchased from Vetec (Duque de Caxias, Brazil).

A standard stock solution at a concentration of 1000.0 mg L^{-1} was prepared by dissolving chlorothalonil (Sigma-Aldrich, Steinheim, Germany, 99.3% w/w) in acetonitrile. The working solution containing chlorothalonil at a concentration of 100.0 mg L^{-1} was prepared by diluting the standard stock solution with the same solvent, acetonitrile. These solutions were stored in a freezer at –20 °C.

The following reagents were used to quantify the ozone gas: sulfuric acid (96.0% w/w, Vetec, Rio de Janeiro, Brazil), potassium iodide (99.0% w/w, Vetec, Rio de Janeiro, Brazil), sodium thiosulfate (99.0% w/w, Carlo Erba, Milan, Italy), and soluble starch (99.6% w/w, Êxodo Científica, Hortolândia, Brazil). Sodium hydroxide (99.0% w/w, Vetec, Rio de Janeiro, Brazil) and phenolphthalein (> 99% w/w, Merck, Darmstadt, Germany) were used to determine the acidity of the table grapes.

Extraction and analysis of the pesticide residue

Chlorothalonil residues were extracted, in triplicate, according to the method described by Morais *et al.*[13] with slight modifications. Briefly, 3.0000 g of table grapes were subjected to solid-liquid extraction with low temperature partition (SLE/LTP) using 6.5 mL of acetonitrile, 1.0 mL of distilled water and 1.5 mL of ethyl acetate. The mixture was stirred for 15 min on a shaker table (Tecnal TE 420, São Paulo, Brazil) at 25 °C and 200 rpm and allowed to cool in the freezer (Consul, model 280, São Paulo, Brazil) at approximately –20 °C for 3 h for phase separation. A 1.8 mL aliquot was removed from the extract for chromatographic analysis. The same method was used for the extraction of chlorothalonil from the skin and pulp of the table grapes, but with an additional 1.0 mL of water in the case of the skins.

Chlorothalonil residues were detected and quantified by a gas chromatograph (model GC-2014, Shimadzu, Kyoto, Japan) equipped with an electron capture detector (GC/ECD). Chromatographic separation of the analytes was performed using an HP-5 capillary column (Agilent Technologies, Palo Alto, USA) with a stationary phase composed of 5% diphenyl and 95% dimethylpolysiloxane (30 m × 0.25 mm, 0.1 μm film thickness) and nitrogen (Air Products, São Paulo, Brazil, 99.999% purity) as the carrier gas at a flow rate of 1.2 mL min^{-1}. The temperatures of the split/splitless injector and the detector were 280 and 300 °C, respectively. The column temperature was initially 200 °C and was heated at 20 °C min^{-1} to 290 °C and held for 4.5 min. One microliter of sample was injected into the chromatograph at a split of 1:5. The total analysis time was 9.0 min.

Chlorothalonil residues were identified by comparing the retention time of the peak present in the extracts of the samples with the standard retention time (4.7 min), quantified by the matrix-matched method.

Method validation

The maximum residue limit (MRL) of chlorothalonil on table grapes established by Brazilian legislation, 5.00 mg kg^{-1}, was considered in the validation of this method to ensure that the method is appropriate for the determination of residues within the concentration range.

The evaluated performance parameters for the method include linearity, selectivity, precision (repeatability and intermediate precision), accuracy/recovery, limit of detection (LOD) and limit of quantification (LOQ) of the method. The limits of detection and quantification were calculated by the ratio between the standard deviation of the coefficient of the linear equation and the slope of an analytical curve with low chlorothalonil concentrations. The resulting value was multiplied by 3.3 to obtain the LOD and by 10 to obtain the LOQ, according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.[14]

The linearity of the method was evaluated by using another analytical curve obtained by the analysis of the extracts of samples spiked with concentrations of chlorothalonil ranging from 0.5 to 10.0 mg kg^{-1} (n = 7 points).

The precision was expressed in terms of repeatability and intermediate precision. To determine repeatability, samples were spiked in replicates of six at three concentrations (2.5, 5.0 and 7.5 mg kg^{-1}), corresponding to 0.50 × MRL, 1.00 × MRL and 1.5 × MRL. To determine the intermediate precision, samples were spiked in replicates of six at three concentrations (2.5, 5.0 and 7.5 mg kg^{-1}) on three consecutive days. The results were expressed as the coefficient of variation.

The accuracy was determined from recovery assays in which known quantities of the analyte had been added to the sample in replicates of six at three different concentrations (2.5, 5.0 and 7.5 mg kg^{-1}). The results were expressed by the recovery percentage.

Ozone gas generation and quantification

Ozone gas was obtained from an ozone generator developed by Ozone & Life (São José dos Campos, Brazil). For the generation of ozone gas, industrial-grade oxygen with a purity of 99.5% (Linde Gases, Canoas, Brazil) was fed into the generator and received a dielectric-barrier discharge (DBD).

The iodometric method was used to quantify the ozone gas concentration. In this method, the ozone gas was bubbled into a solution of 20 g L^{-1} KI. KI solution that had been exposed to ozone gas was then mixed with a solution of 0.5 mol L^{-1} H_2SO_4 until the pH was 2.[15] The iodine formed was titrated with a standard solution of $Na_2S_2O_3$ 5.00 × 10^{-3} mol L^{-1} using a starch indicator (5 g L^{-1}). Ozone gas concentration was calculated by the stoichiometric equation, i.e., the number of moles of iodine formed is directly proportional to the number of moles of ozone gas to oxidize.

Application of fungicide to table grapes

Freshly harvested table grape samples (1.0 kg, cultivar Italy), free of pesticide residues, were immersed for 3 min in 1.0 L of aqueous solution containing 0.27 or 1.5 g of the commercial fungicide. This product contained 850 g kg^{-1} of the active ingredient, chlorothalonil. The samples were left to dry in a ventilated area for 12 h. Some of these samples were submitted in triplicate to SLE/LTP, and the extracts were analyzed by GC/ECD to determine the concentration of chlorothalonil on the table grapes prior to ozone gas treatment (control).

Removal of chlorothalonil from spiked table grapes

The table grapes spiked with chlorothalonil (1 kg) were immersed in 5.0 L of distilled water maintained at 15 °C in a PVC chamber (20 cm diameter and 65 cm height, Figure 2). Ozone was continuously bubbled at gas concentrations of 2 and 3 mg L^{-1}, separately, through a spiral disperser located at the bottom of the treatment chamber with a continuous flow of 2.0 L min^{-1} for 60 min, in water. As the ozone ran through the dispenser, the gas was expelled through the holes present along the length of the spiral; thus, the gas bubbles were evenly distributed into the water inside the chamber. All ozone treatments were performed in triplicate.

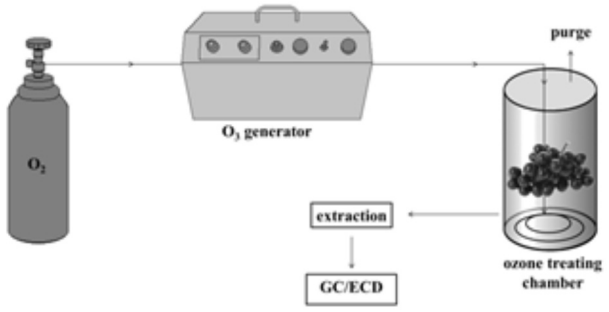

Figure 2. Schematic diagram of the experimental setup.

Part of the treated sample was submitted in triplicate to SLE/LTP, and the extracts were analyzed by GC/ECD to determine the concentration of chlorothalonil on the table grapes after treatment with ozone gas. Part of the grapes was peeled to allow the assessment of the removal of the pesticide in the pulp and skin of table grapes separately. The remaining samples were stored for the assessment of fruit quality after treatment.

Effect of ozone gas on the quality of the table grapes

Ozone gas treated samples and control samples were stored at 1 °C, and the qualitative characteristics of the fruits were evaluated periodically (1st, 7th, 14th, 21st, 28th, 35th, 42nd and 49th days after ozone gas application).

The effects of ozone gas on the postharvest quality of the table grapes were investigated by measuring the soluble solids (SS), weight loss, pH and titratable acidity (TA), according to the Instituto Adolfo Lutz methods.[16] The acidity was determined by titrating the juice from the pulp with a standardized solution of 1.00 × 10^{-1} mol L^{-1} NaOH, and the pH measurement was performed by the immersion of the electrode in the table grape sample prepared to determine the acidity. The soluble solids content of the table grapes was determined by a manual refractometer (model 107, Biobrix, São Paulo, Brazil).

The color of table grapes was assessed with a model CR 400 Minolta colorimeter (Ramsey, USA) using the Commission Internationale de l'Eclairage (CIE) system, with direct reading of the reflectance of the coordinates "L*" (lightness), "a*" (from green to red) and "b*" (from blue to yellow). With the values of these coordinates, it was possible to calculate parameters related to color saturation, chroma (equation 1) and the total color difference, ΔE (equation 2).[17,18]

$$\text{Chroma} = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

Statistical analysis

The validation data were subjected to analysis using descriptive statistics based on the parameters of the measurement of the central tendency (mean) and the

measurement of dispersion (standard deviation, relative standard deviation and coefficient of variation). The descriptive statistics were calculated using Excel (Microsoft Corp., Redmond, USA). Chromatographic data were analyzed using GC solution (Shimadzu, Kyoto, Japan). The data obtained from the tests to assess table grape quality and the effect of ozone gas concentration on the degradation of the chlorothalonil were subjected to analysis of variance (ANOVA) using Statistica 8.0 (StatSoft Corp., Tulsa, USA). A $p < 0.05$ was considered statistically significant.

Results and Discussion

Method validation

To evaluate the selectivity of the method, it was applied both to pesticide-free table grapes and those spiked with chlorothalonil. Both groups were then subjected to extraction and analysis, and their chromatograms were compared. No interference in the retention time of the analytes was observed (Figure 3).

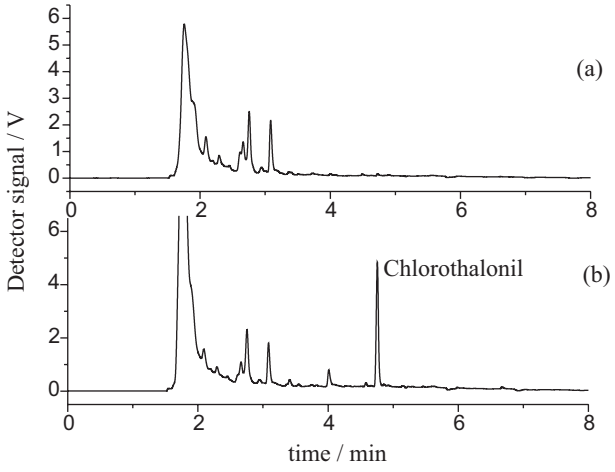

Figure 3. Chromatograms of extracts obtained from table grape samples (a) free from chlorothalonil, (b) containing 5 mg kg^{-1} chlorothalonil, t_R = 4.7 min.

To assess the linearity of the method, we obtained the linear regression fit (y = 1120019.8x + 4257553.4) of the analytical curve from 0.5 to 10.0 mg kg^{-1} chlorothalonil. Seven concentration levels were employed, giving a correlation coefficient (r) of 0.99. This value indicates the high linearity of the method for chlorothalonil.[14] The LOD and LOQ of the method estimated from the parameters of the analytical curve were 0.12 and 0.37 mg kg^{-1}, respectively. The LOQ obtained is far below the MRLs established by the National Health Surveillance Agency (ANVISA, 5.0 mg kg^{-1}), Codex Alimentarius (3.0 mg kg^{-1}) and the European Union (EU, 3.0 mg kg^{-1}).

The repeatability was evaluated at three concentration levels (2.5, 5.0 and 7.5 mg kg^{-1}), and the values of the coefficient of variation (CV) were 5.5, 3.7, and 2.7%, respectively. The intermediate precision was evaluated by the CV obtained from the performance of analyses on three consecutive days. In each test, six samples at each concentration level (2.5, 5.0 and 7.5 mg kg^{-1}) were analyzed by the same analyst, in the same laboratory, under the same conditions, and the following CV values were obtained: 9.2, 4.1 and 4.1%, respectively. The recoveries were 84 ± 5% (2.5 mg kg^{-1}), 109 ± 4% (5.0 mg kg^{-1}) and 102 ± 3% (7.5 mg kg^{-1}). For the analysis of the pesticide residue, the analytical procedure should be able to recover, at each concentration level, 70 to 120% on average, with a precision of CV < 20%.[14] Because the values obtained are within this range, it can be concluded that the recovery and coefficient of variation for chlorothalonil are suitable.

Removal of chlorothalonil residues in table grapes by ozone gas

The chlorothalonil residue quantified in table grape samples before ozone gas treatment were proportional to the concentration of the contaminated solution. Due to the characteristics of the table grape samples, such contamination was not uniform. That is, the residue remaining on the fruit ranged between 38.1 and 82.7 mg kg^{-1} for table grape samples dipped into the chlorothalonil solution of 1.28 g L^{-1} (Table 1) and between 3.5 and 8.0 mg kg^{-1} for table grape samples dipped into the solution of 0.23 g L^{-1} (Table 2). The removal of chlorothalonil residue was significantly affected ($p < 0.05$) by the fungicide contamination. Increased initial pesticide concentration led to increased degradation, regardless of the concentration of bubbled ozone gas.

Table 1. Ozone gas treatment of table grapes spiked with 1.28 g L^{-1} chlorothalonil solution for 60 min

Treatment	Chlorothalonil / (mg kg^{-1})		Removal / %
	Prior to treatment	After treatment	
O_3 / (2.0 mg L^{-1})	54.7 ± 1.04	38.3 ± 1.07	29.9
	55.9 ± 0.97	38.3 ± 0.17	31.5
	76.9 ± 3.59	29.3 ± 1.89	61.9
O_3 / (3.0 mg L^{-1})	38.1 ± 0.08	28.3 ± 2.04	25.6
	45.6 ± 2.86	29.2 ± 1.55	36.0
	82.7 ± 2.86	26.9 ± 0.63	67.4

To evaluate the effect of the water and bubbles on the removal, two experiments were conducted using pure oxygen instead of ozone. The total amount of residue

Table 2. Ozone gas treatment of table grapes spiked with 0.23 g L^{-1} chlorothalonil solution for 60 min

Treatment	Chlorothalonil / (mg kg^{-1})		Removal / %
	Prior to treatment	After treatment	
O_3 / (2.0 mg L^{-1})	5.2 ± 0.19	3.4 ± 0.26	35.5
	5.6 ± 0.30	3.6 ± 0.19	36.8
	8.0 ± 0.02	4.3 ± 0.12	45.8
O_3 / (3.0 mg L^{-1})	3.5 ± 0.12	2.6 ± 0.15	24.9
	4.0 ± 0.10	2.9 ± 0.17	26.3
	4.6 ± 0.01	1.7 ± 0.08	62.6

on the unwashed control table grapes was determined to be 49 ± 11 mg kg^{-1}. Almost 13% of the chlorothalonil residue was removed from the fruit by the water bubbled with oxygen. The percent of residue reduction from the fruit immersed in water bubbled with pure oxygen was less than that from the fruit immersed in water bubbled with ozone.

The present results agree with the results obtained by Chen *et al.*[1] The authors concluded that after the ozone treatment of two vegetables (Chinese white cabbage and greenstem bok choy), the chlorothalonil residual met the Standards for Pesticide Residue Limits in Foods. Kusvuran *et al.*[19] also evaluated the removal of chlorothalonil residue using ozone. In their ozonation studies, the removal efficiency of the pesticide residues with ozone depended on the structural properties of pesticides and matrices (lemon, orange and grapefruit).

Furthermore, Karaca *et al.*[20] reported that cold storage of table grape stemmed berries in an atmosphere enriched with 0.3 μL L^{-1} ozone markedly accelerated the rates of decline of fenhexamid, cyprodinil and pyrimethanil. Gabler *et al.*[21] reported that the reduction of these fungicides on "Ruby Seedless" grapes after a single exposure to 10000 μL L^{-1} ozone fumigation under pulsing vacuum for 1 h at 5 °C were 68.5, 75.4 and 83.7%, respectively.

Effect of ozone treatments on the concentration of fungicide in the pulp and skin of fruit

To verify the fungicide distribution in the fruit and the ability of ozone gas to degrade the residue, the effects on the pulp and the skin of the table grapes were evaluated separately (Table 3).

As expected, fruit contamination was predominantly observed in the skin (between 9.0 and 13.7 mg kg^{-1}). However, it was found that the fungicide had penetrated into the fruit and contaminated the pulp. This contamination ranged between 13 and 18% of the skin contamination.

The results of the removal of chlorothalonil residue during treatments with 2.0 and 3.0 mg L^{-1} ozone gas were statistically equal ($p < 0.05$). On average, 60% of the chlorothalonil was removed from the pulp and skin of table grapes.

Effect of ozone gas application on table grape quality

The quality parameters of the control samples and samples treated with ozone gas at concentrations of 2.0 and 3.0 mg L^{-1} were compared with ANOVA tests (Tables 4 and 5).

Acidity (Figure 4 and 5a) and, consequently, fruit pH (Figure 5b) were the only parameters evaluated by ANOVA that showed a significant difference between the control samples (untreated) and the samples treated with 2.0 mg L^{-1} ozone gas during storage. A *post hoc* Tukey test showed a significant difference in the acidity values after the 6th week in control table grapes, with a linear increase, whereas no change was observed in this parameter in table grapes treated with ozone gas. This result shows the increased quality preservation of the fruit treated with ozone at a gas concentration of 2 mg L^{-1}, with no significant changes in other parameters. Acidity and pH remained constant for a longer period compared to the values of untreated fruit.

Table 3. Contamination distribution and fungicide removal from table grapes dipped in 0.23 g L^{-1} chlorothalonil solution and treated with ozone gas for 60 min

Treatment	Chlorothalonil / (mg kg^{-1})				Removal from the pulp / %	Removal from the skin / %
	Prior to treatment		After treatment			
	Pulp	Skin	Pulp	Skin		
O_3 / (2.0 mg L^{-1})	1.17 ± 0.03	8.97 ± 0.35	0.67 ± 0.01	4.24 ± 0.20	43.34	52.70
	1.35 ± 0.06	10.00 ± 0.95	0.51 ± 0.03	4.69 ± 0.07	61.96	53.16
	1.75 ± 0.07	13.65 ± 2.00	0.60 ± 0.10	5.28 ± 0.80	65.69	61.31
O_3 / (3.0 mg L^{-1})	1.97 ± 0.06	10.87 ± 0.02	0.42 ± 0.02	3.38 ± 0.24	78.86	68.91
	1.49 ± 0.04	11.83 ± 0.74	0.53 ± 0.04	3.61 ± 0.24	64.76	69.52
	1.34 ± 0.03	7.82 ± 0.82	0.71 ± 0.04	3.40 ± 0.27	47.39	56.50

Table 4. ANOVA of the effect of the treatment with 2 mg L^{-1} ozone gas on table grape quality

	Main effects	Df	F-ratio	P-value
ΔE	Ozone gas concentration	1	1.2036	0.275897
	Storage time	7	3.0761	0.006385[a]
	Residual	80	–	–
Chroma	Ozone gas concentration	1	0.002	0.967162
	Storage time	7	2.038	0.060246
	Residual	80	–	–
SS	Ozone gas concentration	1	2.811	0.097505
	Storage time	7	13.571	0.000000[a]
	Residual	80	–	–
TA	Ozone gas concentration	1	40.329	0.000000[a]
	Storage time	7	39.625	0.000000[a]
	Residual	80	–	–
pH	Ozone gas concentration	1	29.3	0.000001[a]
	Storage time	7	30.8	0.000000[a]
	Residual	80	–	–
Weight loss	Ozone gas concentration	1	0.438	0.510125
	Storage time	7	162.989	0.000000[a]
	Residual	80	–	–

[a]Significant at $p < 0.05$; df = degree of freedom; ΔE = total color difference; TA = titratable acidity; SS = soluble solids.

Table 5. ANOVA of the effect of the treatment with 3 mg L^{-1} ozone gas on table grape quality

	Main effects	Df	F-ratio	P-value
ΔE	Ozone gas concentration	1	0.55409	0.458833
	Storage time	7	2.88348	0.009736[a]
	Residual	80	–	–
Chroma	Ozone gas concentration	1	107.654	0.000000[a]
	Storage time	7	0.767	0.616652
	Residual	80	–	–
SS	Ozone gas concentration	1	94.87	0.000000[a]
	Storage time	7	31.24	0.000000[a]
	Residual	80	–	–
TA	Ozone gas concentration	1	68.803	0.000000[a]
	Storage time	7	52.075	0.000000[a]
	Residual	80	–	–
pH	Ozone gas concentration	1	203.479	0.000000[a]
	Storage time	7	53.145	0.000000[a]
	Residual	80	–	–
Weight loss	Ozone gas concentration	1	29.073	0.000001[a]
	Storage time	7	223.860	0.000000[a]
	Residual	80	–	–

[a]Significant at $p < 0.05$; df = degree of freedom; ΔE = total color difference; TA = titratable acidity; SS = soluble solids.

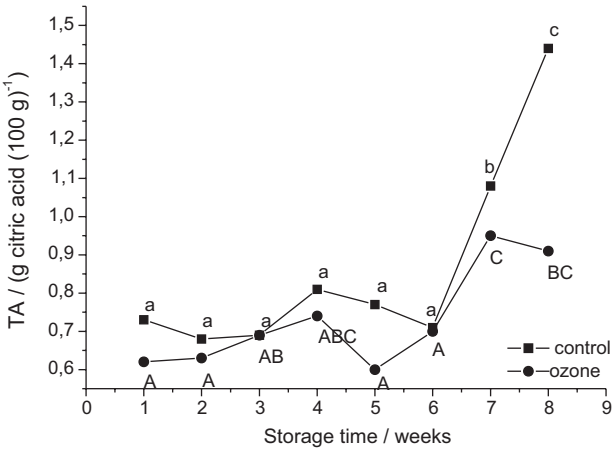

Figure 4. Effect of ozone gas treatment (2.0 mg L^{-1}) on the titratable acidity (TA) of table grapes during 8 weeks of storage. Same letters do not differ by ANOVA with the *post hoc* Tukey test ($p < 0.05$).

However, the treatment with 3 mg L^{-1} ozone gas varied significantly ($p < 0.05$) in most evaluated parameters (Figure 5). Total color difference was the only parameter that was not affected (Figure 5f).

Several studies show favorable aspects of the exposure of table grapes to ozone. Sarig *et al.*[22] evaluated the exposure of table grapes to gaseous ozone and showed that, in addition to its sterilization effect, ozone increases post-harvest fruit resistance. Palou *et al.*[23] showed that gray mold can be completely inhibited in table grapes stored for 7 weeks at 5 °C if they are subjected to a continuous treatment of 0.3 μL L^{-1} ozone. The physiological response of the fruit to treatment with this ozone at low concentration was measured by weight loss, which was not significantly different from that of the control.

Conclusions

Ozone treatment was suitable for removing chlorothalonil residue from table grapes. This strategy allowed 60% of the chlorothalonil to be removed from table grape skin and pulp. The treatment of table grapes with ozone gas at 2 mg L^{-1} did not significantly change the quality parameters: soluble solids, weight loss, color

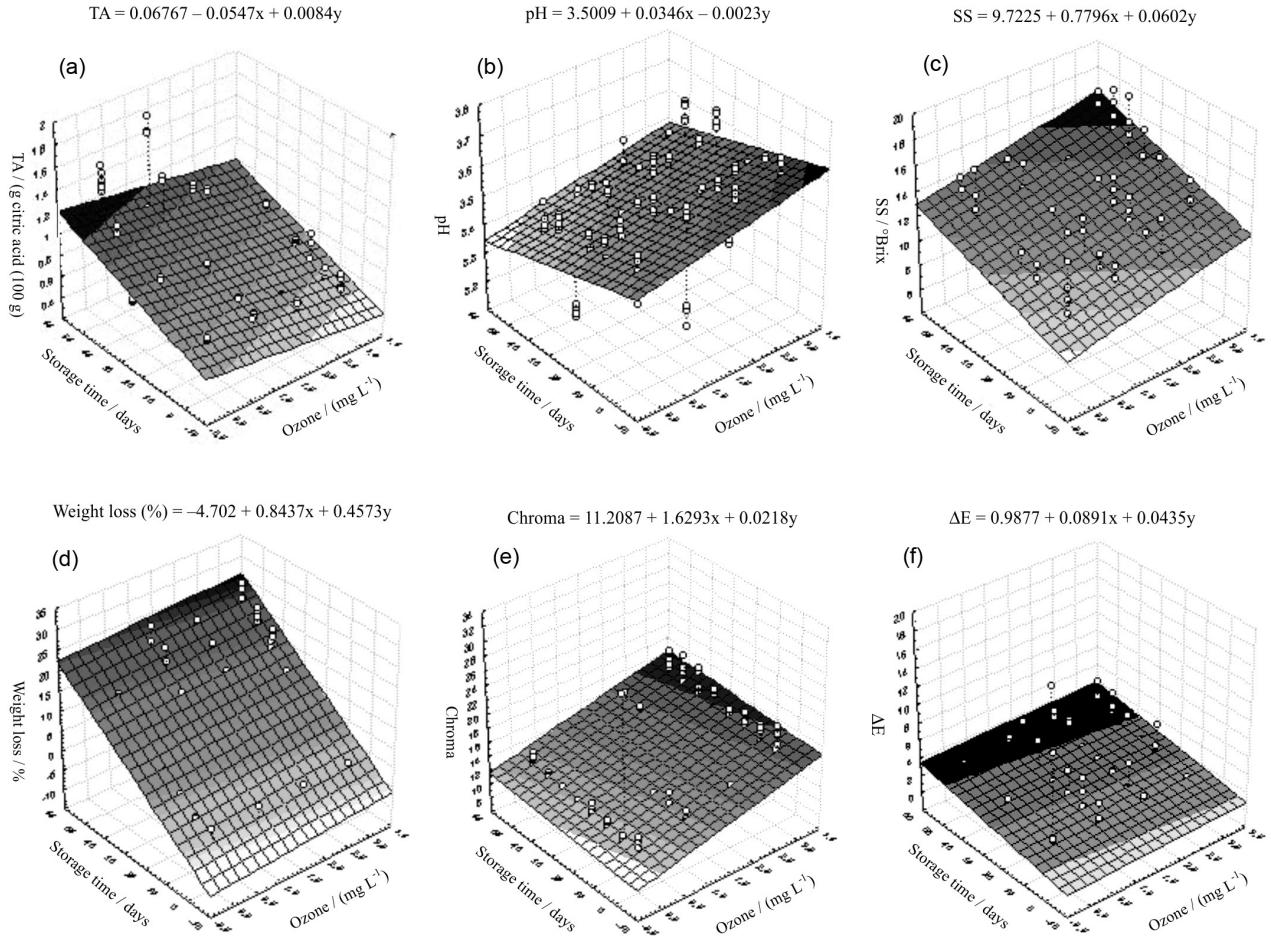

Figure 5. Response surface of the (a) titratable acidity (TA), (b) pH, (c) soluble solids (SS), (d) weight loss, (e) color saturation (chroma) and (f) total color difference (ΔE) of ozonated grapes stored at 1 °C.

intensity, and total color difference. However, this treatment did significantly affect titratable acidity and, consequently, the table grape pH. Treatment with 2 mg L^{-1} of ozone gas prevented acidity from increasing during storage and allowed longer lasting fruit quality.

Acknowledgments

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