Disinfectant of pummelo (*Citrus Grandis* L. Osbeck) fruit juice using gaseous ozone

Nor Nadiah Abdul Karim Shah¹ · Nur Amira Mohd Supian¹ · Nadia Aida Hussein¹

Revised: 7 September 2018 / Accepted: 2 November 2018 / Published online: 21 November 2018
© Association of Food Scientists & Technologists (India) 2018

Abstract This work studied the effectiveness of gaseous ozone disinfection on pummelo (*Citrus Grandis* L. Osbeck) fruit juice components. Unfiltered and filtered pummelo fruit juices were treated with gaseous ozone for up to 50 min with ozone concentration fixed at 600 mg/h. A microbiological and physicochemical properties analysis were conducted on the ozone-treated fruit juices samples. It was found that the survival rate of aerobic bacteria, yeast and mold in unfiltered pummelo fruit juice were higher compared to filtered juice, as the juice components acted as protective barriers to the microorganisms. The microorganisms' inactivation in pummelo fruit juices was also observed to have increased as the ozone treatment time increased. Significant effects on total colour difference, ascorbic acid content, and total phenolic content were also observed over increased ozone-treatment time. However, ozone was shown to be ineffective in activating PME activity in both types of juice. The experimental results of this study indicated that pummelo fruit juice components had significant effects on the effectiveness of gaseous ozone, however, the degree of the effects depends on the different fruit components (total soluble solids, total phenolic content). As a conclusion, filtered juice showed better quality characteristics in comparison to unfiltered juice post-ozone treatment.

Keywords Gaseous ozone · Pummelo fruit juice · Fruit juice components · Aerobic plate count · Antioxidant activity

Introduction

In recent attempts to revamp the state of national agriculture production, the Malaysian Ministry of Agriculture revised the agro-food policy covering the period from 2011 to 2020. The policy listed pummelo as one of the 15 potential tropical fruits that is expected to generate national gross income (for all 15 fruits) of RM 21.44 billion in 2020 (DOA 2012). One of the objectives in realizing the policy is to increase the income of entrepreneur farmers through diversification of fruit products, subsequently, increasing Malaysia's productivity and volume of export trade.

Pummelo is an under-exploited citrus fruit in Malaysia, known to be the largest of all citrus fruits which can grow as large as 30 cm in diameter and weigh up to 9 kg (DOA 2012). The current production of pummelo fruit in Malaysia is recorded at 12,000 metric tons (DOA 2012) and it is grown mostly in the state of Johor and Perak. This non-seasonal citrus fruit have also been known to be mildly-resistant towards Citrus Greening Disease (CDG), and it is more resistant than grapefruit varieties. Kumar et al. (2015) in her study reported that pummelo fruit contains a number of beneficial bioactive compounds such as flavonoids, carotenoids, phenolics, vitamin C and anthocyanin. It also contains high amount of vitamin A, potassium, beta-carotene, folic acid and fiber, which have been known to demonstrate anti-cancer, anti-inflammatory, anti-tumour and blood clot inhibition activities. The colour of the flesh of the fruit varies from white, light yellow, pink or rose-red and the taste varies from sweet sour or spicy

✉ Nor Nadiah Abdul Karim Shah
nadiahkarim@upm.edu.my

¹ Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

bitter-sweet aftertaste in the mouth. Pummelo fruit has an expected ratio of 2:1 (w/v) (unpublished data) of producing juice and a total brix to acid ratio of 12.54 [Florida's commercial juice standard: 13–18 (Kimball 1999)]. Its natural resistance towards pests, means cultivation is easier leading to higher productivity, and lower production cost. These characteristics make pummelo fruit a promising candidate for production of a desirable and healthy juice for human consumption, further giving Malaysia an attractive opportunity to expand the fruit's influence and marketability in the international market of juices.

Currently, heat treatment process is the most commonly used method for microorganisms and enzymes inactivation which will extend the shelf life of juices. Thermal pasteurization process of fruit juices typically involves heating the product to 90–95 °C for 15–20 s (Torlak 2014). However, the high energy treatment usually diminishes vitamins, essential nutrients, and food flavors in the product (Barbosa-Canovas and Bermudez-Aguirre 2011). Various studies have proven that pasteurization using heat caused detrimental effects on juice quality. Nutritional contents, chemical, antioxidant and sensory attributes have been reported to have been directly affected by the heat. Moreover, it is cost-prohibitive for small juice producers. Thus, a non-thermal method of pasteurization has been experimented to treat citrus juice to have a longer shelf-life without compromising on the fresh-like quality of juice and an added advantage is its ability in deactivating spoilage and pathogenic microorganisms. Non-thermal technologies such as ozone, ultraviolet (UV-C), pulsed electric field (PEF) and high-pressure processing (HPP) have been extensively studied and applied in liquid foods pasteurization to achieve a minimum of 5-log reduction of pertinent pathogens, to comply with the USFDA regulations (FDA 2004). However, the popularity of ozone has been dismal due to the negative image projected by ozone and very few studies have been done to investigate the effect of ozonation on quality attributes on tropical fruit juices, even though the capital cost of ozone is the lowest among other non-thermal technologies.

Ozone (O₃) is a triatomic molecule, consisting of three oxygen atoms. It is an allotrope of oxygen that is much less stable than the diatomic O₂. Ozone has gained interests in regards to food safety as ozone is 1.5 times stronger than chlorine and is effective over a wider spectrum of microorganisms (Patil and Bourke 2012). It is also known that ozone leaves no chemical residue and it degrades to molecular oxygen upon reaction or natural degradation. It is a colorless gas that is readily detectable at the range of 0.01–0.05 ppm (Patil and Bourke 2012). When ozone is produced it will decay rapidly, because ozone is an unstable compound with a relatively short half-life. Ozone quickly degrades to oxygen in pure water and even more

rapidly in impure solutions, such as fruit juices that contains various soluble and insoluble solids (Guzel-Seydim et al. 2004). In 2001, United States Food and Drug Administration (USFDA) approved the usage of ozone in the gaseous and aqueous phases as an antimicrobial agent for treatment, storage and processing of foods (Khadre et al. 2001). Ultraviolet radiation (188 nm wavelength), corona discharge, and cold plasma methods can be used to initiate free radical oxygen formation and, thereby generate ozone. Corona discharge method chosen in this study is the most popular type of ozone generator for most industrial and personal uses. They are typically very cost-effective and do not require an oxygen source other than the ambient air.

Due to the highly reactive nature of ozone, it is necessary to understand the key parameters in order, to optimize its application for sterilizing liquid food products. Restaino et al. (1995) in their study, stated that effectiveness of ozone against microorganisms depends not only the concentration of ozone used but also on the residual ozone in the medium and various environmental factors such as medium pH, temperature, humidity, additives such as sugars and the amount of organic matter surrounding the cells. Increase iteration of the complex components of fruit juice such as pectin and sucrose may provide protective effects to the bacterial populations, making ozone sterilizing-behavior ineffective in disinfection of pertinent microorganisms. Williams et al. (2004) reported a low efficiency of ozone to inactivate *E. coli* in orange juice in presence of organic matter and ascorbic acid. The impact of different compounds such as sugars, fibers, ascorbic acid and other organic matter in the dissolution rate and availability of ozone have created a protective effect in some components.

Medium pH is also an important intrinsic factor of ozone efficacy in disinfection process. Cullen et al. (2010) stated that decreasing pH is able to increase ozone efficiency and ozone decomposition rate changes drastically with changes in pH. Pummelo fruit juice pH was recorded at 4.00 ± 0.02 (Shah et al. 2015) which makes pummelo fruit juice the best candidate to be disinfected using ozone. Mechanisms of ozone decomposition are complex and are dependent on factors such as type of radicals formed and organic matter present in the medium that initiate, promote or inhibit the radical chain reaction. Hence, it is difficult to generalize that a particular concentration of ozone at a given time will always be effective in inhibiting microorganisms in the food product. Additionally, the conventional methods of relying on the acidity of their product to ensure microbiological safety is irrelevant as several incidents of food-borne illnesses have been associated with spoiled acidic fruit juices.

While the sterilizing action of ozone has been studied extensively on orange juice (Patil et al. 2009; Tiwari et al. 2008a, b; Williams et al. 2004; Angelino et al. 2003), there is little information about the effects of solid components of tropical fruit juice, specifically pummelo fruit juice, on the bactericidal activity of ozone. This study aims to prolong the shelf-life of pummelo fruit juice using ozone with minimal qualitative changes by focusing on the effect of juice solid components on ozone disinfection effectiveness. Thus, the objectives of this study are (1) to evaluate ozone efficiency towards bacterial inactivation (2) to determine the effects of ozone on physicochemical properties and antioxidant content and (3) to evaluate the shelf-life of disinfected pummelo fruit juice.

Materials and methods

Pummelo fruit juice preparation

Pummelo (*Citrus Grandis* L. Osbeck) fruit juice was extracted using screw type extractor (KAAE, Malaysia). The filtered pummelo fruit juice was prepared by filtering the juice through tea filter to reduce the amount of pulp content. The juices were then kept in a dark glass bottle to be stored in a freezer at a temperature of $-20\text{ }^{\circ}\text{C}$ until further experimental research. Once thawed in a chiller of $4 \pm 1\text{ }^{\circ}\text{C}$, the preliminary analyses were done to establish the initial value of each physicochemical and antioxidant characteristics of pummelo fruit juice.

Ozone treatment

Ozone gas was generated in a closed system using water ozonizer (Model SY-004, Taiwan) by corona discharge method in a 200 mL beaker (Fig. 1). The fixed ozone output concentration at 600 mg/h was measured using an ozone sensor (Model 200 Series, Aeroqual, New Zealand). Ozone gas was pumped directly into the pummelo juice ($4 \pm 1\text{ }^{\circ}\text{C}$) for up to 50 min through the food-grade

silicone tube into the beaker and stirred using magnetic stirrer (100 rpm) to ensure the ozone molecules were completely mixed with the samples. As the ozone generator has a time limit of 50 min (as a safety precaution), ozone processing time was set in the range of 0 and 50 min and considered as ozone parameter. Untreated pummelo fruit juices (control) were stored at $4 \pm 1\text{ }^{\circ}\text{C}$ in sterile dark glass bottles to protect them from light. All experiments were carried out in triplicate and analyses were performed immediately after processing.

Microbiological analysis

The enumeration of survived bacteria was determined by referring the procedures in Chapter 3 of Bacteriological Analytical Manual (Maturin and Peeler 2001). Difco™ Plate Count Agar (Difco Laboratories, Becton, Dickinson and Company Sparks, USA) and Difco™ Malt Extract Agar (Difco Laboratories, Becton, Dickinson and Company Sparks, USA) were used to promote the growth of microorganisms. One tenth milliliter of pummelo fruit juice samples was streaked on the culture medium and incubated for 24–48 h at $37\text{ }^{\circ}\text{C}$ (APC) and $25\text{ }^{\circ}\text{C}$ (YMC). Treatments were performed in triplicate and results were expressed as colony-forming units (CFU) per milliliter.

Physicochemical and antioxidant analysis

Total colour difference

The triplicate values of *L* (whiteness), *a* (redness/greenness), and *b* (yellowness/blueness) of each pummelo juice sample were determined using Hunter Lab Ultra Scan Pro (D65, 28 Hunter Lab Assoc Lab Inc, Reston, VA, USA) color spectrophotometer. It was calibrated using white reference tiles ($L = 97.55$; $a = -0.11$; $b = -0.04$) and light trap. Then, the color values were calculated with formula adapted from Tiwari et al. (2009a, b);

$$\text{TCD} = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad (1)$$

Fig. 1 Schematic diagram of ozone treatment system

Ozone fixed concentration	600 mg/hr
Ozone generator time limit	50 minutes
Ozone generator power and frequency	8W, 60 Hz
Pipe type, diameter and length	Food-grade silicone, 0.5 cm, 0.5 m

where L_0, a_0, b_0 = Initial values obtained from untreated juice.

Pectin methylesterase (PME) activity

PME activity of pummelo juice was measured using the Kimball (1999) method. PME activity was expressed as PME_u/mL which represents μ -equivalent of acid liberated per min per mL of test sample at pH 7.7 and 30 °C. Pectin solution (1%) was prepared using 0.1 M sodium chloride solution at the temperature of 50 °C. Then, 10 mL of juice sample is added to 40 mL aliquot substrate solution at 30 °C. The pH of the solution was adjusted to pH 7.0 by adding 2 N sodium hydroxide solution and was readjusted again until 7.7 with 0.05 N sodium hydroxide solution. Next, 0.1 mL of 0.05 N sodium hydroxide solution was added until pH of solution returned to 7.7 again, during which time was measured. The pectin methylesterase activity was calculated using Eq. 2;

$$\text{Activity} \left(\frac{\text{PMEu}}{\text{mL}} \right) = \frac{(\text{mL NaOH})(\text{Normality of NaOH})}{(\text{mL juice})(\text{reaction time})} \times 10^3 \quad (2)$$

Ascorbic acid (AA) content

Two 6-dichlorophenol-indophenol titration method adopted from Amador (2011) were used in determining ascorbic acid content in samples of pummelo fruit juice. 0.5% sodium 2,6-dichlorophenol-indophenol dye and 3% acid stabilization solution were prepared prior to the test. Then 2 mL of juice sample, blank (distilled water), or standard of 1 mg/mL of ascorbic acid solution was added into 5 mL of acid stabilization solution. The mixture was titrated rapidly with the dye solution until a light but distinct rose pink color persists for at least 5 s. The vitamin C or ascorbic acid content was obtained from the following equation and expressed as mg/100 mL;

Ascorbic Acid

$$= \frac{(\text{mL of titrant for sample}) - (\text{mL titrant for blank})}{(\text{mL titrant for standard}) - (\text{mL titrant for blank})} \times 100 \quad (3)$$

Total phenolic content (TPC)

The methods for determination of total phenolic content were referenced from Alothman et al. (2010). Samples were centrifuged at 5000 rpm for 5 min at 4 °C. One milliliter of juice sample was mixed with 1 mL Folin–Ciocalteu reagent and 10 mL of 20% sodium carbonate

before being diluted to 100 mL with distilled water. The mixture was mixed thoroughly and incubated for 1 h at room temperature in the dark prior to the measurement. UV Visible Spectrophotometer (Ultraspec 3100 Pro, Amersham Pharmacia Biotech, UK) was then used to measure the absorbance of mixture at 765 nm. Data for total phenolic content were obtained from the calibration curve prepared with gallic acid at concentration of 0–500 mg/L and are expressed as Gallic acid equivalents (GAE).

Statistical analysis

The statistical data were analysed via Full Factorial methodology utilizing Design Expert Software (Version 6, Stat-Ease Inc., MN, USA). Two independent variables: type of pummelo juice (filtered and unfiltered) and processing times (0, 10, 20, 30, 40 and 50 min) were used to investigate their influences on the inactivation of microorganisms and physicochemical properties of treated pummelo fruit juices. The experiments were performed in triplicate and the data were analyzed by using Analysis of Variance (ANOVA).

Results and discussion

Microbiological analysis

The antibacterial activity of ozone has also been attributed to its diffusion capability through biological membranes. The reactivity of ozone is ascribed by the activity of hydroxyls ($\cdot\text{OH}$), hydroperoxy ($\cdot\text{HO}_2$) and superoxide radicals ($\cdot\text{O}_2^-$), which are responsible for microbial inactivation (Cullen et al. 2010). In this study, filtered and unfiltered pummelo fruit juice samples underwent microbiological test to examine the presence of aerobic bacteria, yeast and mold before and after being treated with gaseous ozone. Figure 2a shows the bactericidal effect of ozone treatment against aerobic bacteria in both types of juice. The total aerobic plate count for unfiltered pummelo fruit juice showed 2.3 log reductions (from 3.1 log to 0.8 log CFU/mL), whereas, filtered sample showed 6.1 log reductions (from 7.3 log to 1.2 log CFU/mL). A non-linear (downward concavity) trendline can be seen from the aerobic bacteria reduction curves and this is in contrast to the norms of microbial inactivation which has generally been determined using pseudo-first and first order kinetic models. These mechanistic models implicitly assumed that all members in a population behave in the same manner and inactivation of a single macromolecule is due to one circumstance. The difficulty of survival curves in fitting the log linear model is due to the formation of a shoulder or tail during microbial inactivation which accounts for the

Fig. 2 Interaction graphs of types of juice and treatment time on **a** total aerobic plate count in unit CFU/mL and **b** yeast and mold count in CFU/mL

biological variation that can be represented by Weibull model (Cullen et al. 2010). The shape factor, β for both types of juice, is less than 1, indicating the formation of tail (Van Boekel 2002). The occurrence of a tail in the survival curve is generally attributed to inherent resistance or resistance acquired during ozonation. β value of less than 1 could also signify that the remaining bacterial populations have the ability to resist the ozone treatment and to initiate a secondary growth during storage. An unpublished data showed that an aerobic plate count growth of 4 log was seen at Week 8 (unfiltered) and 2 log at Week 10 (filtered). The reaction of ozone molecule with microorganisms and the presence of organic materials could be the reasons for formation of tail during microbial inactivation in this study. The aerobic plate count result showed the R^2 value of 0.80 and RMSE of 0.55, which demonstrates that Weibull model was a good fit for data analysed in the experiment.

The results also show that inactivation rate decreases over treatment time depending on the presence of solid components of juice, with the initial microbial counts differing significantly between filtered and unfiltered samples. This proves that the rate of effectiveness of ozone is dependent on both the type of juice and ozone treatment time ($p < 0.05$). 5 log reduction time, t_{5d} , in unfiltered sample is 6 min at 10 mg/min of ozone concentration, whereas, filtered sample was found with 3 log of aerobic plate count even before the experiment starts. Whereas, in a

study done by Patil (2010) showed that *E. coli* ATCC 25922 in an unfiltered orange juice was reduced to 6 log after 11 min of ozone treatment at a concentration of 8.64 mg/min. Hence, ozone concentration played an important role to achieve the desired inactivation at a minimum exposure time, even for juice with higher juice components.

Total yeast and mold count (YMC) was also found to decrease as the ozone treatment time increased, as illustrated in Fig. 2b. Filtered pummelo fruit juice with its initial value of YMC of 5 log decreased to 1.3 log (reduction of ~ 4 log). Whereas, YMC of unfiltered juice showed a reduction of 6 log ($p < 0.05$, $R^2 = 0.97$) with its t_{5d} of 7.2 min. The results indicate that the organic matter contents in pummelo fruit juice has an influence on the presence of microorganisms and ultimately the efficiency of ozone inactivation. These results further support the previous study on the impact of organic matter present in unfiltered pummelo fruit juice on the efficacy of ozone treatment. Patil (2010) reported in his study the inactivation of *E. coli* took a longer time in orange juice with pulp content compared to zero or low pulp content of orange juice. Kim et al. (2003) also states that the rate of inactivation of the microorganisms is greater in ozone demand-free systems than when the medium contains oxidizable organic substances. Pummelo fruit juice contains high vitamin C ~ 78 mg/mL which is related to the amount of

ascorbic acid present, and this organic material could also act as protective barrier for microbial cell against ozone. Thus, proving that the organic matter with high ozone present in pummelo fruit juice may compete with the microorganisms, resulting in a higher number of microbial count (after disinfection).

Microbial resistance is also dependent on the acidic environment of fruit juice which has an impact on ozone efficiency. Patil (2010) demonstrated that ozone inactivation of *E. coli* was much faster at low pH than at higher pH values. Therefore, acidic environment of pummelo fruit juice (pH of ~ 4.0) can be considered as a suitable matrix for ozonation in terms of antibacterial effectiveness. The effect of pH on antibacterial effectiveness of ozone, similar to temperature, is mainly attributed to the fact that the ozone decomposition occurs faster in higher pH aqueous solutions. The effect of temperature on bacterial effectiveness to ozone in liquids is mainly attributed to the fact that the solubility and decomposition rate of ozone changes substantially with changes in temperature (Tiwari and Muthukumarappan 2012). This study was performed at 20 °C to prevent quick decomposition of ozone as recommended by Patil (2010), as temperature decreases ozone become more soluble and more stable with a decrease in the decomposition rate. Therefore, a fall in the temperature increases availability of ozone in the aqueous medium and subsequently, its efficacy (Torlak 2014).

Ozone processing times (0–50 min) may also contribute to the inactivation of microorganisms in pummelo fruit juice. The results obtained in this study shows that at 50 min, the total count of surviving microorganism had decreased from 10^{10} to 10^4 (unfiltered) and 10^2 CFU/mL (filtered). With the limitation of ozone generator to produce ozone up to 50 min, the overall pattern of the inactivation study shows that the effectiveness gaseous ozone to inactivate microorganisms is higher with prolonged ozone exposure ($p < 0.05$). Patil et al. (2009) reported that the direct application of ozone is effective for the inactivation reduction of *E. coli* in orange juice, but they observed that the inactivation rate is strongly dependent on the soluble solid content of fruit juice. The results in this study are in agreement with previous research where the characteristics of the microbial strain itself could influence the ozone efficiency and that bacteria are more sensitive than yeasts and fungi. Moore et al. (2000) stated that yeast cells are more resistant towards ozonated water as the yeast cell walls provide adequate cell protection to retard the oxidation process of ozone gases from damaging the plasma membrane. Thus, it can be concluded that increasing the processing time and eliminating or minimizing intrinsic factors of the juice (pH and solid content) could increase the efficacy of ozone gases to penetrate directly into the

inner cell promoting cell lysis and eventually death of pertinent microorganisms.

Physicochemical and antioxidant analysis

The results of various dependent variables, namely, total color difference (TCD), pectin methylesterase (PME) activity, ascorbic acid content and total phenolic content (TPC) are presented in Fig. 3.

Tiwari et al. (2008a, b) reported, the differences in perceivable color can be analytically classified as very distinct ($\text{TCD} > 3$), distinct ($1.5 < \text{TCD} < 3$), and small differences ($\text{TCD} < 1.5$). In this study, TCD for unfiltered juices are classified as very distinct as the range lies between 0 to 6.5 and filtered juices is categorised as distinct. The interaction between both factors, types of juice and ozone processing time on TCD is shown in Fig. 3a. The graph illustrates that there are significant changes of color in both treated pummelo fruit juices. The p value on the type of juice is 0.0015 which indicates significance on the values of TCD. However, ozone processing time is considered not significant to influence the changes in color as the p value is 0.0822. This finding is contrary to findings of previous research studies which indicates that ozone treatment time does influence the color degradation of blackberry juice (Tiwari et al. 2009a, b). The authors state that the ozone molecule and hydroxyl radicals produced may attack the conjugated double bond and lead to the partial oxidation of organic acids, aldehydes and ketone. Filtered pummelo fruit juice may have lost its solid components that played an important part on the lightness value (L^*) of the juice. L^* value was observed to significantly increase in the unfiltered juice sample, compared to the filtered juice sample. Implicitly, the ozone reaction on the pulp-less filtered juice sample had a minimal effect on the total color difference in comparison to the unfiltered juice sample. Nevertheless, color changes induced by ozone treatments at doses used in this study, although significant, were too small to impair pummelo fruit juice quality.

Pectin methylesterase (PME) is an enzyme that has a major impact on fruit juice processing as it affects the clarity of pummelo fruit juice. Inactivation of PME is desirable as the cloud appearance in the juice is preferred by consumers. p value obtained from the analysis of variance showed that only ozone treatment time factor affected PME activity of ozone-treated pummelo juice significantly (p value < 0.0001). Moreover, PME activity of unfiltered juice was seen to increase as ozone treatment time increased as shown in Fig. 3b. The range of PME activity for unfiltered juice was found to be between 0.02 to 0.10 PME μmL while for filtered juice it is between 0.04 to 0.07 PME μmL . These results differed from a previous study by Rodoni and Vicente (2010), where a clear

Fig. 3 Interaction effects of types of juice and ozone treatment time on **a** total color difference, **b** pectin methylesterase activity, **c** ascorbic acid content, and **d** total phenolic content

decrease in pectin methylesterase was found for ozone-treated tomato fruit. Similarly, Tiwari et al. (2009a, b) found no significant changes in cloud value of orange juice which implies there is inactivation of PME in the fruit juice sample. The increment of PME activity in unfiltered pummelo fruit juice might be explained by abiotic stress activity as oxidation from ozone concentration increases in the sample. Ozone competes with organic matter (pulp) to inhibit the active enzyme pectin methylesterase reaction of pectin forming calcium pectate complexes (Croak and Corredig 2006). Further proving that ozone-treated filtered pummelo juice for 0–50 min of processing time has little effect in inhibition of enzyme pectin methylesterase, and this finding is in line with the study of Tiwari et al. (2009a, b).

Ascorbic acid degradation reactions are often responsible for important quality changes that occur during the storage of foods, limiting shelf-life. The main factors affecting ascorbic acid in orange juice include temperature, salt and sugar concentration, pH, oxygen, light, metal catalysts and initial ascorbic acid concentration (Al-Zubaidy and Khalil 2007). Ascorbic acid content for ozone-treated pummelo juice was shown to decrease

gradually from 0 to 50 min with values ranging from 77.78 mg/100 mL to 54.76 mg/100 mL for filtered juice and 78.57 mg/100 mL to 51.59 mg/100 mL for unfiltered juice. The maximum losses of ascorbic acid for filtered and unfiltered juices were recorded at 29.60% and 34.33% respectively. The ascorbic acid content in pummelo fruit juice is significantly influenced by both independent variables; type of juice and ozone processing time which gives the p value of 0.0063 and < 0.0001 respectively. Figure 3c illustrates the decreasing trend in ascorbic acid content for both types of juice over ozone-treatment time. These results indicate that ozone exposure or concentration affected the content of ascorbic acid during a period of 50 min. The results of ascorbic acid content is in agreement with the previous study by Allothman et al. (2010) which reported that the longer the ozone exposure time, the more ascorbic acid was oxidized. Tiwari et al. (2008a, b) also found that the ascorbic acid content of freshly squeezed orange juice decreases from 41.59 to 12.70 mg/100 mL after 10 min of ozone treatment. In addition, the unfiltered juice shows higher ascorbic content due to the higher amount of pulp compared to filtered juice. The lack

of pulp presence in the filtered juice could also be the possible cause of lower ascorbic acid content.

The losses of ascorbic acid content in this study might be related to the direct reaction of ozone or indirect reaction of secondary oxidant free radicals (Cullen et al. 2010) on the activity of several enzymes such as ascorbate peroxidase (APX) and ascorbate oxidase (AO) (Yeoh et al. 2014). Ozone is well known to have the strongest oxidation potential (+ 2.07 eV) (Kim et al. 2003), and it may interact with the enzymes ascorbate oxidase (AO). AO may promote the oxidation of ascorbic acid into dehydroascorbic acid (DHA). Then, the instability of DHA is further degraded by ozone and this leads to the reduction of ascorbic acid (Yeoh et al. 2014). The degradation of ascorbic acid during ozone treatment could be due to direct reaction with ozone or indirect reactions of secondary oxidants such as $\cdot\text{OH}$, $\text{HO}^2\cdot$, $\cdot\text{O}_2^-$, and $\cdot\text{O}_3^-$. Such secondary oxidants may lead to electrophilic and nucleophilic reactions occurring with aromatic compounds that are substituted with an electron donor having high electron density on the carbon compounds in ortho- and para-positions. This leads to the oxidative disintegration of ozonide and formation of carbonyl compounds, while oxidative workup leads to carboxylic acids or ketones. Ozone attacks $\cdot\text{OH}$ radicals, preferentially to the double bonds in organic compounds leading to the formation of unstable ozonides which subsequently disintegrate. Another factor that could have contributed to the degradation of ascorbic acid is the activation of ascorbate oxidase. This enzyme is activated under stress conditions, such as chemical exposure. Ascorbate oxidase has been known to promote the degradation of ascorbic acid to dehydroascorbic acid (Alothman et al. 2010).

Ozone concentration also significantly affected the total phenolic content in ozone-treated pummelo juice as the p value for ozone processing time was 0.0004 ($p < 0.05$). The highest TPC was found from ozone-treated pummelo juice for ozone processing time of 10 min for both unfiltered and filtered juice (247.5 mg/L GAE and 224.17 mg/L GAE respectively). The lowest TPC was recorded for processing time of 40 min for unfiltered juice (151.67 mg/L GAE) and for processing time of 50 min for filtered juice (144.17 mg/L GAE). Figure 3d also shows the interaction between the two factors (types of juice and ozone processing time) on TPC of the juice. Decreasing trends of TPC over time was observed for unfiltered juice. This finding is in agreement with the study of Torres et al. (2011) where decreasing of total phenols for ozone-treated apple juice was observed for processing time of 0–10 min. Decrease in PME was due to the strong oxidation potential of ozone (+ 2.07 eV) which influenced the chemical reaction involved in the degradation of the total phenols. These could be direct reactions of ozone with the target

compound or its intermediates and radical reaction between hydroxyl radicals produced through ozone decomposition catalyzed mainly by hydroxide ion, OH^- (Torres et al. 2011; Cullen et al. 2010).

However, TPC for filtered juice increased as processing time increased until 30 min of processing time where TPC started to degrade. The result shows the same pattern found by Alothman et al. (2010), where total phenol and flavonoid contents of pineapple and banana fruits increased significantly when exposed to ozone for up to 20 min. The increase in the phenolic content might have been caused by cell wall modification that occurred during ozone exposure, which might free some of the conjugated phenolic compounds in the cell wall. The auto-decomposition of ozone is accompanied by the production of numerous free radicals. Therefore, the by-products of ozone decomposition were scavenged by the phenolic compounds in the fruits, to different extents, which might have led to the reduction of phenolic contents after 30 min of ozone exposure. Phenolic compounds are particularly susceptible to ozone attack. According to Yeoh et al. (2014), ozone reacts very efficiently on degradation of aromatic compounds of phenolic content. The molecular ozone action on the aromatic compound favors the formation of hydroxylated and quinone compounds because the formation of aliphatic compounds originates from rupture of the aromatic ring.

A possible reason for significant increases of these phenolic compounds can also be attributed to various chemical reactions. These reactions can be direct reactions of ozone with the target compound or its intermediate radicals (produced by the decomposition of ozone catalysed mainly by hydroxide ions). Another reason may be due to the hyper-reactivity of ozone that inhibits several enzymes such as polyphenol oxidase (PPO) and peroxidase (POD) which are responsible for the oxidation of phenolic content (Yeoh et al. 2014). It may also be related to the strong oxidation potential of ozone derived from the nascent oxygen atom (Tiwari et al. 2009a, b).

Shelf life analysis

Based on Fig. 4a, the graphs plotted show that the storage period of 12-weeks affected the total colour difference (TCD) of both unfiltered and filtered ozone-treated pummelo juice. Throughout 12-weeks of storage time TCD values increased for both types of juices. The increasing pattern of TCD values was due to the strong oxidizing activity of ozone which reacted with bioactive compound in fruit juice that was responsible in the visible colour of juice (Rawson et al. 2011). However, for untreated unfiltered juice, a significant decreasing of TCD values was observed during the 12th week of storage period. It may be due to browning of juice which contributed to the increase

Fig. 4 Effect of storage time towards **a** total color difference and **b** pectin methylesterase activity (PMEu/L)

in lightness (L^*) and decreasing of TCD values that results from the action of a group of enzymes called polyphenol oxidase (PPO) or known as enzymatic browning (Cullen et al. 2010).

Graphs plotted in Fig. 4b show the trend of PME activity over 12 weeks of shelf life. Both results show the increment trend of PME activity throughout the 12 weeks for unfiltered as well as filtered juice, as similar to the trend in Fig. 3b. The ascending pattern of PME activity was probably due to the scavenging activity of PME towards ozone free radicals. PME activity was seen to increase from 0.02 to 0.25 PME μ /mL and from 0.05 to 0.11 PME μ /mL for unfiltered and filtered juice, respectively. Hence, it shows that the higher PME activity for unfiltered juice was due to the amount of pectin within the juice. As enzyme PME de-esterifies pectin (Cullen et al. 2010), hence lower pectin content leads to lower enzymatic activity of PME.

Based on Fig. 5a, over the 12 weeks of storage period, it shows that there is significant degradation in ascorbic acid content for both unfiltered and filtered ozone-treated pummelo juice. However, there was no significant variation in content of ascorbic acid between both types of juice. The ascorbic acid content for unfiltered juice was 75.93 mg/100 mL and decreased to 24.87 mg/100 mL

(67% reduction) after 12-weeks of storage. Whereas, ascorbic acid content in filtered juice at first was 78.04 mg/100 mL and it decreased to 23.81 mg/100 mL (70% reduction).

Nevertheless, the amount of ascorbic acid found in this study post-UVC treatment is comparable to the U.S. Recommended Daily Allowance (USRDA) which recommends that any fruit juice to have a minimum of 0.25 mg/mL (Ting et al. 1980). Graph in Fig. 5a shows the minimum recommended level of vitamin C content for both unfiltered and filtered juice. Thus, based on the graphs, samples that were treated with 40 (unfiltered) and 50 min (filtered) ozone-treatment time deteriorated below the minimum level recommended; indicating the end of shelf life of the juice samples.

Figure 5b shows the increasing trend of TPC observed from 0th week to 8th week until degradation of TPC which started to occur from 10th week to 12th week. This trend applies to both unfiltered and filtered pummelo juice. The degradation of TPC was likely due to auto-decomposition of ozone which causes the production of free radicals. This can cause the phenolic compounds to initiate their protective roles by scavenging on free radicals produced by the ozone and as a result the polyphenol content of pummelo juice decreased (Allothman et al. 2010).

Fig. 5 Effect of storage time towards **a** total phenolic content (mg/GAE L) and **b** ascorbic acid content (mg/100 mL)

Conclusion

The effects of ozone on pummelo fruit juice were non-linear and highly dependent on processing conditions and the food matrix. Ozone treatment was found to be effective in reducing total aerobic plate count and yeast and mold in both types of pummelo fruit juice. With ozone concentration of 600 mg/h and different reduction time, t_{5d} were recorded for filtered and unfiltered juice samples. Model parameter also showed that, as ozone processing time increased, the survival of aerobic bacteria, yeast and mold decreased. However, as shown in this study, ozone treatment also affected the physicochemical and antioxidant properties of pummelo juice, where TPC and ascorbic acid content was shown to decrease significantly. In conclusion, ozone treatment at less than 10 min (ozone concentration of 600 mg/h) gave optimum results in term of microbiological (5 log reductions) and physicochemical effects on filtered pummelo fruit juice. Thus, it could be concluded that ozone has the potential to be used as a non-thermal pasteurization to produce a safe and high quality juice. However, extrinsic and intrinsic parameters in this study—ozone processing time, ozone concentration, medium temperature, solid contents and pH should be considered prior to industrial adoption. Achieving microbial inactivation using optimised points may mitigate losses in nutritional quality. Further validation studies are still needed to

determine the critical limits (ozone concentration and time parameters) for effective treatment in terms of bactericidal activity and to retain bioactive compounds of tropical fruit juice products.

Acknowledgements The authors would like to acknowledge the financial support provided by Universiti Putra Malaysia under the IPM Grant No. 9491300.

References

- Allothman M, Kaur B, Fazilah A, Bhat R, Karim AA (2010) Ozone-induced changes of antioxidant capacity of fresh-cut tropical fruits. *Innov Food Sci Emerg Technol* 11(4):666–671
- Al-Zubaidy MMI, Khalil RA (2007) Kinetic and prediction studies of ascorbic acid degradation in normal and concentrate local lemon juice during storage. *Food Chem* 101(1):254–259
- Amador JR (2011) Juice analysis: ascorbic acid by indophenol titration. In: Amador JR (ed) *Laboratory manual: procedures for analysis of citrus products*, 6th edn. John Bean Technology Corporation Inc., Chicago, pp 82–84
- Angelino PD, Golden A, Mount JR (2003) Effect of ozone treatment on quality of orange juice. In: *IFT annual meeting book of abstracts*. Chicago, IL
- Barbosa-Canovas GV, Bermudez-Aguirre D (2011) Why nonthermal technologies. In: Zhang HQ, Barbosa-Cánovas GV, Balasubramaniam VM, Dunne CP, Farkas DF, Yuan JTC (eds) *Nonthermal processing technologies for food*. Wiley, Chichester
- Croak S, Corredig M (2006) The role of pectin in orange juice stabilization: effect of pectin methylesterase and pectinase

- activity on the size of cloud particles. Food Hydrocol 20:961–965
- Cullen PJ, Valdramidis VP, Tiwari BK, Patil S, Bourke P, O'Donnell CP (2010) Ozone processing for food preservation: an overview on fruit juice treatments. Ozone Sci Eng 32(3):166–179
- DOA (2012) Department of Agriculture Malaysia, Pomelo: Varieties/clones. <http://www.doa.gov.my/main.php?Content=sections&SubSectionID=390&SectionID=5&CurLocation=315&IID=>. Accessed 9 June 2012
- FDA (2004) Juice HACCP: hazards and controls guidance, guidance for industry, 1st edn. Division of Plant Product Safety
- Guzel-Seydim ZB, Greene AK, Seydim AC (2004) Use of ozone in food industry. Lebensm Wiss Technol Food Sci Technol 37(4):453–460
- Khadre MA, Yousef AE, Kim J (2001) Microbiological aspects of ozone applications in food: a review. J Food Sci 66(9):1242–1252
- Kim JG, Yousef AE, Khadre MA (2003) Ozone and its current and future application in the food industry. Adv Food Nutr Res 45:167–218
- Kimball D (1999) Citrus processing. A complete guide, 2nd edn. Kluwer Academic, New York
- Kumar D, Antonius H, Lamers H, International B, Ratna B, Bioversity S (2015) Phytochemical evaluation of pummelo fruits (*Citrus grandis*) in India for enhancing marketing opportunities. Indian J Plant Genetic Resour 28:50–54
- Maturin L, Peeler JT (2001) Aerobic plate count. In: Bacteriological analytical manual, 8th edn, pp 1–9. <https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063346.htm>. Accessed 30 Oct 2016
- Moore G, Griffith C, Peters A (2000) Bactericidal properties of ozone and its potential application as a terminal disinfectant. J Food Prot 63(8):1100–1106
- Patil S (2010) Efficacy of ozone and ultrasound for microbial reduction in fruit juice. Doctoral Dissertation. Dublin Institute of Technology. <http://arrow.dit.ie/sciendoc>. Accessed 15 Feb 2017
- Patil S, Bourke P (2012) Ozone processing of fluid foods. In: Novel thermal and non-thermal technologies for fluid foods. Elsevier, pp 225–261
- Patil S, Bourke P, Frias JM, Tiwari BK, Cullen PJ (2009) Inactivation of *Escherichia coli* in orange juice using ozone. Innov Food Sci Emerg Technol 10(4):551–557
- Rawson A, Patras A, Tiwari BK, Noci F, Koutchma T, Brunton N (2011) Effect of thermal and non thermal processing technologies on the bioactive content of exotic fruits and their products: review of recent advances. Food Res Int 44(7):1875–1887
- Restaino L, Frampton EW, Hemphill JB (1995) Efficacy of ozonated water against various food-related microorganisms. Appl Environ Microbiol 61(9):3471–3475
- Rodoni L, Vicente AR (2010) Effect of short-term ozone treatments on tomato (*Solanum lycopersicum* L.) fruit quality and cell wall degradation. J Agric Food Chem 58(December):594–599
- Shah NNAK, Rahman RA, Shamsudin R, Adzahan NM (2015) Effects of pectinase clarification treatment on phenolic compounds of pummelo (*Citrus grandis* L. Osbeck) fruit juice. J Food Sci Technol Mysore 52(8):5057–5065
- Ting SV, Huggart RL, Ismail MA (1980) Color and processing characteristics of Star Ruby grapefruit. Proc Fla State Hortic Soc 93:293–295
- Tiwari BK, Muthukumarappan K (2012) Ozone in fruit and vegetable processing. In: O'Donnell C, Tiwari BK, Cullen PJ, Rice RG (eds) Ozone in food processing, 1st edn. Wiley, Chichester, pp 55–74
- Tiwari BK, Muthukumarappan K, O'Donnell CP, Cullen PJ (2008a) Modelling colour degradation of orange juice by ozone treatment using response surface methodology. J Food Eng 88(4):553–560
- Tiwari BK, Muthukumarappan K, O'Donnell CP, Cullen PJ (2008b) Kinetics of freshly squeezed orange juice quality changes during ozone processing. J Agric Food Chem 56(15):6416–6422
- Tiwari BK, O'Donnell CP, Muthukumarappan K, Cullen PJ (2009a) Anthocyanin and colour degradation in ozone treated blackberry juice. Innov Food Sci Emerg Technol 10(1):70–75
- Tiwari BK, O'Donnell CP, Patras A, Brunton N, Cullen PJ (2009b) Anthocyanins and color degradation in ozonated grape juice. Food Chem Toxicol 47(11):2824–2829
- Torlak E (2014) Efficacy of ozone against *Alicyclobacillus acidoterrestris* spores in apple juice. Int J Food Microbiol 172:1–4
- Torres B, Tiwari BK, Patras A, Wijngaard HH, Brunton N, Cullen PJ, Donnell CPO (2011) Effect of ozone processing on the colour, rheological properties and phenolic content of apple juice. Food Chem 124(3):721–726
- Van Boekel M (2002) On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int J Food Microbiol 74(1):139–159
- Williams RC, Sumner SS, Golden DA (2004) Survival of *Escherichia coli* O157:H7 and *Salmonella* in apple cider and orange juice as affected by ozone and treatment temperature. J Food Prot 67(11):2381–2386
- Yeoh WK, Ali A, Forney CF (2014) Effects of ozone on major antioxidants and microbial populations of fresh-cut papaya. Postharvest Biol Technol 89:56–58