

Total Phenols, Antioxidant Activity and Microbiological Quality of Ozone Sanitized Blackberry (*Rubus* spp. L.)

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Abstract: Regular research conducted has associated the consumption of fresh fruits to beneficial effects on health, but microbiological quality must be ensured. The objective of this study was to verify the effect of ozone as a sanitizing agent and evaluate the impact of treatment on the content of phenolic compounds, antioxidant activity, color and microbiological quality of blackberry (*Rubus* spp. L.) Tupy cultivar. The total coliforms and at 45°C, *Salmonella*, yeasts and molds, content of total phenolic compounds, antioxidant activity, color and pH of the samples were evaluated. The values obtained for total phenols, antioxidant activity and color test were not significantly different ($p > 0.05$) between the control treatment, and those where the ozone was applied for 30s and for 3 min. However, sanitization with a solution of sodium hypochlorite caused a significant reduction ($p \leq 0.05$) for the *hue* angle in relation to the *in natura* sample (control). It was concluded that the treatment in which ozone was used as a sanitizer for 3 min, was the most effective in reducing the microbial load of yeasts and molds, followed by the treatment with sodium hypochlorite solution and ozone for 30 s.

Key words: Antioxidant activity, blackberry, ozone sanitization

INTRODUCTION

In recent decades, research in human nutrition has shown that a balanced diet rich in fruits and vegetables, especially *in natura*, is beneficial to health and may reduce the risk of incidences of various diseases (Bastos, 2006). As a result, the horticultural market is showing changes due to consumer awareness. According to the Apas (2010), sales of fruits and vegetables in the large supermarket chains accounted for 8-12% of total sales in Brazil. The country is one of the three largest producers of fruits, with 41 million tons in 2009, of which 47% were destined for the fresh fruit market (IBGE, 2010).

Among the various options of fruit species with good prospects for cultivation and marketing, blackberry (*Rubus* spp. L.) has shown significant growth area in recent years in Rio Grande do Sul state (main Brazilian producer), and high potential for production in other states with similar climatic characteristics. The low production cost, ease of handling, ruggedness, low use of pesticides and great potential for incorporation into food products are some advantages of this fruit (Antunes, 2002).

The blackberry is a small highly appreciated fruit due to its intense acid-sweet taste and color ranging from dark

red to black, it has a high content of phenolic compounds mainly anthocyanins (Wang *et al.*, 2008; Koca and Karadeniz, 2009; Acosta-Montoya *et al.*, 2010). Among the anthocyanins, the gallic and ellagic acids stand out (Sellappan *et al.*, 2002) and in smaller amounts the epicatechin (Hassimotto *et al.*, 2008) and quercetin (Wang *et al.*, 2008; Hassimotto *et al.*, 2008). Research shows that the blackberry has a high antioxidant capacity (Wang and Jiao, 2000; Halvorsen *et al.*, 2002), showing high correlation with the content of phenolic compounds higher than 0.95% (Sellappan *et al.*, 2002).

According to Antunes (2002) phenolic compounds, especially flavonoids (anthocyanins) have scavenging action of free radicals (antioxidant activity) (García-Alonso *et al.*, 2004), with anti-mutagenic and anticarcinogenic effect on human strains of cervical, colon, oral, breast, prostate and lung cancer (Ding *et al.*, 2006).

Fruits *in natura*, present a higher content of phenolic compounds, however, they can easily be contaminated by a wide range of microorganisms, mainly because they are grown, harvested, transported and handled in open environments. In the U.S., fruits and vegetables are related to disease out breaks caused by enteropathogenic

Escherichia coli (about 20%) (Silva *et al.*, 2003). In addition to diseases caused by *Escherichia coli*, *Salmonella*, *Shigella*, *Listeria*, protozoa, helminths and hepatitis A virus can also be transmitted by these vegetables (Nascimento *et al.*, 2003).

The environment is the first contaminant, because the soil is rich in Gram positive bacteria and fungi, which contaminate food either directly or are transported by wind or insects. The rain carries earth to crops grown near the ground, increasing the microbial load, as well as moisture and favoring fungal growth up to 72% (Brackett, 1999).

Despite the germicidal efficiency of chlorine against bacteria and some viruses, the potential toxicity of the byproducts of chlorination makes the process less attractive. The chlorinated compounds have some disadvantages that limit their use increasingly, both in water treatment and in the food industry, because the chlorination can lead to the formation of organochlorine compounds, trihalomethanes (THMs) and haloacetic acids, which have the mutagenic, toxic and carcinogenic potential in water, food or contact surfaces (Prestes, 2007).

An alternative to the use of chlorine has been the application of ozone. The antimicrobial action of ozone is due to attack by oxidation of glycolipids, glycoproteins and amino acids of the microbial wall, altering cell permeability and causing rapid lysis of cells subjected to this treatment. It also has an effect on sulfhydryl groups of enzymes, causing the collapse of the cellular enzyme activity. It has the action on the nuclear material of microorganisms by changing the purine and pyrimidine bases of nucleic acids, as occurs with some viruses, in addition to changing its polypeptide chains capsid protein (Kim *et al.*, 1999).

The advantage of using this compound, is that ozone breaks down quickly to a nontoxic product (O_2), leaving no residue in food and environment, so it is described as a product that has no deleterious effects in humans under normal conditions of use (Prestes, 2007), allowing the intake of ozonized food without risks to health. In the U.S., ozone is not only approved as a sanitizing agent but is also recognized as a food additive (FDA, 2001). The objective of this study was to assess the effect of ozone as a sanitizing agent and evaluate the influence of their application on the content of phenolic compounds, antioxidant activity and color and microbiological quality of blackberry (*Rubus* spp. L.) Tupy cultivar.

MATERIALS AND METHODS

Raw material: The blackberry (*Rubus* spp. L.) used was Tupy cultivar, grown in the municipality of Morro Redondo-RS-Brazil. The fruits were harvested by the

producer at the commercial maturity stage (black color fruit) during the period of November 2009 to January 2010, and immediately frozen (-18°C in polyethylene packages of low density without prior sanitization). For the experiments, the fruits were thawed at room temperature for a period of 3 h.

Sanitization of fruits: The study was carried out between November 2009 to February 2010 in the Food Quality Control Laboratory of Federal University of Rio Grande, and in the Chromatography Laboratory of Federal University of Pelotas, RS, Brazil. The fruits were divided into four batches of approximately 2 kg, where in one of them sanitation was not performed, another batch was subjected to sanitizing with chlorine, and two other lots were sanitized by using ozone.

Sanitizing with chlorine: The sanitization of the berries was performed by immersing the fruit in a solution of sodium hypochlorite (NaClO) at 200 ppm of active chlorine, in fruit:solution proportion of 1:5 (w/v) for 10 min, pH 6.5; 20°C (Ahvenainen, 1996).

Sanitizing with ozone: The ozonated water was generated from the bubbling of ozone gas in water for 3 min, resulting in a concentration 0.6 mg/L (0.6 ppm), following the procedure described by Chiattoni (2010). Ozone generator (OZ This model, TLS 6A) borrowed from OZ Engenharia Ltda, Brazil was used. Part of the fruits were immersed in ozonated solution for 30 s and another part immersed in ionized solution for 3 min, both at 5°C , fruit:solution proportion of 1:5 (w/v).

Physical-chemical and microbiological: physical-chemical and microbiological analysis of the fruits, *in natura* (control), were carried out on all batches, those not washed with water, those subjected to sanitation in chlorine solution and those sanitized by ozonated solutions.

Physical-chemical analysis: The pH of the samples was determined in potentiometer (Hanna Instruments, HI 221, USA) according to the methods described in the Analytical Standards of Adolfo Lutz Institute (Pregnotatto and Pregnotatto, 1985). The color analysis was measured by employing a colorimeter (Minolta, CR 300, USA). The results were expressed by the coordinates L^* ; a^* and b^* , with subsequent use in calculating the hue angle ($h^\circ = \arctan(b^*/a^*)$). L^* ranges from 0 (completely black) and 100 (completely white) a^* between - 80 (green) and +100 (red) and b^* between -50 (blue) and 70 + (yellow). The determination of total phenolic content was performed using the Folin-Ciocalteu method, according to Swain and Hillis (1959). The absorbance was measured in a

spectrophotometer (Ultrospec, 2000, Pharmacia Biotech, England) at 765 nm. The results were calculated using a standard curve of gallic acid and expressed in mg per 100 g sample. The antioxidant activity was determined against the DPPH radical (2,2-diphenyl-1-picryl-hydrazyl) according to the method described by Brand-Williams *et al.* (1995). The absorbance was measured in a spectrophotometer (Ultrospec, 2000, Pharmacia Biotech, England) at 517 nm after 24 h of reaction. The antioxidant activity was calculated by a standard curve of Trolox and expressed as equivalent antioxidant activity to the Trolox.

Microbiological analysis: All samples were submitted to microbiological analysis for the determination of total coliforms and coliforms at 45°C, detection of *Salmonella* sp. and enumeration of yeasts and molds, according to the methodologies proposed by ICMSF (1983) and Silva *et al.* (2001). For the determination of total coliforms and coliforms at 45°C, 1 mL aliquots of each dilution were inoculated in series of three tubes containing 9 mL of Lauryl Sulfate Tryptose (LST) broth, with inverted Durham tube. The tubes were incubated at 35°C for 24-48 h. From the positive reading of the tubes, confirmatory tests were performed for total coliform in Lactose Brilliant Green Bile Broth at 35°C for 24-48 h and 44.5°C in EC broth for 24 h. In the detection of *Salmonella* sp. lactose broth was used as a means of pre-enrichment and 25 g of sample were homogenized in 225 mL of this diluent. The inoculated medium was incubated at 37°C for 24 h. Next, selective enrichment was performed in selenite cystine broth and tetrathionate. Later, biochemical identifications were made according to the methodology of the ICMSF (1983). For yeast and mold count the surface plating method was used with dilutions of 10^{-1} and 10^{-2} on potato dextrose agar supplemented with 0.2 mg/mL chloramphenicol. Aliquots of 100 μ L were seeded on the surface of the agar and the plates were incubated at 22°C for 3 to 5 days according to ICMSF (1983). Microbiological tests were performed in triplicate.

Statistical analysis: Measurements of color, pH, total phenols and antioxidant activity were performed in triplicate and the results submitted to analysis of variance (ANOVA), followed by mean comparison tests (Tukey) at 5% significance level. Before performing ANOVA it was necessary to check if the data had normal behavior (Kolmogorov-Smirnov) and if the variances were equal (homoscedasticity) to those analyzed by the Levene test (Triola, 1999).

RESULTS AND DISCUSSION

Color, total phenolics and antioxidant activity: Table 1 shows the mean values for color, total phenolic content and antioxidant activity of the samples. Through statistical analysis it is observed that the results were normal by the Kolmogorov-Smirnov test and

homoscedasticity by Levene's test, so it was possible to perform ANOVA. It can be seen in Table 1 that the samples submitted to treatments under which ozone was used for 30 s to 3 min, showed less loss of color, not significantly different ($p>0.05$) among themselves in relation to the control sample. In the sanitization with sodium hypochlorite, the value found for the Hue angle was significantly lower ($p\leq 0.05$) compared to other treatments. This loss may be related to the degradation of pigments such as flavonoids (anthocyanins) constituents of the shell. According to Terci (2004), anthocyanins can be oxidized and bleached in the presence of oxidizing agents such as sodium bisulfite and sodium hypochlorite. Hurst (1995) notes that the use of solutions with higher concentrations of free residual chlorine (50-200 mg/L) causes the discoloration of vegetables. Although Forney *et al.* (2007) relate the use of ozone with the discoloration of the surface of plants exposed to ozone for 2 or 4 days, they found no significant differences in the treatment of carrots in ozonated chamber. Peres *et al.* (1999) studied the effect of ozone in fruits, and found that there is no undesirable effect on the color, Saring *et al.* (1996) also observed no negative effect on the pigmentation of grapes using ozone as a sanitizer. In the study of Kuskoski *et al.* (2005), values were observed for total phenols in mulberry (*Morus nigra*, 118.9 mg/100 g) similar to the content found in this study (127.36 to 134.41 mg/100 g). From the data, it is observed that the control sample and samples treated with ozone were not significantly different ($p\leq 0.05$) on the content of total phenols. However, in the treatment with sodium hypochlorite, there was a higher amount of total phenols (134.41 mg/100 g sample) being statistically ($p\leq 0.05$) higher than others. This increased concentration of phenolic compounds may be associated with weight loss of the fruit (Antunes *et al.*, 2006) that led to concentration of these substances, through loss of fluid from the tissues by solubilization of protopectin, which are responsible for the texture of fruits and vegetables, by the partial hydrolysis caused by high concentrations of chlorine solution (200 ppm active chlorine) used (Regis *et al.*, 2006). Regis *et al.* (2006) submitted strawberries to the sanitation step in different concentrations of chlorine (0, 100, 150 and 200 ppm) at 5°C for 10 min and found that strawberries treated at 200 ppm had greater mass loss in relation to other treatments, due to physiological damage caused by the high concentration of chlorine on the surface of the

Table 1: Color, total phenols content, antioxidant activity of blackberry.

Treatments	pH	Color (h°)*	Total phenols* (mg/100 g)	Antioxidant activity* (μ M/g)
Control	2.9 ^a	178.46 \pm 0.62 ^a	127.36 \pm 1.62 ^a	87.49 \pm 2.97 ^a
Ozone 30 s	3.0 ^a	176.69 \pm 0.81 ^a	127.78 \pm 0.68 ^a	87.69 \pm 0.39 ^a
Ozone 3 min	2.9 ^a	175.75 \pm 1.14 ^a	128.75 \pm 0.16 ^a	88.14 \pm 0.96 ^a
Chlorine	2.9 ^a	172.51 \pm 1.5b ^b	134.41 \pm 0.47 ^b	88.91 \pm 0.60 ^a

*: Values represent the mean \pm SD of three determinations; different letters: in the same column, indicate significant differences ($p<0.05$)

Table 2: Values of *Salmonella* sp., total coliforms, coliforms at 45°C, molds, yeast in sanitized blackberry

	Treatments				
	Control	Chlorine (200 ppm /10 min)	Ozone 30 s	Ozone 3 min	RDC/AN VISA
<i>Salmonella</i> sp. in 25 g	Abs.	Abs.	Abs.	Abs.	Abs.
Total coliforms (MPN/g)	nd	nd	nd	nd	-
Coliforms at 45°C (MPN/g)	nd	nd	nd	nd	5.0×10 ² (a)
Molds (CFU/g)	6.0×10 ²	2.0×10 ²	3.7×10 ²	1.5×10 ²	-
Yeast (CFU/g)	2.2×10 ⁴	4.5×10 ³	5.2×10 ³	3.0×10 ³	-

(a): RDC n° 12 de 02/01/2001/ANVISA; MPN: Most Probable Number; CFU: Colony Forming Units; Abs: Absence; nd: not detected

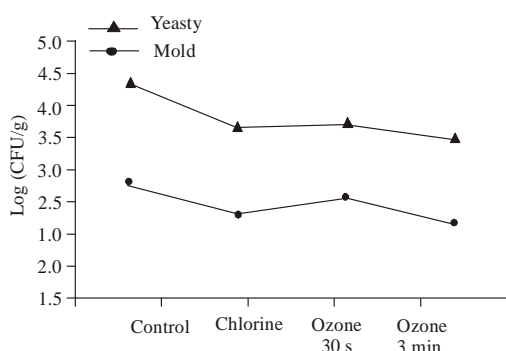


Fig. 1: Count of molds and yeasts in blackberry using different treatments

fruit. It can be observed that the use of chlorine and ozone concentrations in this study did not affect negatively the antioxidant activity of fruit, since there was no significant difference ($p \geq 0.05$) between different treatments, compared to the control. According to Kuskoski *et al.* (2005), the value of antioxidant activity for the frozen blackberry (-15°C) was 82.6 $\mu\text{M/g}$ this being in agreement with the values found in this study (87.49-88.91 $\mu\text{M/g}$).

Microbiological analysis: Table 2 shows the results for the coliform counts and total coliforms at 45°C, molds and yeasts and detection of *Salmonella* sp. obtained from the blackberry samples: control, sanitized with sodium hypochlorite (200 ppm) and sanitized with ozone during 30 s to 3 min. As per the data in Table 1 the pH value found for the control samples and the different treatments was close to 3.0, indicating the acidic character of this fruit. With this, there was no fostering of the multiplication of micro-organisms, especially pathogens thus being in accordance with the standards required by legislation (Brasil, 2001). These conditions lead to the development of spoilage micro-organisms such as molds, yeasts, bacteria and other lactic acid-tolerant micro-organisms (Siqueira and Borges, 1997), which was observed in the results in Table 2. In all samples for the

different treatments, the presence of molds and yeasts was observed, and the values related to the yeasts were higher when compared to those found for molds (Fig. 1). This is due to a faster multiplication of these micro-organisms. All sanitizing agents used in this study were effective in reducing the load of yeasts and molds of the sample, when compared with the control sample. The treatment that used ozone as a sanitizing agent for 3 min was more effective in reducing microbial counts of yeasts and molds, followed by the treatment with sodium hypochlorite solution. The sanitization with ozone for 30 s was less effective among the treatments tested. The sanitization with ozone for 3 min resulted in a reduction of 0.6 and 0.87 logarithmic cycles of the population of yeasts and molds respectively over the control sample. Smilanick *et al.* (1999) employing a contact time of 2 min and ozone concentration equivalent to 1.5 mg/L achieved a reduction of fungal flora from 95 to 100%. In the use of ozone for 3 min there was no survival of micro-organism. These same researchers sanitizing strawberries in ozone immersion at 4 mg/L for 2 min achieved a reduction of 91% of the population of yeasts and molds. However the immersion of grapes in 10 mg/L of O_3 for 1-4 min reduced the population of fungi in only 50% (Smilanick *et al.*, 1999). Farooq and Akhlaque (1983) observed that the yeast population decreased by 2 logarithmic cycles when exposed to 0.26 ppm ozone for 1.7 min. Gimenez (2003) found that it would be more efficient sanitizing melons for 3 min, compared to a time of 5 min, since a longer exposure allowed the ozone to react with other tissue components of the fruit, decreasing its bacteriocinogenic action, suggesting working at higher concentrations of 6.5 mg/L of O_3 with times less than 3 min.

CONCLUSION

From this study it can be concluded that the application of ozone as a sanitizing agent, under the conditions tested for the blackberry (*Rubus* spp. L.) of the Tupy cultivar did not result in significant differences ($p < 0.05$) for color, total phenol content and antioxidant activity. The treatment that used ozone as a sanitizing agent for 3 min was more effective in reducing microbial counts of yeasts and molds, followed by the sanitization treatment with sodium hypochlorite and ozone for 30 s. To check the efficiency of ozone as a sanitizing agent for fungi and yeasts, it is suggested for future studies testing different concentrations from 0.6 mg/L and fixing the time at 3 min.

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