JULIANE LAUREANO

APPLICATION OF OZONE IN THE MICROVINIFICATION IN REPLACEMENT OF SULPHITE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Engenharia Agrícola, para obtenção do título de *Doctor Scientiae.*

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APROVADA: 22 de setembro de 2015.

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(Orientadora)

“La più grande soddisfazione nella vita è fare quello
che la gente crede che tu non sia in grado di fare”

“Il successo non è definitivo, il fallimento non è fatale:
ciò che conta è il coraggio di andare avanti.”
(Winston Churchill)

Dedico

Ao amigo que me trouxe aqui e a quem eu não salvei a vida...
Marcelo Pereira Coelho

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BIOGRAFIA

JULIANE LAUREANO, filha de Geraldo Eloi Laureano e Sirlene de Fátima Macedo Laureano, nasceu em 23 de abril de 1985, na cidade de Viçosa – MG.

Estudou grande parte do ensino fundamental na E.E. Cônego José Ermelindo de Souza, em Araponga - MG, concluindo-o na E.M. Ministro Edmundo Lins, em Viçosa - MG, no ano de 1999.

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LAUREANO, Juliane, D.Sc., Universidade Federal de Viçosa, setembro de 2015. **Application of ozone in the microvinification in replacement of sulphite.** Orientador: Lêda Rita D'Antonino Faroni. Coorientadores: Paulo Henrique Alves da Silva, Maria Cristina Dantas Vanetti e Paulo Roberto Cecon.

O dióxido de enxofre (SO_2) é o aditivo mais frequentemente utilizado para evitar a deterioração de vinhos. No entanto, ele pode causar alterações sensoriais no produto final e apresentar riscos à saúde humana. O ozônio, por sua vez, tem sido proposto em diferentes fases da produção de vinho, porém ainda não diretamente no mosto para o controle de micro-organismos. O objetivo desta pesquisa foi investigar a viabilidade do ozônio como substituto ao SO_2 em algumas etapas da linha de produção de vinhos. Para isso, os trabalhos foram realizados em duas etapas. Na primeira etapa, analisou-se o efeito do ozônio (0 a 7,5 $mg.L^{-1}$, durante 10 min) sobre micro-organismos de importância enológica (*Saccharomyces cerevisiae, Lactobacillus casei, Leuconostoc mesenteroides, Pediococcus acidilactici* e *Gluconobacter oxydans*), *in vitro,* em comparação ao tratamento com SO_2. O ozônio foi mais eficaz que o SO_2 na redução das populações de bactérias lácticas (BAL), permitindo a permanência das leveduras, mesmo na presença dos nutrientes da uva e nas condições de vinificação. O ozônio causou também a redução das bactérias acéticas (BAC), porém, a produção de biofilme pode ter afetado os resultados. Na segunda etapa dos trabalhos foram produzidos vinhos com uvas var. Syrah, pelo processo de microvinificação. O ozônio foi aplicado em diferentes concentrações (2 a 12 $mg.L^{-1}$) e tempos de tratamento (5 a 25 min), nos dias 1º, 6º e 16º da fermentação alcoólica. Foi realizado um tratamento testemunha, empregando-se metabissulfito de potássio, e um controle, sem adição de ozônio ou SO_2. Leveduras, BAL e BAC foram quantificadas logo após os tratamentos. As análises de antocianinas, parâmetros de cor, concentração de polifenóis totais, atividade antioxidante e compostos voláteis foram realizadas após o fim da fermentação alcoólica. Leveduras, BAL e BAC não foram afetadas pelos tratamentos com ozônio, assim como as antocianinas totais e poliméricas, densidade de cor, cor decorrente das antocianinas poliméricas, características cromáticas, polifenóis totais e atividade antioxidante. Os vinhos produzidos com maiores concentrações de ozônio adquiriram características de vinho envelhecido, apresentando diminuição nas antocianinas monoméricas, aumento no percentual da cor decorrente das antocianinas poliméricas e aumento na tonalidade amarelo/amarronzada, o que pode contribuir para reduzir o tempo necessário de envelhecimento para que o vinho atinja a coloração desejada. Entre os compostos voláteis analisados, o acetaldeído atingiu o limite de detecção sensorial em todos os tratamentos. Em alguns vinhos tratados com ozônio por 15 e 25 min, o metanol estava presente em quantidade acima do permitido pela legislação Brasileira. Estes compostos voláteis são importantes para a qualidade sensorial dos vinhos e tais alterações devem ser levadas em consideração antes de se empregar o ozônio como um substituto do sulfito na produção de vinho.

ABSTRACT

LAUREANO, Juliane, D.Sc., Universidade Federal de Viçosa, September, 2015. **Application of ozone in the microvinification in replacement of sulphite.** Adviser: Lêda Rita D'Antonino Faroni. Co-advisers: Paulo Henrique Alves da Silva, Maria Cristina Dantas Vanetti and Paulo Roberto Cecon.

Sulfur dioxide (SO_2) is the additive that is most frequently used to avoid wine spoilage. However, it can cause sensorial alterations in the final product and presents risks to human health. Ozone has been proposed at different stages of wine production. However, it has not yet been used directly in the must to control microorganism growth. Therefore, the purpose of this research was to investigate the viability of ozone as substitute for SO_2 in some stages of wine production. For this, experiments were carried out in two parts. First, the ozone effect (0 to 7.5 $mg.L^{-1}$ for 10 min) on microorganisms with enological importance *(Saccharomyces cerevisiae, Lactobacillus casei, Leuconostoc mesenteroides, Pediococcus acidilactici,* and *Gluconobacter oxydans) in vitro* in comparison with SO_2 treatment was analyzed. Ozone was more effective in reducing the population of lactic bacteria (LAB) compared with the SO_2 treatment, even in the presence of grape nutrients and under winemaking conditions. Ozone also caused a reduction in acetic bacteria (AAB). However, biofilm production may have affected the results. In the second part, wines were produced with Syrah grapes by microvinification. Ozone was applied at different concentrations (2 – 12 $mg.L^{-1}$) and treatment times (5 – 25 min) at days 1, 6 and 16 of fermentation. A standard treatment was performed employing potassium metabisulfite. The control was performed without O_3 or SO_2. The yeasts, LAB and AAB were quantified after the treatments. The analyses of anthocyanins, color parameters, phenolic content, antioxidant activity and volatile compounds were performed after the end of alcoholic fermentation. The yeasts, LAB and AAB were not affected by the treatments with ozone, nor were the total and polymeric anthocyanins, color density, polymeric color, chromatic characteristics, polyphenol content and antioxidant activity. Wines produced with higher ozone concentrations acquired aging characteristics, presented decreased monomeric anthocyanins, increased percentage of polymeric color and increased yellow/brown shades and tonality. This suggests that the use of ozone as a substitute of sulfite may confer aging characteristics to wine in terms of color and may contribute to reducing the time needed for maturation. Of the analyzed volatile compounds, acetaldehyde reached the sensorial detection limit in all the treatments. In some wines treated with ozone for 15 and 25 min, methanol was present in quantities above those permitted by Brazilian law. These volatile compounds are important for the sensory quality and safety of wines. Such changes should be taken into account before using ozone as a substitute for sulfite in wine production.

INTRODUÇÃO GERAL

O vinho é uma bebida alcoólica amplamente consumida em todo o mundo e de grande valor comercial, e o seu consumo moderado é cada vez mais recomendado devido às suas propriedades funcionais (Garaguso e Nardini, 2015). É definido pelo *Office International de la Vigne et du Vin* como a bebida resultante da fermentação alcoólica total ou parcial de uvas frescas, esmagadas ou não, ou de mostos de uvas (OIV, 2015).

Na transformação do mosto em vinho ocorrem complexos processos bioquímicos, envolvendo interações entre leveduras, bactérias láticas e acéticas (Andorrà et al., 2010). As leveduras, essenciais à produção do vinho, são responsáveis pela conversão do açúcar em álcool por meio da fermentação alcoólica (Manfroi, 2002). As bactérias láticas participam da fermentação malolática do vinho, mas podem também causar danos sensoriais ao mesmo (Montersino et al., 2008). As bactérias acéticas, por sua vez, são capazes de contaminar uvas, mostos e vinhos, oxidando o etanol em ácido acético (Barata et al., 2012). Estas últimas são aeróbias estritas e, portanto, necessitam de oxigênio para realizarem a fermentação acética. Multiplicam-se em maior quantidade na parte superior do vinho, formando um “véu” conhecido como "mãe do vinagre". O crescimento excessivo de bactérias acéticas pode provocar modificações na composição do mosto, alterando o crescimento das leveduras durante a fermentação alcoólica e causando alterações sensoriais no produto final (Flanzy, 2003).

Para inibir as bactérias indesejáveis ao processo fermentativo, a prática tradicionalmente empregada nos estabelecimentos vinícolas é a sulfitação com metabissulfito de potássio ($K_2S_2O_5$), o qual libera o gás sulfuroso (SO_2) quando dissolvido em água. Esse gás possui ação seletiva sobre as leveduras, funciona como antioxidante e auxilia na extração do pigmento das cascas (Rizzon, 1996). Em geral, uma concentração de SO_2 molecular superior a 0,8 $mg.L^{-1}$ é suficiente para prevenir a multiplicação de bactérias no vinho (Zoecklein, 2001). No entanto, essa recomendação é frequentemente negligenciada, uma vez que os sulfitos e seus derivados são adicionados em excesso para garantir a completa e acelerada remoção de oxigênio e com isso, evitar a oxidação de alguns compostos do vinho (Araújo, 2006). Tal prática pode oferecer riscos à saúde dos consumidores.

Na tentativa de conciliar a adição de sulfito no processo de produção de vinho com o consumo seguro dessa bebida, órgãos nacionais e internacionais estabeleceram limites máximos de sulfito considerados adequados. A este respeito, a Resolução RDC nº 34 da ANVISA (Agência Nacional de Vigilância Sanitária), de 9 de março de 2001, estabelece que o limite máximo permitido de sulfito total em vinhos (expresso em SO_2) é 350 $mg.L^{-1}$. Já a Organização Mundial de Saúde (OMS) recomenda um consumo máximo diário de SO_2 equivalente a 0,7 mg kg^{-1} de peso corpóreo (Machado et al., 2009). Assim, uma pessoa de 70 kg terá um limite diário de 49 mg de ingestão de anidrido sulfuroso. No entanto, ao ingerir meia garrafa de vinho com 150 $mg.L^{-1}$, poderá estar ingerindo 56 mg de SO_2, o que está acima do limite recomendado pela OMS.

Hoje há uma tendência de restrição ao uso de sulfitos como aditivos, tanto em vinho como em outros produtos alimentícios, devido à sua ingestão estar associada a inúmeros efeitos adversos à saúde dos consumidores (Machado e Toledo, 2006; García-Ruiz et al., 2011, Artés-Hernández et al., 2007). Além disso, os sulfitos apresentam alta corrosividade sobre metais (Cayuela et al., 2009) e em altas doses podem causar alterações sensoriais no produto final (Guzzon et al., 2013). Tais desvantagens impelem pesquisadores à busca por métodos alternativos para a conservação do vinho.

O ozônio tem sido empregado como um importante agente para garantir a segurança microbiológica de alimentos (Palou et al., 2001; Tiwari et al., 2009b; Patil et al., 2011; Cayuela et al., 2009; Kim et al., 1999). Ele se destaca por sua alta reatividade, difusividade e decomposição espontânea em produtos não tóxicos (gás oxigênio - O_2) (Pirani, 2011). Aliado a isto, o ozônio pode ser gerado no próprio local de aplicação, podendo ser usado de forma contínua ou intermitente, aplicado sob a forma gasosa ou dissolvido na água (Simões, 2012; Palou et al., 2003; Mohammad et al., 2009). A aplicação do ozônio tem sido proposta em diferentes estágios da fabricação de vinho, na sanitização de equipamentos, utensílios, barris e garrafas (Pascual et al., 2007). Porém, não são encontrados estudos detalhados sobre a efetividade do ozônio contra os micro-organismos, quando aplicado diretamente ao vinho, suas possíveis modificações na qualidade da bebida e os riscos associados ao seu uso (Guzzon, 2013).

Cada micro-organismo possui sensibilidade intrínseca em relação à ozonização e sua suscetibilidade varia de acordo com o pH do meio, temperatura, umidade e presença de ácidos, surfactantes e açúcares (Kim et al., 1999). Além disso, se utilizado em doses inadequadas, o ozônio pode oxidar compostos orgânicos, resultando em descoloração e deterioração de componentes de sabor dos produtos (Kim et al., 1999). A este respeito foi relatada a degradação de antocianinas e cor em sucos de uva, morango e amora preta por Tiwari et al. (2009a, 2009b, 2009c).

Nesse contexto, se fazem importantes os estudos para avaliar a viabilidade do uso do ozônio diretamente na produção de vinho, como substituto ao $SO_{2,}$ no controle de micro-organismos e seus possíveis efeitos sobre a qualidade do produto final. O objetivo deste trabalho foi avaliar o uso do ozônio gasoso em substituição ao sulfito em algumas etapas da linha de produção de vinho.

Esse trabalho se divide em quatro capítulos. O primeiro trata-se do efeito do ozônio sobre a viabilidade de leveduras, bactérias lácticas e acéticas, *in vitro*, em condições de vinificação. O segundo capítulo se refere ao efeito do ozônio sobre esses mesmos micro-organismos, quando aplicado em diferentes etapas da fermentação alcoólica, além do seu efeito sobre as antocianinas e características de cor do vinho produzido. No terceiro capítulo foi analisado o efeito do ozônio sobre polifenóis totais, atividade oxidante e compostos voláteis do vinho. O quarto capítulo trata do efeito do ozônio e da água eletrolisada sobre a textura de uvas de mesa e viníferas, em um trabalho desenvolvido na Universidade de Turim, Itália.

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ARTIGO 1

EFFECT OF OZONATION ON YEAST, LACTIC ACID BACTERIA AND ACETIC ACID BACTERIA VIABILITY, *IN VITRO,* UNDER WINEMAKING CONDITIONS

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Significance and impact of the study: The effect of ozone applied directly to must on the control of microorganisms that affect winemaking was investigated for the first time. The results support the efficacy of ozone in reducing microorganisms even in the presence of grape components, and we compare the results to the use of sulfite. The study provides important information for developing safer and more environmentally friendly alternatives to replace the use of sulfite in wine production.

Abstract

The purpose of this research was to investigate the effect of ozone *in vitro* on yeast, lactic bacteria and acetic bacteria viability, under winemaking conditions. The microorganisms were inoculated in sterile grape juice (pH 3.5 and 20 ºBrix), and the juice was subjected to ozonation at 0 to 7.5 mg L^{-1}, for 10 min. A control treatment was conducted with SO_2 (potassium metabisulfite 80 mg L^{-1}). Cell quantification was performed before and after treatments. Ozone was more effective in reducing the population of lactic acid bacteria compared to SO_2 treatment, even in the presence of grape nutrients under winemaking conditions. The higher ozone concentration (7.5 mg L^{-1}) was the most efficient in reducing lactic acid bacteria; the bacteria were reduced by more than four log cycles. All of the ozone concentrations tested allowed the maintenance of the fermenting yeast. Ozone caused a reduction of acetic bacteria; however, the production of a biofilm may have affected the results.

Keywords: ozone, lactic bacteria, spoilage bacteria, sulfite, wine

1. Introduction

Winemaking can be understood as a complex biochemical process involving interactions between yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) in grape juice (Pérez-Martín et al. 2015; Andorrà et al. 2010). Yeasts are most important because of their role in alcoholic fermentation (Bartowsky 2009).

Only a few genera of LAB commonly grow in grape must and wine, mainly *Oenococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. (Barata et al. 2012; König and Fröhlich 2009). LAB have great relevance to enology due to their participation in malolactic fermentation (Lonvaud-Funel 1999). When well controlled, LAB reduce wine acidity and contribute to aroma and flavor (Kántor et al. 2014). *Oenococcus oeni* is the most desirable species for malolactic fermentation development, and no important sensorial defect has been attributed to it (Lonvaud-Funel 1999). However, some LAB from other genera may be responsible for many wine spoilage problems that compromise quality and value (Kántor et al. 2014).

AAB are considered spoilage bacteria in wines (Bartowsky 2009), but their population is typically low on healthy grapes (10^2–10^3 colony forming units per gram (CFU g^{-1})). In grapes with gray rot, AAB populations can reach up to 10^5-10^6 CFU g^{-1}, comprising mainly *Acetobacter* spp. and *Gluconobacter* spp. (Barata et al. 2012; Kántor et al. 2015), and *Gluconobacter oxydans* is the most representative species (Bartowsky and Henschke 2008). AAB are able to spoil the wine by ethanol acidification, and their growth on grapes and must could modify the must composition and, by consequence, the ecological equilibrium of yeast and LAB growth during alcoholic and malolactic fermentation (González-Arenzana et al. 2015). Wines contaminated by AAB have characteristic volatility, sourness like vinegar on the palate, a range of acetic, nutty, sherry-like, solvent or bruised apple aromas and frequently reduced fruity characteristics, which have negative effects on wine quality (Bartowsky et al. 2003; Andorrà et al. 2008).

Sulfur dioxide (SO_2) is the additive most frequently used to control microorganisms during winemaking. However, currently, there is a tendency to restrict the use of SO_2 in wine processing due the risks to human health and because high doses can cause sensorial alterations in the final product (Artés-Hernández et al. 2006, 2007; García-Ruiz et al. 2011; Guzzon et al. 2013). This restriction has an important impact on the microbial stability of wines (Guzzon et al. 2013), motivating the development of alternative methods for microbial control.

Ozone is a powerful antimicrobial agent, and it has been approved by the FDA as a direct additive to food since 2001 (Öztekin et al. 2006; Patil et al. 2011; Segat et al. 2014; Feliziani et al. 2014). Ozone has a non-specific action and has been shown to be active against all microbiological forms: fungi, bacteria, virus and spores (Khadre et al. 2001; Mylona et al. 2014; Petry et al. 2014; Torlak 2014; Dev Kumar et al. 2016). Its high reactivity ensures the complete disappearance of its residues in the environment after just a few minutes of treatment (Khadre et al. 2001; Botondi et al. 2015).

Ozonation has been proposed at different stages of wine production to reduce the spoilage microbiota in grapes and to provide sanitation of barrels, tanks, hoses and bottles (Coggan 2003; Hester 2006; Guillen et al. 2010; Guzzon et al. 2013), but no information is available about the use of ozone directly in the must. Moreover, relatively little information is available about its effects on microorganisms when applied in the presence of grape nutrients. It is known that organic substances present in the food matrix can compete for ozone and provide protective effects to microorganisms (Guzel-Seydim et al. 2004; Joshi et al. 2013).

The purpose of this research was to investigate the *in vitro* the effect of ozonation on yeast, LAB and AAB viability in the presence of grape nutrients and under conditions usually found in the early stages of wine production.

2. Results and discussion

After 10 min of ozone treatment, the initial number of viable LAB decreased as ozone concentration increased. The mean bacterial counts are shown in Figure 1. *Lactobacillus casei* had initial counts ranging from 5.5 to 5.8 log CFU mL^{-1}, and reductions in bacterial counts of 2.36, 3.83 and 4.04 log cycles were observed after treatments with 2.5, 5 and 7.5 mg L^{-1} of ozone, respectively. The reductions were significantly greater, according to Dunnet's test ($P < 0.05$), than those obtained using SO_2. Although bacteria from the genus *Lactobacillus* participate in malolactic fermentation, inhibiting their growth can improve the quality of wine because they convert glycerol into 3-hydroxypropionaldehyde (3-HPA) during anaerobic fermentation. The accumulation of 3-HPA can affect wine quality due to its spontaneous conversion to acrolein under winemaking conditions. This substance is not only dangerous for living cells; it is also related to the development of unpleasant bitterness when combined with polyphenols in wine (Bauer et al. 2010).

Pediococcus acidilactici was reduced by 1.3, 3.6 and 4.6 log cycles at ozone concentrations of 2.5, 5 and 7.5 mg L^{-1}, respectively, and these were reductions greater ($P < 0.05$) than those that occurred upon treatment with SO_2. *P. acidilactici* is another lactic acid bacterium that performs malolactic fermentation and produces exopolysaccharide (EPS) (Bartowsky 2009). Wines with high EPS have elevated viscosity, which is sometimes observed during vinification or after bottling. Such wines cannot be sold due to their abnormal viscosity, which represents a serious problem for wine producers and merchants, resulting in considerable economical loss (Montersino et al. 2008).

Leuconostoc mesenteroides had its cell counts reduced to 4 and 4.6 log CFU mL^{-1} by treatment with 5 and 7.5 mg L^{-1} of ozone, respectively, which differed from the results of SO_2 treatment ($P < 0.05$). Montersino et al. (2008) found that *L. mesenteroides* strains isolated from grape juice or wine are also EPS producers, and they are related to increased wine viscosity that develops inside the bottle. The higher effectiveness of ozone in reducing EPS-producing microorganisms demonstrates its potential as a sulfite substitute that avoids the viscosity problem in wines.

Treatment with 0 mg L^{-1} of ozone (only O_2) showed a slight reduction in LAB (Figure 1a,c). This can be explained by the low affinity of these microorganisms to aerobic conditions (Guzzon et al. 2013).

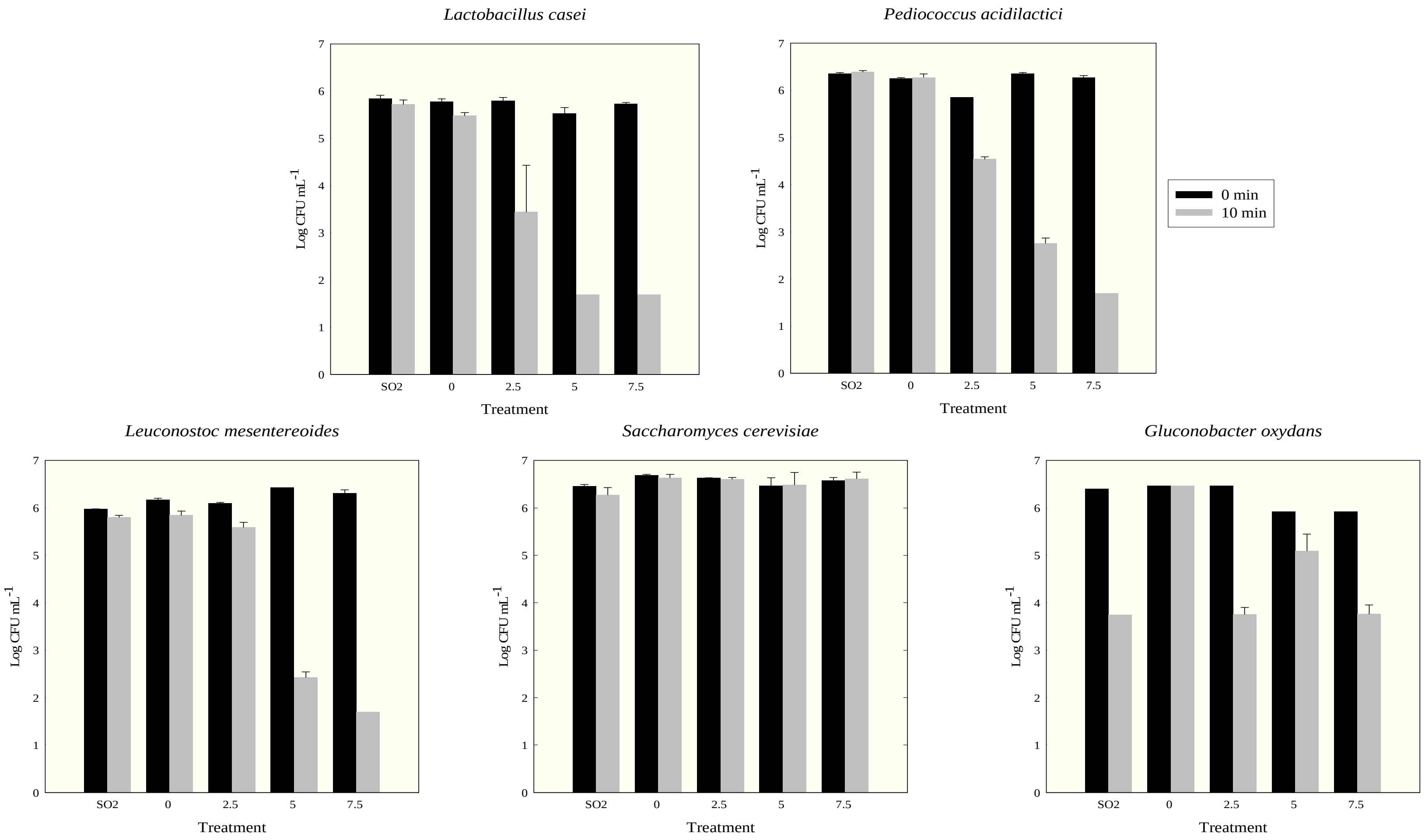

Figure 1. Mean values counts in log CFU mL^{-1} and mean standard error, after 0 e 10 min of ozonation. The treatments 0, 2.5, 5 and 7.5 refers to ozone concentration, in mg L^{-1}.

Practically no reduction of *S. cerevisiae* viability was observed under any ozone treatment performed for 10 min. Pascual et al. (2007) found that yeasts are more resistant to ozone than bacteria. However, Guzzon et al. (2013) found that 7 mg L^{-1} of ozone completely inactivated 10^6 CFU mL^{-1} of *S. cerevisiae* in distilled water when applied for 30 min. This shows that increasing the time of exposure to ozone may strongly inhibit microorganisms, and it suggests that ozone may be more effective in the absence of organic compounds. Considering the importance of *S. cerevisiae* to winemaking, the yeast resistance to ozone concentration that is enough to eliminate undesirable bacteria is an important result. *S. cerevisiae* is responsible for the conversion of sugar to alcohol (González-Arenzana et al. 2015), and bacteria that grow during alcoholic fermentation can compete for nutrients or produce secondary metabolites, restricting the growth of fermentative yeasts (Fleet 2003). Therefore, the inhibitory effect of ozone on bacteria promotes the predominance of yeasts during the initial step of winemaking. This favors yeast fermentation.

G. oxydans had its viability reduced by ozone at 2.5 and 7.5 mg L^{-1} in the same proportions as caused by SO_2 (between 2.2 and 2.7 log cycles). There was no significant difference between the reductions ($P > 0.05$). Treatment with 0 mg L^{-1} of ozone (only O_2) did not affect the viability of *G. oxydans* because the species is strictly aerobic (Bartowsky and Henschke 2008). Treatment with 5 mg L^{-1} of ozone caused lower reduction in *G. oxydans* viability (in order to 0.8 cycles log) than treatment with SO_2 ($P < 0.05$). The response of this AAB to ozone was also different from that shown by LAB. *G. oxydans* is a biofilm producer, as evidenced by a white ring that formed in the neck of the bottle at the interface of the wine and the headspace of air (Bartowsky and Henschke 2008). Guzzon et al. (2013) found that there is a correlation between ozone resistance and biofilm activity. Once formed, the exocellular matrix that characterizes the biofilm can promote the resistance of the cells to ozone. Tristezza et al. (2010) stated that biofilms may cause an increase in tolerance to biocides and antibiotics (100-fold greater resistance) compared to the same microorganisms in a free-floating state. AAB species are catalase-positive, and the presence of this enzyme may allow cells to detoxify themselves and become more resistant to oxidative stress (Serrazanetti et al. 2009). This would allow them to resist the effects of reactive oxygen species formed during ozone treatments (Tiwari et al. 2009).

Ozone acts initially on the cell membrane, and its antimicrobial action is due to oxidation of glycolipids, amino acids and glycoproteins in the cell wall, which changes the membrane permeability and causes rapid lysis. Ozone also attacks sulfhydryl groups in enzymes, disabling certain cellular enzyme activities. Moreover, its action on the nuclear material of microorganisms alters the purine and pyrimidine bases of nucleic acids (Silva et al. 2011). In addition to the molecular ozone action, the antimicrobial activity may also be to due oxidative stress initiated by the intermediate radicals $^{\bullet}OH$, $^{\bullet}O_2^-$, $HO_2^{\bullet}$ and $^{\bullet}O_3^-$ that result from ozone decomposition (Serrazanetti et al. 2009; Tiwari et al. 2009).

The effect of ozone on microbial cells can change depending on the substrate that the cells are living on. Guzzon et al. (2013) verified that ozone activity is reduced in the presence of organic matter. Guzel-Seydim et al. (2004) evaluated the efficacy of ozone in reducing bacterial populations in the presence of food components and hypothesized that ozone more readily oxidizes the substrate components than the bacterial cells. In our study, we observed that ozone was effective in killing bacteria, allowing the maintenance of yeast viability, even in the presence of organic compounds from grapes.

Regression equations were adjusted for cells counts as a function of ozone concentrations to analyze the differences between ozone treatments (Table 1). The adjusted models showed a significant linear effect of ozone on *L. mesenteroides* and *P. acidilactici* viability ($P < 0.10$ and $P < 0.05$, respectively), i.e., the populations decreased linearly with increasing ozone concentrations. The effect of ozone on *L. casei* had a quadratic adjustment, reaching a minimum point at 5.41 mg L^{-1} of ozone. The reduction of *G. oxydans* populations with increasing ozone concentration had a square root effect, with the minimum at 5.29 mg L^{-1} of O_3. The effect of ozone on *S. cerevisiae* survival was not significant and the estimative of cells was equal the overall mean for any ozone concentration. The respective curves that estimate the number of viable cells after treatment are shown in Figure 2.

Table 1. Regression equation for cells count (y, CFU mL^{-1}) vs. ozone concentration (x, mg L^{-1})

Microorganism	Equation	R^2
L. casei	$\hat{y} = 5.6015 - 1.3153^{\square}x + 0.1216^{\circ}x^2$	0.961
L. mesenteroides	$\hat{y} = 6.2275 - 0.6238^{*}x$	0.891
P. acidilactici	$\hat{y} = 6.1471 - 0.6208^{**}x$	0.988
S. cerevisiae	$\hat{y} = 6.5859$	-
G. oxydans	$\hat{y} = 6.3889 - 1.9688^{\square}\sqrt{x} + 0.4281^{\Delta}x$	0.677

* Significative to 5%; ** significative to 1%; $^{\square}$ significative to 10%; $^{\circ}$ significative to 15%; $^{\Delta}$significative to 20%

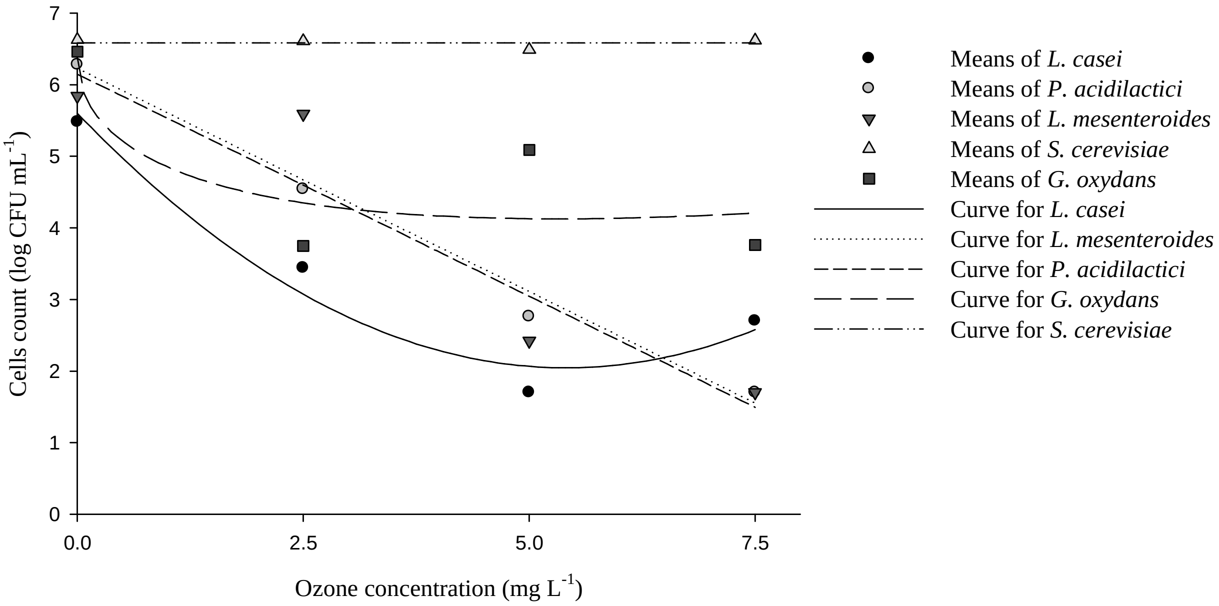

Figure 2. Curves to estimative of cells (CFU mL^{-1}) vs. ozone concentration (mg L^{-1})

In general, the results confirmed that gram-positive bacteria are more sensitive to ozone than yeasts, as previously verified by Pascual et al. (2007). Ozone as a biocide has an advantageous mechanism action, which destroys the microorganism through cell lysis and does not lead to microorganism resistance (Pascual et al. 2007).

In conclusion, ozone was more effective in reducing the populations of LAB compare to SO_2 treatment, even in the presence of grape nutrients under winemaking conditions. The highest ozone concentration tested (7.5 mg L^{-1}) was the most effective, reducing the population by more than four log cycles while still allowing survival of fermenting yeast. Although the results of ozone effectiveness on AAB have shown a reduction in microbial cell number, the production of a biofilm by *G. oxydans* may have affected the results. More studies are needed to find ways to eliminate the interference of biofilms when ozone is used to control acetic acid bacteria.

3. Materials and methods

3.1. Preparation of grape juice

Fresh Niagara Rosada grapes (*Vitis labrusca* L. x *Vitis vinifera* L.), cultivated in Southeast Minas Gerais, Brazil, were split from bunches and crushed using a domestic juice extractor (Arno, São Paulo, Brazil). Juice was filtered on a cotton cloth to remove skin, seeds and pulp. The total soluble solids content was adjusted to 18 ºBrix with granulated sugar cane, and the must was frozen at -18 ºC until processing. Approximately 500 mL of frozen juice were thawed for 4 hours at 20 ºC, centrifuged at 15300 × *g* in a Beckman J2-MI Centrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA) for 10 min and autoclaved (121 ºC, 1.5 kgf for 15 min). Sterilized juice was maintained at 8 ± 2 ºC until use. The final grape juice pH was 3.5.

3.2. Preparation of inoculum

The microorganisms were obtained from the Tropical Foundation André Tosello (Campinas, SP, Brazil), Bioagro collection (BIOAGRO, Department of Microbiology, Federal University of Viçosa, MG, Brazil) and the Indupropil Commerce (Ijuí, RS, Brazil) (Table 2). Microorganisms were cultured in growth media according to the requirements of different species, as described in Table 2.

Cells were centrifuged in a Beckman J2-MI Centrifuge at 3020 × *g*, for 10 min at 10 ºC. The pellet was suspended in sterile peptone saline solution (0.1% peptone, 0.85% NaCl) and centrifuged twice. After two washes, the pellet was re-suspended in sterile peptone saline solution, and cell density was determined by measuring absorbance at 600 nm in a BioMate5 spectrophotometer (THERMO Scientific, Woburn, MA, USA), using a calibration curve that was previously prepared, as described by Begot et al. (1996). Then, the cell suspension was diluted to approximately 10^8 CFU mL^{-1}.

Table 2. List of microorganisms studied and culture conditions for each one

Microorganism	ATCC	Cultured conditions
Lactobacillus casei	1465*	MRS (Himedia); 37 °C - Microaerophilia (5% CO_2)
Leuconostoc mesenteroides	10830	MRS (Himedia); 28 °C - Microaerophilia (5% CO_2)
Pediococcus acidilactici	8042	MRS (Himedia); 30 °C - Microaerophilia (5% CO_2)
Gluconobacter oxydans	23774	Manitol Agar (Himedia); 28 °C
Saccharomyces cerevisiae	NA**	PDA (Himedia); 30 ºC

*CCT; **Not available – commercial product (Refer. Blastosel FR 95)

3.3. Ozone treatment

Experiments were carried out in a 500 mL tube with a gas diffuser (Figure 3). Ozone gas was generated using an ozone generator (O&LM, Ozone and Life, São José dos Camps, SP, Brazil), by corona discharge. Pure oxygen was supplied by an oxygen cylinder (White Martins Ltda, Juiz de Fora, MG, Brazil), and the flow rate of 0.35 $L.min^{-1}$ was controlled using an oxygen flow regulator. Grape juice samples (200 mL) inoculated with each microorganism (10^6 CFU mL^{-1}) were ozonated. For each replication of the experiment, the same amount of inoculum was added to a constant volume of each substrate. Ozone concentrations of 0 – 7.5 mg L^{-1} were applied for 10 min at 20 ± 2 ºC. Grape juice contains large amounts of organic matter, which did not permit us to measure dissolved ozone in the liquid phase.

Because of this, the ozone concentration in the system was recorded by an iodometric method (Rice 2012). Residual ozone was destroyed using an Ozone Destroyed Unit (O&LM). A control treatment was made using potassium metabisulfite at 80 mg L^{-1} as the source of SO_2 (Rizzon et al. 1996).

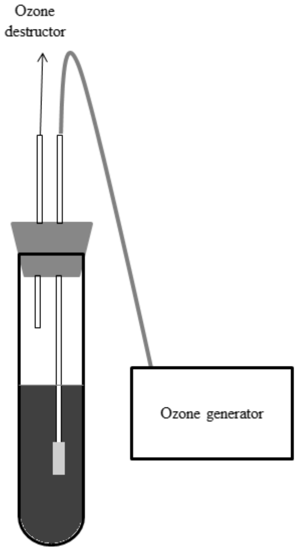

Figure 3. Scheme of ozone application system

3.4. *Microbiological analyses*

Microbiological analyses were carried out by mixing 1 mL of each sample with 9 mL sterile saline peptone solution. A series of solutions from 10^{-1} to 10^{-5} were made. Lactic acid bacteria were plated (MRS agar – Himedia, Mumbai, India) using the microdot method (Morton 2001) and incubated at 37 ºC for 24 h under microaerophilic conditions. The spread plate method (Jett et al. 1997) was used for acetic acid bacteria (WL agar – Himedia) and yeast (PDA – Himedia) followed by incubation at 28 ºC for 4 days and 30 ºC for 5 days. CFU were counted, and the results were reported as log CFU mL^{-1}. All samples were analyzed before and after treatments, in two replicates. Due to sterilization of the juice before use, the counts at time 0 min represented the total population of each microorganism.

3.5. Statistical analyses

The experiment had a completely randomized design with five treatments and two replicates. Statistical analyses were performed using the statistical software package SAEG 9.0 (Federal University of Viçosa). To compare the effect of ozone on microorganism populations to the effect of SO_2 (control treatment) Dunnet's test ($P < 0.05$) was performed. The results for ozone treatments were also used for regression analysis. The models were chosen based on the significance of the regression coefficients (using Student's t-test at a level of 15% probability), using the determination coefficient (R^2), and by considering biological phenomena. The graph was generated using SigmaPlot 10.0.

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Conflict of interest

The authors declare no conflict of interest.

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ARTIGO 2

ANTIMICROBIAL ACTIVITY OF OZONE TREATMENT DURING ALCOHOLIC FERMENTATION AND ITS EFFECT ON THE ANTHOCYANINS AND COLOR OF RED WINE

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Abstract

This work investigated the effect of ozone on yeasts, lactic bacteria (LAB) and acetic bacteria (AAB) during fermentation, as well as its effect on the anthocyanins content and color of wine compared with sulfite. Wines were produced with a Syrah cultivar grape for 15 days. Ozone was applied at different concentrations (2 - 12 $mg \cdot L^{-1}$) and times (5 – 25 min) at days 1, 6 and 16 of fermentation. Analyses of anthocyanins and color parameters were performed at the end of fermentation. The yeasts, LAB and AAB were not affected by the ozone treatments. The wines treated with higher ozone concentrations acquired characteristics of mature wine, presenting with decreased monomeric anthocyanins, and increased percent of polymeric color, yellow/brown shades and tonality. This suggests that the use of ozone as a substitute for sulfite may confer aging characteristics to wine in terms of color and may contribute to reducing the time for maturation.

Keywords: Ozonation; color; anthocyanin; winemaking, sulfite

1. Introduction

Transforming grape must to wine is a complex biochemical process involving interactions between yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Andorrà, Landi, Mas, Guillamòn & Esteve-Zarzoso, 2008). Represented by its flavor and aroma, wine quality is influenced by the metabolism of these microorganisms (Swiegers, Bartowsky & Henschke, 2005). The role of yeast is to perform fermentation by converting sugars into ethanol (Suárez-Lepe & Morata, 2012). LAB influences wine flavor. However, their growth has also been correlated to producing off-flavors and increasing wine viscosity in the bottle (Montersino, Prieto, Munoz & De Las Rivas, 2008). Similarly, AAB growth generates undesirable compounds, such as acetic acid and ethyl acetate, which have a negative effect on wine quality (Andorrà et al., 2008).

Sulfur dioxide (SO_2) application and yeast inoculation are well-established practices in winemaking to restrict the growth of endogenous yeasts and bacterial populations (Andorrà et al., 2008). Despite its well-established ability to control microorganisms, numerous adverse effects on human health have been linked to the ingestion of SO_2 (Garaguso & Nardini, 2015). Moreover, SO_2 is highly corrosive to metals (Cayuela, Vázquez, Pérez & García, 2009), which compels researchers to search for alternative methods to control microorganisms during the fermentation step.

After FDA approval, the direct use of ozone (O_3) in food has been frequently studied as a postharvest treatment and a method for surface decontamination of fruits and vegetables (Tiwari, O'Donnell, Patras, Brunton & Cullen, 2009a, b; Cayuela et al., 2009; Torres et al., 2011; Botondi, De Sanctis, Moscatelli, Vettraino, Catelli & Mencarelli, 2015). Because ozone is effective against all microbiological forms

(Khadre, Yousef & Kim, 2001), it could be used to control or eliminate undesirable microorganisms in wine production. Relatively low concentrations of ozone and short contact times are adequate to reduce microbial numbers. Additionally, ozone spontaneously decomposes to molecular oxygen, resulting in no toxic residues (Tiware et al., 2009a). Ozone results in controlled oxidation that can be sensorially beneficial to red wine by enhancing and stabilizing its color and reducing its astringency (Oliveira, Ferreira, Freitas & Silva, 2011). However, doses sufficient to eliminate some microorganisms may cause prejudicial sensorial modifications to the wines. Tiware et al. (2009a) found that ozone caused a significant reduction in color and anthocyanin content when applied to grape juice.

Normally, the color of a wine is the first characteristic perceived by a consumer. Consequently, it conditions a global sensorial analysis of the wine. In young red wines, the color is determined by monomeric anthocyanins, which are pigments mainly located in the skin of grape berries (He et al., 2012). During maturation, polymeric pigments more stable to pH changes are formed (Gutiérrez, Lorenzo & Espinosa, 2005). The nature of anthocyanins, their extraction during winemaking and their final content influence the sensory characteristics of wine and, as a result, their acceptance by a consumer (Giacosa, Marengo, Guidoni, Rolle & Hunter, 2015). Moreover, this compound presents potential benefits for human health and disease prevention (Tiware et al., 2009b).

There is no information available on the effects of ozone on microorganisms during wine fermentation. There is also no information on the possible changes in the anthocyanin content and color of wine caused by ozone. Knowing the strong oxidant power of ozone and its effects on grape juice, in which ozone affected the color and anthocyanin content (Tiware et al., 2009a), it is expected that ozone causes degradation of anthocyanin content and, thus, changes in color of the wine. However, such supposed effects need to be confirmed.

The purpose of this work was to investigate the effect of ozone on yeasts, LAB and AAB during fermentation, as well as on the anthocyanin content and parameters of color of red wine.

2. Materials and Methods

2.1. Grapes

The study was performed with *Vitis vinifera* L. cultivar Syrah grown at a vineyard located in South-East Brazil (Minas Gerais State). Grapes were manually harvested with approximately 17 °Brix and transported to the laboratory. After the grapes were manually crushed and destemmed, the must was maintained at -18 ºC until winemaking. This practice is considered a valid research tool for producing experimental wines outside of vintage or in distant places of a vineyard (Schmid, Li, Liebich, Culbert, Day & Jiranek, 2007).

2.2. Winemaking procedure

Wines were produced using microvinification. Approximately 4 L of frozen must was thawed overnight (12 h) for each treatment. Musts received the first-stage of treatments (day 1) (as described in the following section). They were then placed into a stainless steel tank for fermentation. Active dry yeasts (*Saccharomyces cerevisiae,* Blastosel FR 95, Indupropil, Ijuí, Brazil) were added in the proportion of 40 g per 100 L of must. On the second day, granulated sugar cane was added to the must (54 $g \cdot L^{-1}$) to standardize the must at 18 ºBrix.

The soaking period of the skins was 6 days. After this, musts without skins were transferred to a flask glass with hydraulic stopper, where the second stage of treatments was performed (day 6). Alcoholic fermentation was a total of 15 days. When complete (at 16 day), a second solids separation method and application of the last stage of treatments were performed. All processes were performed at 20 ºC.

The progression of alcoholic fermentation was monitored daily by measuring the sugar content of the fermenting must with each treatment. A digital hand-held refractometer with temperature compensation (Pal-3 model, Atago Corporation, Tokyo, Japan) was used. Malolactic fermentation was not made. Bottling was performed manually at 16 day in bottles of 750 mL that were sealed with cork stoppers. The bottles were stored in a dry and ventilated area at room temperature that was protected from light.

2.3. Ozonation

Ozone treatments were carried out (at day 1, 6 and 16) in a mechanically agitated bioreactor with a capacity of 8 L. Ozone gas was produced by a generator (O&LM model, Ozone & Life, São José dos Campos, Brazil) that was fed by an oxygen cylinder (White Martins, Juiz de Fora, Brazil). Extrinsic parameters of ozone concentration (2 – 12 $mg\cdot L^{-1}$) and treatment time (5 – 25 min) were varied. The gas flow rate was 0.37 $L\cdot min^{-1}$. Ozone concentration was monitored by the Iodometric method (Rice & Bridgewater, 2012). Two trials were performed for each ozone concentration and treatment time.

To compare this method with a traditional method for vinification, a testimony treatment was performed by employing potassium metabisulfite ($K_2S_2O_5$) (80 $mg\cdot L^{-1}$ must). At 6 and 16 days, $K_2S_2O_5$ was applied to the wines to correct for free sulfur dioxide content in 30 mg·L^{-1}. A control treatment was also carried out without ozone or SO_2.

2.4. *Microbiological analyses*

For microbiological analysis, aliquots of 1 mL were withdrawn at days 1, 6 and 16 of fermentation, immediately after treatments with ozone. The aliquots were serially diluted in a sterile saline peptone solution (0.1% peptone, 0.85% NaCl). A surface spread method was used for the quantification of yeasts (PDA – Himedia) and AAB (WL agar – Himedia), which were incubated at 30 ºC for 5 days and 28 ºC for 4 days, respectively. To determine LAB quantity, aliquots of 20 μL were plated (MRS agar – Himedia) using a microdot method (Morton, 2001) and incubated at 37 ºC for 24 h in ambient microaerophilic conditions. The different colonies from each plate were analyzed using an optical microscope by Gram staining and tested for catalase production. Gram-positive and catalase negative cells were considered to be LAB. Gram-negative and catalase positive cells were considered to be AAB. Colony-forming units (CFU) were counted as specified by the *Organisation Internationale de la Vigne et du Vin* (OIV) (OIV, 2014). All samples were analyzed in duplicate. The viable numbers of cells obtained were transformed into log10 before statistical analysis and were expressed as CFU·mL^{-1}.

2.5. *Enological parameters*

Total soluble solid concentrations (in ºBrix) were measured using a digital hand-held refratometer. The pH was determined by potentiometry using digital pH meter (DM-20 model, Digimed, Weilheim, Germany). The alcoholic content and relative density 20/20 °C was determined by a portable digital densimeter (DMA 35 model, Anton Paar, Haryana, India) and was expressed in % v/v at 20 ºC and g·cm^{-3}, respectively. Total acidity and volatile acidity were determined by titrimetry in accordance with official methods proposed by OIV (2014).

2.6. Anthocyanins determination

Total anthocyanin, polymeric and monomeric was determined using a method proposed by Cliff, King and Schlosser (2007) with modifications. Sample wines had their pH adjusted to 3.6 using 2 N NaOH or 2 N HCl and were centrifuged (J2-MI, Beckman Instruments Inc., Palo Alto, USA) at 1087 × g for 15 min at 20 ºC. As the first step, aliquots were diluted 1:10 with model wine (5 g·L^{-1} tartaric acid, 12% ethanol, pH 3.6). The absorbance was measured at 520 nm (A_{dil}) with a spectrophotometer (BioMate5, Thermo Scientific, Woburn, USA) using a 10 mm cuvette. In the second step, aliquots of 4 mL of each sample were mixed with 320 μL of 5% SO_2 (v/v) (as $NaHSO_3$ solution). The absorbance was measured using 1 mm cuvette (A_{SO2}). The results were expressed in absorbance units (a.u.), taking into account the dilution and the different thickness of cuvettes ($A_{dil} = Abs \times 4$ and $A_{SO_2} = Abs \times 2.16$).

The different anthocyanins fractions were determined by equations 1 to 3.

$$\text{Total anthocyanin} = A_{dil} \qquad \text{Eq. 1}$$

$$\text{Polymeric anthocyanin} = A_{SO_2} \qquad \text{Eq. 2}$$

$$\text{Monomeric anthocyanin} = A_{dil} + A_{SO_2} \qquad \text{Eq. 3}$$

2.7. Polymeric color analysis

The measurement of polymeric color is very useful to evaluate the color quality of products containing anthocyanins (Danişman, Arslan & Toklucu, 2015). Methods described by Giusti and Wrolstad (2001) were used to determine color density, polymeric color (color as a result of polymeric anthocyanins) and percent of polymeric color. Samples were diluted with distilled water to have an absorbance reading under 1.0 at 420 and 520 nm. To a test tube containing 3.0 mL of wine, 0.2 mL of $K_2S_2O_5$ 20% (bisulfite-bleached sample) was added. A control sample was performed with 0.2 mL of water. Samples were measured at $\lambda = 420$, 520, and 700 nm. Color density was calculated using a control sample according to Eq. 4:

$$\text{Color density} = \left[\left(A_{520} - A_{700}\right) + \left(A_{420} - A_{700}\right)\right] \times \text{dilution factor} \qquad \text{Eq. 4}$$

Polymeric color was determined using a bisulfite-bleached sample, according Eq. 5:

$$\text{Polymeric color} = \left[\left(A_{520} - A_{700}\right) + \left(A_{420} - A_{700}\right)\right] \times \text{dilution factor} \quad \text{Eq. 5}$$

Percent of polymeric color was determined from color density and polymeric color (Eq. 6).

$$\text{Percentage of polymeric color} = \left(\frac{\text{polymeric}}{\text{color density}}\right) \times 100 \qquad \text{Eq. 6}$$

2.8. Chromatic characteristics

To determine the wine color two methods were used: a CIELAB method and a spectrophotometric absorbance of the wine at 420, 520, and 620 nm (using a 1 mm cuvette).

Values of the CIELAB parameters lightness (L^*), red-greenness (a^*) and yellow-blueness (b^*) were obtained using a colorimeter (Color Quest II Spera, Hunter Lab, Reston, USA). Chromaticity (C^*) and hue angle (h^*) values were calculated according to equations 7 and 8, respectively, that were proposed by Ayala, Echávarri and Negueruela (1997). Data were processed with MSCV® software (Ayala et al., 2001).

$$C^* = \sqrt{(a^{*2}) + (b^{*2})} \qquad \text{Eq. 7}$$

$$h^* = \text{arctg}\left(\frac{b^*}{a^*}\right) \qquad \text{Eq. 8}$$

From the spectrophotometric absorbance of the wine at 420, 520, and 620 nm, color intensity and tonality were determined using equations 9 and 10:

$$\text{Color intensity} = A_{420} + A_{520} + A_{620} \qquad \text{Eq. 9}$$

$$\text{Tonality} = \frac{A_{420}}{A_{520}} \qquad \text{Eq. 10}$$

2.9. Statistical analyses

This experiment was a completely randomized design with 11 treatments and two repetitions. To compare the effect of ozone on microorganism populations, descriptive statistics were used. To compare the effect of ozone on anthocyanins and color parameters in relation to the effect of SO_2 and the control treatment, Dunnet's test was performed ($P < 0.05$). Dates for ozone treatments were submitted in a regression analysis. Models were chosen based on the significance of regression coefficients using Student's t-test at level until 15% of probability, on a determination coefficient (R^2), and on biological phenomena. Three-dimensional curves of the response surface were performed using SigmaPlot version 10.0. Pearson correlation coefficients were calculated to determine significant correlations between the studied parameters. All statistical analyses were performed using SAEG version 9.0 software (Federal University of Viçosa).

3. Results and Discussion

3.1. Ozone effects on the microorganisms during fermentation

The results of the viable cells number of yeasts, LAB and AAB analyzed at days 1, 6 and 16 of fermentation, after the treatments, are shown in Table 1.

The population of yeasts on the must at the first day after the treatments ranged from 5.5 to 6.4 log CFU·mL^{-1}. At day 6 of fermentation, an increase in the yeast population in the must of the control, SO_2 and ozone treatments at 7 mg·L^{-1} for 5, 15 and 25 min was observed. At the last day of fermentation (day 16), there was a downward trend of viable cells with a verified variation from under 3 to 5.6 log CFU·mL^{-1}. This reduction at 16 day did not largely vary between the treatments. Such behavior probably indicates natural death of the yeasts that follows the reduction of available sugar and increase of ethanol concentration (Guzzon, Nardin, Micheletti, Nicolini & Larcher, 2013), which is not dependent on the treatment performed.

The AAB count on first day after the treatments ranged from 4.3 log CFU·mL^{-1} in the must treated with SO_2 to 5.5 log CFU·mL^{-1} in the untreated must. The results of the ozone treatments had intermediate values. However, at day 6 of fermentation, the population of these bacteria was estimated to be lower than 3 log CFU·mL^{-1} and was inclusive in the control treatment. This population was not detected at 16 day of fermentation by the chosen method. Thus, it was also estimated lower than 3 log CFU·mL^{-1} (detection limit) for all the treatments.

Table 1

Effect of treatments on microorganisms in red wine at days 1, 6 and 16 of fermentation. Results reported as mean values of log CFU·mL^{-1}.

Treatments	**Yeasts**			**Acetic Bacteria**			**Lactic Bacteria**		
(mg·L^{-1} O$_3$ / min)	**1 d**	**6 d**	**16 d**	**1 d**	**6 d**	**16 d**	**1 d**	**6 d**	**16 d**
2 / 5	6.4	5.6	5.1	5.4	<3	<3	<3	<3	<3
2 / 15	6.0	5.4	4.8	5.0	<3	<3	3.6	<3	<3
2 / 25	6.0	5.7	4.8	5.0	<3	<3	3.8	<3	<3
7 / 5	5.8	6.0	5.5	5.2	<3	<3	4.2	<3	<3
7 / 15	5.7	6.5	4.8	5.1	3.3	<3	3.5	<3	<3
7 / 25	5.5	6.2	5.6	4.9	<3	<3	4.0	<3	<3
12 / 5	6.3	6.3	<3	5.4	<3	<3	4.7	<3	<3
12 / 15	6.2	5.3	4.8	5.5	3.0	<3	3.7	<3	<3
12 / 25	6.3	5.8	4.0	5.1	3.3	<3	<3	<3	<3
SO$_2$	6.3	7.7	5.0	4.3	<3	<3	<3	<3	<3
Control	6.2	7.4	5.0	5.5	<3	<3	<3	<3	<3

<3: under quantification limit of the utilized method for count cells

The LAB count ranged between under 3 and 4.7 log CFU·mL^{-1} at the first day of fermentation. This cell population was not recovered on the analyses carried out at 6 and 16 day of fermentation. It was below the detection limit of the adopted procedure (3 log CFU·mL^{-1}) for all the treatments, including the control.

In general, independent of the treatment used, the populations of all the microorganisms were reduced after day 6 of fermentation. In fact, winemaking is conducted in an environment that is stressful for yeasts and bacteria. Under low pH, anaerobic conditions, nutrient scarcity and high alcohol concentrations at the end of fermentation, only a few microorganisms are able of survive and grow (Du Toit, Pretorius & Lonvaud-Funel, 2005). More resistant microorganisms were probably not present on the utilized grapes, eliminated with the solids in the wine transfers or in low numbers under the detection limit of the technique used.

3.2. Enological parameters

The characteristics of the wines produced are shown at Table 2. No significant differences ($P > 0.05$) were observed in the total soluble solids, pH, alcoholic content, density and total acidity. The overall means were 6.05 ºBrix, 3.51, 8.393% v/v at 20 ºC, 0.9860 $g\cdot cm^{-3}$ and 104.13 $meq\cdot L^{-1}$ of tartaric acid, respectively. Only volatile acidity had means significantly lower in all treatments, in relation to treatment with SO_2 (2.51 $meq\cdot L^{-1}$ of acetic acid) ($P < 0.05$). Volatile acidity is derived from acids of the acetic series as a metabolite of bacteria or yeast (Vilela-Moura et al., 2011). The volatile acidity parameter refers to the sanity of the wine. The low values observed after the treatments may to correspond to the low number of acetic bacteria. Further, bound SO_2 is considered bacteriostatic rather than bactericidal (Sumby, Grbin & Jiranek, 2014). Thus, acetic bacteria only inhibited by SO_2 may have regrown and produced acetic acid, which would explain the increase in volatile acidity. The legislation of Brazil determined that the mean values of total acidity are between 55 and 130 $meq\cdot L^{-1}$ and that the volatile acidity at a maximum of 20 $meq\cdot L^{-1}$ (Brazil, 2004). For both parameters, the wines met the Brazilian legislation standards. The alcoholic content was less than that stipulated by legislation, but this was not due to the treatments.

Table 2

Enological properties of wines

Treatments ($mg \cdot L^{-1}$ O_3 / min)	**Total Soluble Solids (ºBrix)**	**pH**	**Alcohol (% v/v at 20 ºC)**	**Density ($g \cdot cm^{-3}$)**	**Total Acidity ($meq \cdot L^{-1}$ tartaric acid)**	**Volatile Acidity ($meq \cdot L^{-1}$ acetic acid)**
2 / 5	6.1	3.51	8.55	0.9857	106.09	1.01*
2 / 15	6.1	3.52	8.65	0.9856	106.21	1.03*
2 / 25	6.0	3.51	8.57	0.9859	104.08	0.89*
7 / 5	6.1	3.53	8.48	0.9861	96.15	0.91*
7 / 15	6.2	3.53	7.88	0.9869	92.16	1.03*
7 / 25	6.1	3.51	8.32	0.9864	93.66	0.96*
12 / 5	6.0	3.51	8.48	0.9858	104.75	0.96*
12 / 15	6.1	3.48	8.75	0.9858	108.91	0.87*
12 / 25	6.0	3.50	7.98	0.9864	107.61	0.94*
SO_2	6.1	3.53	8.28	0.9861	112.34	$2.51^{\dagger}$
Control	6.0	3.51	8.38	0.9855	113.57	1.09*

*Differ from SO_2 treatment in the column by Dunnet test ($P < 0.05$);

†Differ from control treatment in the column by Dunnet test ($P < 0.05$).

3.3. Ozone effects on the anthocyanin content of wine

The means values of total, polymeric and monomeric anthocyanins are listed in Table 3. No significant differences were observed between the ozone treatments and the control or SO_2 treatment by Dunnet's test at 5% of probability in the total and polymeric anthocyanins (overall means 2.04 and 0.33 a.u., respectively). Significant reductions of monomeric anthocyanins ($P < 0.05$) were found in the samples treated with ozone at 7 $mg \cdot L^{-1}$ for 25 min and 12 $mg \cdot L^{-1}$ for 15 and 25 min (1.48, 1.45 and 1.34 a.u., respectively) compared to the control (1.84 a.u.). After the SO_2 treatment (mean 1.76 a.u.), significant differences in monomeric anthocyanins were found for the ozone treatment at 12 $mg \cdot L^{-1}$ for 15 and 25 min. With the ozone treatments, the total and monomeric anthocyanins were affected by the exposure time. They were reduced with the increase of time. The total anthocyanins were no affected by either exposure time or concentration of ozone. In the regression analyses (Table 4), the model adjusted for total anthocyanins indicated a quadratic effect for ozone concentration and a linear effect for exposure time. The response surface was plotted as a function of the ozone concentration and treatment time (Figure 1a). According to the model, for any exposure time, the total anthocyanins reached a minimum at 9.89 $mg \cdot L^{-1}$ of ozone. When a constant ozone concentration was maintained for each minute of exposition to ozone, the total anthocyanins were reduced in 0.0163 a.u. For the monomeric anthocyanins, we found a linear fit for both the concentration and time of treatment (Figure 1b). Each unit of ozone concentration caused a reduction of 0.0337 a.u. in monomeric anthocyanins. Each unit of time of exposure reduced it by 0.0169 a.u. The results of the polymeric anthocyanins were not satisfactory fit to any model and were considered the overall means.

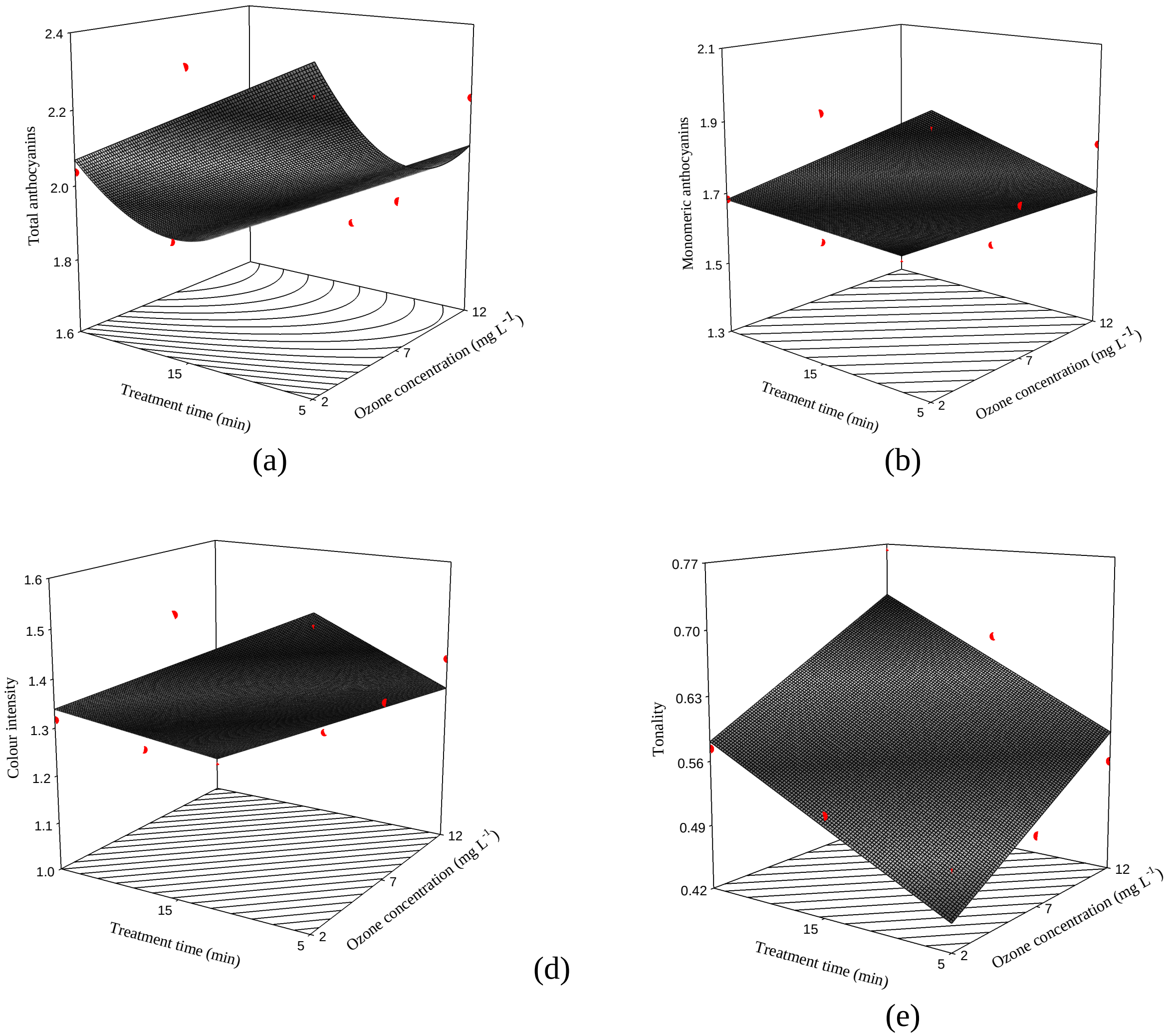
Table 3

Total, polymeric and monomeric anthocyanins, color density, polymeric color and percentage of polymeric color of wines

Treatments ($mg \cdot L^{-1}$ O_3 / min)	**Total anthocyanins ¹**	**Polymeric anthocyanins ¹**	**Monomeric anthocyanins ¹**	**Color density**	**Polymeric color**	**Percentage of polymeric color**
2 / 5	2.31	0.34	1.97	8.89	2.79	31.22
2 / 15	2.35	0.38	1.97	9.14	3.08	33.63
2 / 25	2.04	0.35	1.68	7.98	2.86	35.78
7 / 5	1.99	0.28	1.72	7.53	2.41	31.95
7 / 15	2.04	0.32	1.72	9.45	3.27	34.62
7 / 25	1.76	0.28	1.48*	7.28	2.67	36.65
12 / 5	2.20	0.38	1.82	8.28	2.96	35.74
12 / 15	1.79	0.33	1.45*†	7.92	3.98	49.66*†
12 / 25	1.73	0.40	1.34*†	7.63	3.85	50.03*†
SO_2	2.02	0.26	1.76	8.26	2.58	31.11
Control	2.18	0.34	1.84	8.30	2.47	29.79

*Differ from control treatment in the column by Dunnet's test ($P < 0.05$); †Differ from SO_2 treatment in the column by Dunnet's test ($P < 0.05$);

¹ expressed in absorbance unit (a.u.)



(a) (b) (c) (d) (e)

Figure 1. Response surface plots of effect of ozone concentration (mg·L^{-1}) and treatment time (min) on total anthocyanins (a), monomeric anthocyanins (b), percentage of polymeric color (c), color intensity (d) and tonality (e) of wines

There was a trend in a reduction of the monomeric anthocyanins and increase in the polymeric ones with the intensification of the treatments. The same observation was reported by Cano-López, López-Roca, Pardo-Minguez and Gómez-Plaza (2010), who were studying the effects of micro-oxygenation on wine, and would be characteristic of wine aging. However, in the treatments with ozone at 2 mg·L^{-1} for 25 min, 7 mg·L^{-1} for 25 min and 12 mg·L^{-1} for 15 min, a reduction of both the monomeric and polymeric anthocyanins was observed with the increment of the treatment time. Such behavior may be due the higher oxidant power of ozone, which may have degraded the anthocyanins before the polymerization reactions. According to Xue, Chen and Wang (2008), beyond the ozone, the formation of high-reactive species as its secondary products, such as $\bullet OH$, $HO^{2\bullet}$, $\bullet O_2^-$ and $\bullet O_3^-$, facilitates the degradation of organic compounds. Anthocyanins contain aromatic rings, which may be opened by ozone and/or hydroxyl radicals (generated in an aqueous solution) due the formation of ozonide. Thus, the aromatic ring is broken down, and the degradation rapidly increases (Tiwari et al., 2009b). Moreover, reactions of polymerization and condensation of monomeric anthocyanins normally occur during wine maturation. In some cases, the time may not have been adequate (Oliveira et al., 2011).

3.4. Ozone effects on the polymeric color of wine

The stability of wine color is conferred by pigments which retain their color at the pH of wine and are resistance to bleaching by sulfite (Bindon, Kassara, Hayasaka, Schulkin & Smith, 2014). The results of the parameters of the polymeric color are shown in Table 3. No significant differences ($P > 0.05$) were observed after the ozone treatments in color density or polymeric color in relation to the control or

SO_2 treatment. Color density quantifies the visual appearance of wine and has a strong positive correlation with wine quality (Somers & Evans, 1974). Its values ranged between 7.28 and 9.45. Polymeric color corresponds to the contribution of polymeric anthocyanins and brown pigments from oxidation to color (Wrolstad, 1976). Although there were no significant differences, the values were higher at 12 $mg \cdot L^{-1}$ of ozone at 15 and 25 min. From the observed results, the ozone treatments tested possibly did not cause enough oxidation of the pigments to significantly change the polymeric color of the wines. Good models for this parameter were not found in the regression analyses. Therefore, they were considered similar to the overall means (Table 4).

The percentage of polymeric color presented higher mean values in the treatments with 12 $mg \cdot L^{-1}$ of ozone for 15 and 25 min. They reached up to 50%. These means were significantly ($P < 0.05$) higher than the control and SO_2 treatments (29.79 and 31.11%, respectively). The regression analysis was an adjusted model for the percentage of polymeric color. The response surface was plotted as a function of the ozone concentration and treatment time (Figure 1c). The quadratic effect for ozone concentration ($P < 0.05$) and linear effect for exposure time ($P < 0.01$) were verified (Table 4). According to the adjusted model, the total anthocyanins reached a minimum pf 4.06 $mg \cdot L^{-1}$ of ozone for any exposure time. For each minute of exposure to ozone, the percentage of polymeric color increased by 0.3925 when a constant ozone concentration was maintained.

As expected, a positive correlation was found between the percentage of polymeric color and polymeric anthocyanins content with a correlation coefficient of 0.43. With the monomeric anthocyanins, the correlation coefficient was negative (0.55).

Table 4

Equations adjusted for the parameters (anthocyanins total, polymeric and monomeric, color density, polymeric color, percentage of polymeric color, *L**, *a**, *b**, *C**, *h**, color intensity and tonality) in function of ozone concentration ($mg \cdot L^{-1}$) and treatment time (min) and their respective determination coefficient (R^2)

Parameters	Equation	R^2
Total anthocyanins	$\hat{y} = 2.6753 - 0.1108^{*}x + 0.0056^{\square}x^{2} - 0.0163^{*}z$	0.827
Polymeric anthocyanins	$\hat{y} = 0.3401$	-
Monomeric anthocyanins	$\hat{y} = 2.1721 - 0.0337^{*}x - 0.0169^{*}z$	0.853
Color density	$\hat{y} = 8.2409$	-
Polymeric color	$\hat{y} = 2.9928$	-
Percentage of polymeric color	$\hat{y} = 30.07 - 1.604^{\circ}x + 0.1974^{*}x^{2} + 0.3925^{**}z$	0.651
L*	$\hat{y} = 27.8503$	-
a*	$\hat{y} = 8.1301$	-
b*	$\hat{y} = 1.3504$	-
C*	$\hat{y} = 8.2475$	-
h*	$\hat{y} = 0.1564$	-
Color intensity	$\hat{y} = 1.7114 - 0.0264^{**}x - 0.0128^{**}z$	0.745
Tonality	$\hat{y} = 0.3909 + 0.0127^{**}x + 0.0066^{**}z$	0.811

* Significative to 5%; ** significative to 1%; $^{\square}$ significative to 10%; $^{\circ}$ significative to 15%.

3.5. Ozone effects on the chromatic characteristics of wine

The means values to *L**, *a**, *b**, *C** and *h** are listed in Table 5. There were no significant differences in these parameters ($P > 0.05$) between the ozone treatments and the control or SO_2 treatment. The effect of exposure time on all the parameters was noted when the individual factors were analyzed. The higher means corresponded to the treatments of 25 min. Their increase was in accordance with the increase of ozone concentration. No models were found with a satisfactory fit. Therefore, they were considered the overall means (Table 4).

Due to its strong coloration, the wine normally had low values of luminosity (*L**). The increase of *L** values represented a discreet brightening of the wine. With *a**, an increase of values corresponded to an increase in red color. In this case, *a** ranged from 6.63 to 10.73 for the ozone treatments of 2 $mg \cdot L^{-1}$ for 15 min and 12 $mg \cdot L^{-1}$ for 25 min, respectively. The range of *b** was between 0.72 and 2.26. This value refers to the gain of yellow color and reduction of blue shades. Normally, during maturation in the presence of oxygen, the redness of a young red wine decreases, while the absorbance in the yellow/brown region (near 420 nm) increases (Gao, Girard, Mazza & Reynolds, 1997).

Chromaticity reports the purity of the color. An increase of *C** corresponded to a change from a mix of colors to a more pure color. The hue angle defines the color. For all the wines produced, this value near 0, indicating a basically red color. Treatments with ozone for 25 min had values slightly higher than on the other times (0.17, 0.20 and 0.20 to 2, 7 and 12 $mg \cdot L^{-1}$, respectively). This represented the gain of yellow color

.

Table 5

Parameters of color of wines

Treatments (**mg·L^{-1} O$_3$ / min**)	***L****	***a****	***b****	***C****	***h****	**Color Intensity**	**Tonality**
2 / 5	27.62	6.67	0.82	6.72	0.12	1.57	0.50
2 / 15	27.54	6.23	0.72	6.27	0.11	1.56	0.53*[†]
2 / 25	28.07	8.89	1.57	9.03	0.17	1.32	0.57*[†]
7 / 5	27.87	7.93	1.34	8.05	0.17	1.37	0.50
7 / 15	27.84	7.77	1.31	7.88	0.16	1.38	0.56*[†]
7 / 25	28.23	9.95	2.05	10.16	0.20	1.18*[†]	0.60*[†]
12 / 5	27.77	7.96	1.23	8.05	0.15	1.39	0.54*[†]
12 / 15	27.86	8.17	1.38	8.30	0.15	1.19*[†]	0.67*[†]
12 / 25	28.40	10.73	2.26	10.97	0.20	1.07*[†]	0.77*[†]
SO_2	27.66	7.37	1.01	7.43	0.14	1.49	0.47
Control	27.51	7.78	1.17	7.86	0.15	1.49	0.47

*Differ from control treatment in the column by Dunnet's test ($P < 0.05$);

[†] Differ from SO_2 treatment in the column by Dunnet's test ($P < 0.05$).

The color of wine is mainly influenced by the presence of anthocyanins. For example, the colors orange, salmon, pink and red are due the presence of pelargonidins; magenta and crimson colors are due to cyanidins; and purple, mauve and blue colors are result of delphinidins (Tiwari et al., 2009a). Thus, in the wines treated with ozone, the changes in color may be related to anthocyanin oxidation. Ozone causes the breakdown of conjugated bonds, resulting in oxidative cleavage of chromophores (Xue et al., 2008).

Color intensity refers to quantity of color, considering the contribution of yellow (A_{420}), red (A_{520}) and blue (A_{620}) to total color (Ribéreau-Gayon, Glories, Maujean & Dubourdieu, 2006). In this work, color intensity decreased with the intensification of ozone treatments (Table 4), probably due the reduction in blue shadows and increases the yellow. The means of the treatments with 7 $mg \cdot L^{-1}$ for 25 min, 12 $mg \cdot L^{-1}$ for 15 min and 12 $mg \cdot L^{-1}$ for 25 min (1.18, 1.19 and 1.07, respectively) were significantly smaller ($P < 0.05$) than the means from the control and SO_2 treatments (1.49 for both). Both ozone concentration and exposure time affected the reduction of color intensity. The adjusted model in the regression analyses showed a linear effect for both factors at 1% of probability (Table 4). The response surface plotted for color intensity was a function of the ozone concentration and treatment time and is shown in Figure 1d.

Tonality refers to development of the color to orange shades (Ribéreau-Gayon et al. 2006). Anthocyanin degradation is followed by a loss of the red molecular structure and appearance of the yellow color. Only the means of the treatments with 2 and 7 $mg \cdot L^{-1}$ of ozone for 5 min (0.50 both) did not differ statistically from the control and SO_2 treatments (0.47 both) (Table 4). According Ribéreau-Gayon et al. (2003), the normal values of tonality for a young red wine are between 0.5 and 0.7.

The intensification of treatments caused an increase in wine tonality. The higher values were after the 12 $mg\cdot L^{-1}$ of ozone for 25 min, 12 $mg\cdot L^{-1}$ of ozone for 15 min and 7 $mg\cdot L^{-1}$ of ozone for 25 min (0.77, 0.67 and 0.60, respectively). Similarly, Cano-López, Pardo-Minguez, López-Roca and Gómez-Plaza (2007) studied the micro-oxygenation process and found a higher tonality in the oxidized wine than the control. According, Boulton (2001) found that the increase of tonality and yellow/brown shades is a characteristic of aging of wines. The adjusted model verified linear the effect of both factors ($P < 0.01$). The response surface plotted for tonality was a function of the ozone concentration and treatment time and is shown in Figure 1e. Effects of the individual factors (ozone concentration and exposure time) and their interaction were noted.

In general, the ozone treatments caused slight changes in the Syrah wine in comparison to the SO_2 treatments, considering that ozone is an oxidizing agent and SO_2 is a reducing agent. The alterations were caused after the treatments with higher ozone concentrations. However, the ozone exposure time was more significant.

4. Conclusions

Although neither ozone nor SO_2 affected the yeast, LAB, or AAB populations, it promoted a decrease of monomeric anthocyanins and increase of percent of polymeric color, yellow/brown shades and tonality. The produced wines were young at the time of the analyses. Those treated with higher ozone concentrations acquired the characteristics of a mature wine. This suggests that the use of ozone as a substitute for sulfite may confer aging characteristics to wine in terms of color, thereby reducing the time of maturation.

Others studies are suggested to evaluate the ozone effectiveness on microorganism spoilage of wines and its effect on the sensorial characteristics of wine.

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Conflict of interest

The authors declare no conflict of interest.

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ARTIGO 3

THE EFFECT OF OZONE ON THE POLYPHENOL CONTENT, ANTIOXIDANT ACTIVITY AND VOLATILE COMPOUND CONTENT OF RED WINE

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Abstract

Red wines were produced with the use of ozone at different concentrations (2 - 12 $mg \cdot L^{-1}$) and exposure times (5 - 25 min) during the alcoholic fermentation phase. The effects of these production variables on phenolic content, antioxidant activity and volatile compound content were investigated. Ozone did not cause significant changes in phenolic content, antioxidant activity or the contents of the volatile compounds propanol, isobutyl alcohol, isoamyl alcohol, methanol, acetone or ethyl acetate. However, wines treated with ozone at 2 $mg \cdot L^{-1}$ for 15 min and at 7 and 12 $mg \cdot L^{-1}$ for 25 min had methanol concentrations that were over the limit allowed by Brazilian legislation. Ethyl lactate was present in higher concentrations in all ozonated wines relative to wine treated with SO_2. Acetaldehyde reached the sensorial detection limit for all treatments, and higher values were associated with longer exposure times to ozone. Such changes should be taken into account prior to directly employing ozone in wine production.

Keywords: red wine, ozonation, sulfite, polyphenols, volatile compounds

Chemical compounds studied in this article

ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (PubChem CID: 6871216); Folin-ciocalteu reagent; Gallic acid (PubChem CID: 370); Potassium persulfate (PubChem CID: 24412); Sodium carbonate (PubChem CID: 10340); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (PubChem CID: 40634).

1. Introduction

Wine is one of the most frequently consumed alcoholic beverages in the world, according to data from the World Health Organization (WHO, 2014). In 2014, the expected worldwide production of wine was 271 million hectoliters, and wine consumption was estimated to be 243 million hectoliters (OIV, 2014). This level of production and consumption of wine highlights the importance of its quality to both producers and consumers.

Wine quality, with respect to its sensorial characteristics, depends largely on the polyphenolic compounds extracted from grapes during the winemaking process (Río Segade, Giacosa, Gerbi & Rolle, 2011; Rastija & Medic´-Šaric´, 2009). Polyphenols can, in general, be classified into two main groups: flavonoids (anthocyanins, flavonols and dihydroflavonols) and non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) (Fanzone, Zamora, Jofré, Assof, Gomez-Cordovés & Peña-Neira, 2012). These compounds are responsible for the quality of red wines, influencing their astringency, bitterness and color (Hosu, Cristea & Cimpoiu, 2014). The total amount of polyphenols in red wines is estimated to range from 2000 to 6000 $mg \cdot L^{-1}$ (Quideau, Deffieux, Douat-Casassus & Pouysegu, 2011) and is dependent on various factors, such as the grape variety, vineyard location, vine cultivation practices, harvesting time, the technology used in the winemaking process and aging (Di Majo, La Guardia, Giammanco, La Neve & Giammanco, 2008).

It has been recognized that wine can have beneficial effects on human health when drunk in moderation (Garaguso & Nardini 2015). Based on the high antioxidant capacity of phenolic compounds (Paixão, Perestrelo, Marques & Câmara, 2007), epidemiological studies suggest that long-term consumption of products rich in these compounds offers some protection against the development of diseases such as cancer, diabetes, osteoporosis, and cardiovascular and degenerative diseases (Paixão et al., 2007; Garaguso & Nardini, 2015).

Another important parameter of wine quality is its aroma. Aroma results from a complex combination of grape components and is produced during fermentation and maturation, which give each wine a distinctive character. The aroma of wines is defined by volatile compounds belonging to the following groups: acetates and esters, higher alcohols, fatty acids, ketones and aldehydes (Athanasios, Paul, Argyro, Athanasios & Michael, 2007). The effects of these compounds on aroma have different origins. Varietal aromas depend on the type of grapes; fermentative aromas come from alcoholic fermentation under anaerobic conditions, and the bouquet results from the transformation of the aroma that occurs during aging (Câmara, Alves & Marques, 2006).

In terms of quantity, higher alcohols represent the main group of volatile compounds in many alcoholic beverages. These are alcohols that have more than two carbons and are formed by yeast during the fermentation process from the amino acids or sugars of the grapes. The concentrations of these alcohols are affected by the variety and maturity of the grape berries, which result in quantitative and qualitative differences in the amino acid composition of the must. Depending on their concentrations, higher alcohols may contribute to the aromatic complexity of wine or, in some cases, they may influence its sensorial quality (Cameleyre, Lytra, Tempere & Barbe, 2015).

In addition to yeasts, bacteria also produce important aromatic compounds during wine fermentation; however, some bacterial spoilage in wines produces secondary metabolites responsible for undesirable volatile compounds. The management of wine spoilage by bacteria, to avoid the development of off-flavors, can be performed by adding sulfur dioxide (SO_2) (Bartowsky, 2009). However, this practice is strictly controlled due to the risks to human health associated with SO_2 consumption (Botondi, De Sanctis, Moscatelli, Vettraino, Catelli & Mencarelli, 2015) and the fact that it is highly corrosive to metals (Cayuela, Vázquez, Pérez & García, 2009). In addition, high doses of sulfites can cause sensorial alterations in the final product, perceived as the undesirable aromas of sulfurous gas and of the reduction products hydrosulfate and mercaptans (Garaguso & Nardini, 2015).

The use of ozone (O_3) has been studied as a way to control enologically important microorganisms (Guzzon, Nardin, Micheletti, Nicolini & Larcher, 2013), and it has been proposed for use at different stages of wine production, including the reduction of spoilage-inducing microflora in grapes, and the sanitation of barrels, tanks, hoses and bottles (Guzzon et al., 2013; Guillen, Kechinski & Manfroi, 2010). Some of the known advantages of ozone are its effectiveness against a wide spectrum of microorganisms, its spontaneous decomposition to molecular oxygen, which results in no toxic residues (Botondi et al., 2015), and its *in situ* production. Thus, ozone may be a potential substitute for sulfite in wine production. However, there is no information available about the possible effects caused by ozone on the phenolic content, antioxidant activity or volatile compound content when applied directly to wine.

The objective of this study was to investigate the effect of ozone concentration and application time on the phenolic content, antioxidant activity and volatile compound content of red wine.

2. Materials and methods

2.1. Wine producing

The wines were produced from *Vitis vinifera* L., a Syrah cultivar, grown at a vineyard located in Minas Gerais State, Brazil. The grapes, harvested manually with approximately 17 °Brix, were transported to the Laboratory of Bioprocessing and Fermentation (Federal University of Viçosa, Brazil), manually crushed and destemmed. The must was maintained at -18 ºC until used for wine production.

Approximately 4 L of frozen must was thawed overnight (12 h) under laboratory conditions. The treatments were applied (as described below) and the musts were then placed into stainless steel tanks for fermentation. Active dry yeast (*Saccharomyces cerevisiae,* Blastosel FR 95, Indupropil, Ijuí, Brazil) was added at a ratio of 40 g of yeast per 100 L of must. On the second day, crystalized sugar cane was added to the must (54 $g \cdot L^{-1}$) to standardize it at 18 ºBrix. The soaking period with the skins was 6 days. After this, the wine, without the skins, was transferred to a glass flask with a hydraulic stopper for the second stage of treatments. The alcoholic fermentation phase lasted a total of 15 days. On day 16, the wine was again separated from the solids and the final treatments were applied. All processes were performed at 20 ºC.

The progression of alcoholic fermentation was monitored daily by measuring the sugar content of the fermenting must from each treatment using a digital hand-held refractometer with temperature compensation (Pal-3 model, Atago Corporation, Tokyo, Japan). Malolactic fermentation was not performed. Bottling was performed manually on day 16 in 750 mL bottles that were sealed with cork stoppers. The bottles were stored at room temperature in a dry, ventilated room and were protected from light.

The wines produced had the following properties: 6.05 ± 0.08 ºBrix, pH 3.51 ± 0.02, alcohol content 8.393 ± 0.314% v/v at 20 ºC, density 0.9860 ± 0.0004 $g\cdot cm^{-3}$ and total acidity 104.13 ± 9.26 $meq\cdot L^{-1}$ of tartaric acid.

2.2. *Ozone treatment*

Ozone treatments were carried out in a mechanically agitated bioreactor with capacity of 8 L. Ozone gas was produced by a generator (O&LM model, Ozone & Life, São José dos Campos, Brazil) fed by an oxygen cylinder (White Martins, Juiz de Fora, Brazil). Different treatment parameters for ozone concentration (2, 7 and 12 $mg\cdot L^{-1}$) and treatment time (5, 15 and 25 min) were tested. The gas flow rate was 0.37 $L\cdot min^{-1}$. Ozone concentration was monitored using an iodometric method (Rice & Bridgewater, 2012). Two trials were performed for each ozone concentration and treatment time.

For comparison with a traditional method of winemaking, a standard treatment was performed employing potassium metabisulfite ($K_2S_2O_5$) (80 $mg\cdot L^{-1}$ must). At days 6 and 16, the wines received an application of $K_2S_2O_5$ to correct the free sulfur dioxide content to 30 $mg\cdot L^{-1}$. A control treatment was also carried out without the use of ozone or SO_2.

2.3. *Total phenolic content*

Total phenols were analyzed according to the Folin-Ciocalteu method (Singleton & Rossi, 1965), using gallic acid as the standard. Aliquots of 3 mL of Folin–Ciocalteu reagent (0.2 M) were added to 0.6 mL of wine that was appropriately diluted with distilled water to ensure that the absorbance was in the range of 0.200 - 0.800. The mixture was allowed to react for 3 min, and then 2.4 mL of Na_2CO_3 (7.5% m/v) was added. Samples were incubated for 1 hour at room temperature in the dark, and the absorbance was measured at 760 nm in a spectrophotometer (BioMate5, Thermo Scientific, Woburn, USA), using 10 mm cuvette. A calibration curve obtained using gallic acid (0–75 $mg \cdot L^{-1}$) was used to calculate the total phenolic content of the wines. The analyses were performed in triplicate, and the results are given as mg of gallic acid equivalents per liter (mg $GAE \cdot L^{-1}$).

2.4. Antioxidant activity - ABTS assay

The ABTS assay was used for the quantification of antioxidant activity, as described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999). The $ABTS^{\bullet+}$ method provides excellent consistency and is one of most rapid tests for antioxidant activity. The results are reproducible, and the test functions at various maximums of absorption and has good solubility, which allows for the analysis of lipophilic or hydrophilic compounds (Kuskoski, Asuero, Troncoso, Mancini Filho & Fett, 2005). This method is based on the generation of the highly stable chromophoric cation radical of $ABTS^{\bullet+}$ from a reaction between the ABTS solution and $K_2S_2O_8$. The absorbance of the radical diminishes in the presence of antioxidant components, which allows for the quantification of the antioxidant activity of a sample.

The cation radical $ABTS^{\bullet+}$ was obtained from the reaction of 7 mM ABTS diammonium salt solution with 2.45 mM $K_2S_2O_8$ solution mixed in a 1:1 (v/v) ratio and incubated for 16 h at room temperature in the dark. This solution was diluted in 80% ethanol (v/v) to give an absorbance of 0.700 ± 0.005 at 734 nm. Five different dilutions of each sample were prepared. Then, 0.5 mL of each diluted sample was added to 3.5 mL of the $ABTS^{\bullet+}$ solution and, after 6 min, the absorbance was measured at 734 nm. Trolox solutions with concentrations between 0 and 80 μM were used to obtain the calibration curve, which was used to calculate the reducing capacity of the wine samples. The results were expressed as the Trolox-equivalent antioxidant activity (TEAC) (mM TEAC L^{-1}). All solutions were prepared fresh daily.

2.5. Volatile compounds

The volatile compounds analyzed were propanol, isobutyl alcohol, n-butyl alcohol, sec-butyl alcohol, isoamyl alcohol, methanol, acetone, ethyl acetate, ethyl lactate and acetaldehyde. Samples of wine were distilled in a distillation flask coupled to a condenser. The samples were heated in a heating mantle (model 22E, Fisatom, São Paulo, Brazil) until two-thirds of the clear distillate was collected. Distilled samples were sent to the Laboratory Amazile Biagioni Maia – LABM (Belo Horizonte, Brazil) for analysis. As reported by the LABM, the analyses were performed with a gas chromatographer (model Geração 8000, GCROM, São Paulo, Brazil), equipped with a Megabore Carbowax® 20 M column (30 m x 0.53 mm and

1 µm film thickness) and a flame-ionizing detector using the following parameters: injector temperature, 140 ºC; detector temperature, 180 ºC; ramp-up temperature from 33 ºC to 165 ºC; and an injection volume of 2 µL. The results are expressed in $mg \cdot L^{-1}$.

2.6. Statistical analyses

The experiment used a completely randomized design with 11 treatments and two repetitions. Dunnett's test ($p < 0.05$) was used to compare the effects of ozone treatment on the polyphenol content, antioxidant activity and volatile compound content, in relation to the effects of SO_2 (standard treatment) and to the control. Data for ozone treatments were also analyzed by regression. The regression models were chosen based on the significance of the regression coefficients, as determined by Student's t-test at a 20% probability level, in coefficient of determination (R^2) and in the knowledge of the biological phenomenon. Three-dimensional curves of the response surface were produced using SigmaPlot 10.0. SAEG 9.0 software (Federal University of Viçosa) was used for both statistical analyses.

3. Results and discussion

The means of the results for polyphenolic content, antioxidant activity and volatile compound content of the wine samples are presented in Table 1.

Table 1

Volatile compounds, polyphenolic content and antioxidant activity, for the respective treatments

	Treatments										
	2 $mg.L^{-1}$ O_3			7 $mg.L^{-1}$ O_3			12 $mg.L^{-1}$ O_3			SO_2	Control
	5 min	15 min	25 min	5 min	15 min	25 min	5 min	15 min	25 min		
Polyphenolic content[1]	1803	1861	1708	2243	2462	2212	1852	1837	1915	2075	1998
Antioxidant activity[2]	4.42	4.71	4.04	3.48	4.21	3.71	3.94	4.21	3.93	3.84	4.74
Propanol[3]	62.42	62.79	61.28	60.15	54.18	57.81	59.51	56.49	58.74	45.22	60.51
Isobutyl Alcohol[3]	76.40	73.25	69.19	81.94	72.22	75.08	71.52	64.29	66.00	72.49	77.09
Isoamyl Alcohol[3]	252.29	255.69	251.03	259.60	237.24	248.66	239.93	248.89	241.93	225.00	250.71
n-Butyl Alcohol[3]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Sec-butyl Alcohol[3]	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Methanol[3]	271.04	310.15	226.39	298.56	287.76	313.33	277.81	269.06	304.29	222.68	168.34
Acetone[3]	0.81	0.86	0.51	1.11	1.42	1.33	0.89	1.05	1.00	1.15	0.96
Ethyl acetate[3]	26.08	19.76	17.07	31.43	22.89	27.62	25.61	22.81	20.37	43.79	26.92
Ethyl lactate[3]	8.04†	9.34*†	10.92*†	9.12*†	10.28*†	11.43*†	7.60†	9.80*†	10.91*†	5.48*	7.18†
Acetaldehyde[3]	64.71*	71.41*	83.02*†	54.36*	78.49*	82.86*†	64.53*	90.88*†	81.38*†	61.80*	25.31†

[1] expressed in mg GAE L^{-1}; [2] expressed in mM TEAC L^{-1}; [3] expressed in $mg.L^{-1}$; *Differ from control treatment in the lines by Dunnet test ($p<0.05$); †Differ from SO_2 treatment in the lines by Dunnet test ($p<0.05$); n.d, not detected.

No significant differences (*p* > 0.05) were found between the mean polyphenolic contents of the different ozone treatment relative to the control and SO_2 treatments. Polyphenolic content ranged from 1708 to 2462 mg GAE·L^{-1} (mean of 1997.38 ± 228.29 mg GAE·L^{-1}). Mean polyphenolic contents lower than 2000 mg GAE·L^{-1} in Syrah wines were also found by Ivanova-Petropulos, Ricci, Nedelkovski, Dimovska, Parpinello and Versari (2015). On average, Syrah wines have slightly lower phenolic levels compared to other varieties, such as Merlot and Cabernet Sauvignon (Ivanova-Petropulos et al., 2015; Fanzone et al., 2012). No previous studies are available on the application of ozone to wine, but Torres et al. (2011) showed that 4.8% w/w ozone applied to apple juice for 10 min reduced the total phenolic content by 49.7%. However, Botondi et al. (2015), investigating the use of ozone fumigation for the safety and quality of wine grapes postharvest, concluded that ozone was able to preserve the polyphenol content.

No significant differences (*p* > 0.05) were found between the mean antioxidant activities of the different ozone treatment relative to the control and SO_2 treatments, as demonstrated by the $ABTS^{\bullet +}$ results. The antioxidant activities ranged from 3.48 to 4.74 mM TEAC L^{-1} (mean 4.11 ± 0.39 mM TEAC L^{-1}). Despite the polyphenolic compounds present in wines known to have a high antioxidant capacity (Paixão et al., 2007), in the present study, a low correlation was found between these parameters ($r^2 = 0.138$, $p = 0.27$). Similar results were found by Heinonen, Lehtonen and Hopia (1998) in studying the antioxidant activity of berry and fruit wines and liquors. According the author, the lack of a correlation between the total phenolic content and the antioxidant activity may be, in part, explained by a poor response of anthocyanins in the Folin-Ciocalteu assay. Furthermore, the specific phenolic compounds present in the wines produced in the present study and their interactions with ozone were not known. New compounds with different properties may have been formed, or products of potential interactions with ozone may have interfered with the methods utilized for the quantification of total phenolic content and/or antioxidant activity.

No significant differences ($p > 0.05$) were found between the mean volatile compound contents of the different ozone treatment to the control and SO_2 treatments for propanol (58.10 $mg \cdot L^{-1}$), isobutyl alcohol (72.68 $mg \cdot L^{-1}$), isoamyl alcohol (246.45 $mg \cdot L^{-1}$), methanol (268.13 $mg \cdot L^{-1}$), acetone (1.01 $mg \cdot L^{-1}$) and ethyl acetate (25.85 $mg \cdot L^{-1}$). The N-butyl alcohol content was less than 0.1 $mg \cdot L^{-1}$ for all treatments and sec-butyl alcohol was not detected in any samples. Ortega-Heras, Rivero-Pérez, Pérez-Magariño, González-Huerta and González-Sanjosé (2008) found few changes in volatile compound contents of wine oxidized by micro-oxygenation. In general, it is believed that higher alcohols contribute more to the intensity of the odor of the wine than to its quality (Câmara et al., 2006). According to Cabanis & Cabanis (2000), the normal concentrations of the higher alcohols analyzed are less than 68 $mg \cdot L^{-1}$ for propanol, between 9 and 148 $mg \cdot L^{-1}$ for isobutyl alcohol, less than 490 $mg \cdot L^{-1}$ for isoamyl alcohol, and between 43 and 320 $mg \cdot L^{-1}$ for methanol. However, Brazilian legislation states that wine can have a maximum methanol content of 300 $mg \cdot L^{-1}$ because of its high toxicity (Brasil, 2010). Wines treated with ozone at 2 $mg \cdot L^{-1}$ for 15 min and at 7 and 12 $mg \cdot L^{-1}$ for 25 min had mean methanol contents over 300 $mg \cdot L^{-1}$. Methanol occurs naturally at low levels in fresh fruit juices and is produced in large quantities after the enzymatic degradation of the pectins present in grape skins. During the mashing, fermentation, and aging stages of winemaking, pectin methyl esterase (PME) causes the release of methanol (Hou et al., 2008). The higher content of methanol in wines treated with ozone may indicate an effect of ozone on grape pectin or on PME during fermentation, which must be further studied.

The means values of acetone were below than those normally found in wine (3 to 32 mg·L^{-1}, Hashizume (2001)). The concentrations of ethyl acetate in wines produced varied range 17.07 to 43.79 mg·L^{-1}, being the higher value reached with SO_2 treatment. Ethyl acetate is the most important ester present in wine, and when present at levels between 50 and 80 mg·L^{-1}, it contributes to the aroma of the wine. However, it confers an aggressive smell to wine when present at levels above 160 mg·L^{-1} (Peynaud, 1984). In all wines produced in the present study, the concentrations of ethyl acetate were below the desirable levels.

Mean ethyl lactate content was significantly higher ($p < 0.05$), relative to that of the control (7.18 mg·L^{-1}), in wines treated with ozone at 2 mg·L^{-1} for 15 and 25 min, at 7 mg·L^{-1} for 5, 15 and 25 min, and at 12 mg·L^{-1} for 15 and 25 min (means: 9.34, 10.92, 9.12, 10.28, 11.43, 9.80 and 10.91 mg·L^{-1}, respectively). The mean ethyl lactate content of SO_2 treated wines (5.48 mg·L^{-1}) was significantly lower ($p < 0.05$) than in all other treatments, including the control. However, the values of ethyl lactate reported to potentially influence the overall aroma of wine are over 200 mg·L^{-1} (Ugliano & Moio, 2005). This compound is present in wine as a mixture of enantiomers, which can vary according to the species of microorganisms contributing to the winemaking process (Ugliano & Moio, 2005). Specifically, it has been reported that the (S)-enantiomer is produced in high amounts by *Oenococcus oeni* (Lloret et al., 2002).

Mean acetaldehyde content was significantly higher ($p < 0.05$), relative to that of the control (25.31 mg·L^{-1}), in all ozone treatments. The acetaldehyde content of wines treated with ozone at 2 and 7 mg·L^{-1} for 25 min, and at 12 mg·L^{-1} for 15 and

25 min (means: 83.02, 82.86, 90.88 and 81.38 mg·L^{-1}, respectively) were significantly higher ($p < 0.05$) than that observed with SO_2 treatment (61.80 mg·L^{-1}). Acetaldehyde is a highly volatile compound that originates from yeast metabolism during alcoholic fermentation and from ethanol oxidation (Torres Neto, Silva, Silva, Swarnakar & Silva, 2006). When present in excess, it imparts an undesirable green, grassy and apple-like aroma, which is usually masked by the addition of sulfur dioxide (SO_2) (Osborne, Orduña, Pilone & Liu, 2000). Sensory detection limits for red wines are typically in the range of 40-100 mg·L^{-1}, and when acetaldehyde reaches these levels, consumers may consider the wine spoiled. In the present study, all treatments (except the control) reached the sensorial detection limit of acetaldehyde, which can result in rejection of the product by the consumer. Carlton, Gump, Fugelsang and Hasson (2007) have previously shown that excessive oxidation may result in increased levels of acetaldehyde, which may explain its high values in the ozonated wines.

Regression analyses found models with a satisfactory fit ($R^2 > 0.500$) only for ethyl lactate and acetaldehyde (Table 2). The response surfaces were plotted as a function of ozone concentration and treatment time (Fig. 1 and 2, for ethyl lactate and acetaldehyde, respectively). A quadratic effect of ozone concentration ($p < 0.01$) and a linear effect of exposure time ($p < 0.01$) on ethyl lactate content was found. According to the model, ethyl lactate reached a maximum at 7.02 mg·L^{-1} of ozone at any exposure time. At a constant ozone concentration, each minute of exposure to ozone increased ethyl lactate by 0.1417 mg·L^{-1}. For acetaldehyde, the model found a linear effect of ozone concentration ($p < 0.20$) and a quadratic effect of exposure time ($p < 0.10$). Keeping treatment time constant, each unit increase in ozone concentration caused an increase of 0.5885 mg·L^{-1} in acetaldehyde. At any concentration of ozone, a maximum acetaldehyde level is reached at 21.3 min of treatment. The results of the other parameters' measures did not show a satisfactory fit to any model. There was no effect of the interaction between ozone concentration and treatment time on any of the parameters studied.

Table 2

Regression equations adjusted for the volatile compounds in function of ozone concentration ($mg.L^{-1}$) and treatment time (min) and their respective determination coefficient (R^2)

Parameters	Equation	R^2
Polyphenolic content	$\hat{y} = 1988.65$	-
Antioxidant activity	$\hat{y} = 4.0715$	-
Propanol	$\hat{y} = 59.2636$	-
Isobutyl alcohol	$\hat{y} = 72.21$	-
Isoamyl alcohol	$\hat{y} = 248.3615$	-
Methanol	$\hat{y} = 284.2639$	-
Acetone	$\hat{y} = 0.9984$	-
Ethyl acetate	$\hat{y} = 23.7375$	-
Ethyl lactate	$\hat{y} = 6.4929 + 0.4743^{**}x - 0.0338^{**}x^2 + 0.1417^{**}z$	0.883
Acetaldehyde	$\hat{y} = 41.2161 + 0.5885^{\Delta}x + 3.5957^{*}z - 0.0844^{\square}z^2$	0.517

* Significative to 5%; ** significative to 1%; $^{\square}$ significative to 10%; $^{\Delta}$ significative to 20%

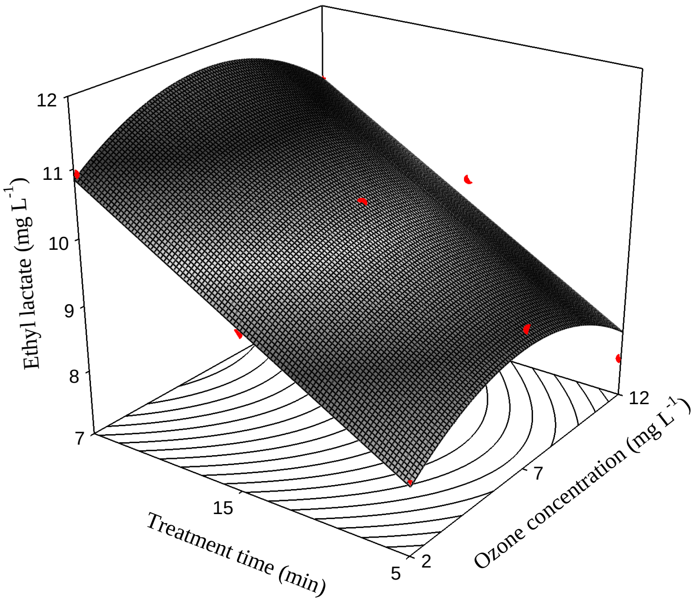

Figure 1. Response surface plots of effect of ozone concentration (mg.L^{-1}) and treatment time (min) on ethyl lactate of wine

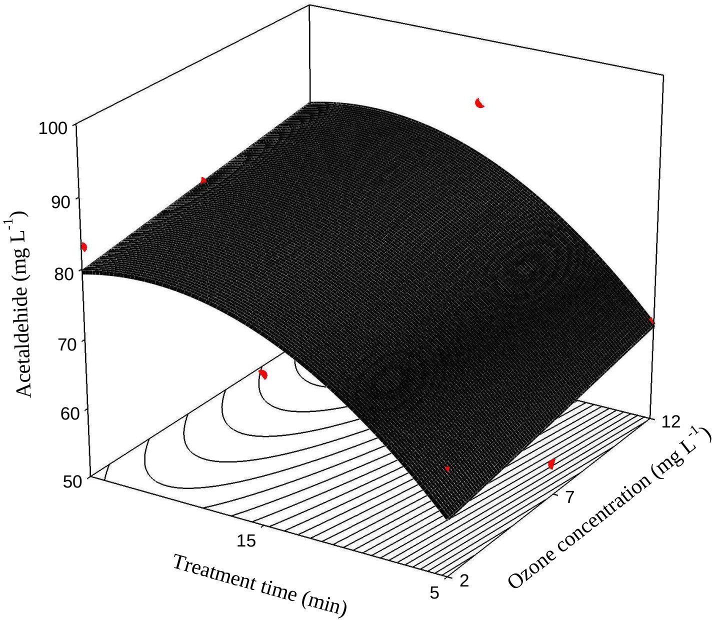

Figure 2. Response surface plots of effect of ozone concentration (mg.L^{-1}) and treatment time (min) on acetaldehide of wine

4. Conclusion

Ozone did not cause a significant change in phenolic content or antioxidant activity and did not change the content of most of the volatile compounds analyzed. However, acetaldehyde reached the sensorial detection limit for all treatments and, for some ozone treatments, methanol was present at mean values over that permitted by Brazilian legislation. These compounds are important for the sensorial quality and safety of wines, and such changes should be taken into account prior to employing ozone as a substitute of sulfite in wine production, giving priority to decreasing the concentration of ozone used and the duration of the treatment times.

Further experiments are needed to elucidate the specific reactions between ozone and various wine components, such as phenolic compounds and pectin, and to determine the toxicity of the products that may be formed.

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ARTIGO 4

EFFECTS OF CONTINUOUS EXPOSURE TO OZONE GAS AND ELECTROLYZED WATER ON THE SKIN HARDNESS OF TABLE AND WINE GRAPE VARIETIES

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Abstract

The effects of continuous exposure to ozone gas (O_3, 30 µL/L, 24 h) and immersion in electrolyzed water (EW, 400 mg/L free chlorine, 10 min) on skin hardness of Italia and Muscat Hamburg table grapes, and Merlot and Barbera wine grapes were investigated and compared with those of control treatments (air and deionized water, DW). Skin hardness was instrumentally evaluated via measurements of skin break energy using a puncture test. For all cultivars and density classes, ozone treatment was more effective in increasing skin hardness than electrolyzed water treatment, although the differences were not always significant. For the same treatment, skin hardening was independent of berry density, and the magnitude of this effect was cultivar-dependent. This skin hardening might positively affect the postharvest life of table grapes. In wine grapes, increases in skin break energy values were related to higher yields and slow extraction kinetics of phenolic compounds during maceration.

Practical Applications

The purpose and the originality of this work were to determine the real impact of continuous exposure to ozone gas and immersion in electrolyzed water on skin hardness in table grape and wine grape varieties. Differences in the treatment effect due to the grape maturity level were studied in berries sorted according to density. For all cultivars and density classes evaluated, ozone treatment was more effective for increasing skin hardness than electrolyzed water treatment although the differences were not always significant. Therefore, the use of ozone gas as alternative sanitizing agent could extend the postharvest life of table grapes and

facilitate the extraction of phenolic compounds from wine grapes during maceration according to increased skin hardness.

Keywords: ozone gas, electrolyzed water, skin hardness, texture analysis, grapes

Introduction

Grapes used for fresh consumption, winemaking and raisin production are among the most cultivated fruits worldwide (FAO, 2013). Grapes are considered perishable fruits because their surfaces are characterized by the coexistence of various microorganisms, including filamentous fungi, yeasts and bacteria, that have different effects on the quality and shelf life of the final product (Barata *et al*. 2012). Table grapes are especially susceptible to fungal infection by *Botrytis cinerea*, which can induce gray mold disease and berry dehydration (Guentzel *et al*. 2010; Rolle *et al*. 2012a). In wine grapes, the growth of yeasts, such as *Brettanomyces* species, can result in off-flavors in wine that are primarily due to the formation of volatile phenols (Kheir *et al*. 2013).

Sulfur dioxide (SO_2) is an effective preservative that is commercially used to control the postharvest decay of table grapes and the microbial ecology of the must. However, its use is increasingly being restricted to avoid issues associated with sulfite residues, SO_2 emissions and berry damage that affects sensory quality (Feliziani *et al*. 2014). Currently, there is a considerable interest in research on alternative, safe and effective sanitizing agents such as ozone and electrolyzed water.

Ozone (O_3) is a powerful antimicrobial agent against a wide spectrum of microorganisms. The mechanism of microbial inactivation is complex because ozone attacks numerous cellular constituents (Khadre *et al*. 2001). In the gaseous and aqueous phases, ozone has previously been already as an environment-friendly alternative to traditional approaches for postharvest fruit and vegetable microbial control due to its high oxidizing power and rapid degradation that leaves not residues (Horvitz and Cantalejo 2014; Karaca and Velioglu 2007; Sengun *et al.* 2014). Another alternative eco-friendly sanitizer is electrolyzed water (EW), which has been used to preserve the quality and safety of fresh-cut fruits and vegetables (Artés *et al*. 2009; Jemni *et al*. 2014). EW has a strong antimicrobial effect against pathogens and spoilage microorganisms due to the combined actions of hydrogen ions, its oxidation-reduction potential and free chlorine (Huang *et al*. 2008). In contrast to bleaching treatments involving hypochlorite, acidic and near-neutral EW contains primarily hypochlorous acid and thus leaves far less chlorine residue on the fruit (Guentzel *et al*. 2010). Therefore, human health and safety issues are minimized. Although the efficacies and sustainabilities of ozone and EW as antimicrobial agents for use in the control of the postharvest decay of grapes have been demonstrated, the effects of these treatments on the grape quality attributes have been minimally studied (Artés-Hernández *et al*. 2007; Botondi *et al.* 2015; Feliziani *et al*. 2014; Smilanick *et al*. 2002).

Texture is one of the most important quality characteristics of fresh fruits and plays a key role in the perceived quality and overall acceptability (Ha *et al*. 2007; Konopacka and Plocharski 2004). In table grapes, firmness and crunchiness are sensory quality traits that are highly appreciated by consumers (Sato *et al*. 2006). Instrumental measurements of the mechanical properties of the whole berry, flesh and skin have been used to objectively and quantitatively assess the textural quality of fresh table grapes (Giacosa *et al*. 2014; Río Segade *et al*. 2013a) and to monitor their postharvest shelf life (Deng *et al*. 2005). Specifically, the skin plays a key role in gas exchange regulation and berry resistance to injury and diseases during harvest and postharvest handling (Battista *et al.* 2015). In wine grapes, skin hardness permits an easy estimation of anthocyanin extraction kinetics with adequate reliability (Rolle *et al*. 2008).

There is no information available about the possible changes to the skin mechanical properties of grapes that are elicited during or after berry treatments with ozone or electrolyzed water. Therefore, the purpose of this study was to evaluate the effects of exposure to ozone gas and submersion in electrolyzed water on the skin hardness of table and wine grapes. The study was performed with *Vitis vinifera* L. cv. Italia and Muscat Hamburg table grapes and Merlot and Barbera wine grapes to assess the effects of the two treatments on fresh grape berries with different skin hardness. Furthermore, the table and wine grape varieties investigated in this study are highly appreciated and commercialized worldwide for fresh consumption and for the production of renowned red wines, respectively.

Materials and Methods

Grape samples

The study was performed in 2014 with one white (Italia) and three red (Muscat Hamburg, Merlot and Barbera) *Vitis vinifera* L. cultivars grown in Italy. Italia and Muscat Hamburg table grapes were purchased from the local market. Barbera and Merlot wine grapes were harvested at an experimental vineyard located in the North-West Italy growing zone (Cuneo province). For each cultivar, approximately 10 kg of berries were randomly picked with short pedicels attached. To define the different maturity levels and to increase the intrasample homogeneity, the berries were sorted according to their density by flotation in saline solutions with sodium chloride concentrations ranging 100 to 190 g/L, which correspond to densities between 1069 and 1125 kg/m^3 (Rolle *et al.* 2012b). The study was performed with berries belonging to the three most representative density classes, which accounted for total relative weights higher than 85% w/w as follows: A = 1069 kg/m^3, B = 1075 kg/m^3 and C = 1081 kg/m^3 for Italia; B = 1075 kg/m^3, C = 1081 kg/m^3 and D = 1088 kg/m^3 for Muscat Hamburg; D = 1088 kg/m^3, E = 1094 kg/m^3 and F = 1100 kg/m^3 for Merlot; and G = 1107 kg/m^3, H = 1115 kg/m^3 and I = 1119 kg/m^3 for Barbera. The sorted berries were washed with water and visually inspected prior to treatment, and those with damaged skins were discarded.

For each cultivar and density class, two sets of 60 sorted berries were randomly selected for application to the ozone and EW treatments. The remaining two sets of 60 sorted berries were used as control samples; one group was exposed to the air, and the other was dipped in deionized water. Subsequently, the berries were placed into open polystyrene boxes in numbered positions. The remaining berries were subdivided into two replicates and used to determine the technological ripeness parameters of the grape juice obtained by manual crushing and centrifugation in a PK 131 centrifuge (ALC International, MI, Italy) for 5 min at 4000 *g* at 20 °C.

Technological ripeness parameters

Total soluble solid concentrations (ºBrix, as SSC) were measured using an Atago 0–32 °Brix temperature compensating refractometer (Atago Corporation, Tokyo, Japan), the pH was determined by potentiometry using an InoLab 730 pH meter (WTW, Weilheim, Germany), and titratable acidity (g/L tartaric acid, as TA) was estimated using the OIV official method (OIV, 2008). Organic acids (citric acid, tartaric acid and malic acid) and reducing sugars (glucose and fructose) (g/L) were determined according to the methods of Giordano *et al*. (2009) using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD) set to 210 nm and a refractive index detector, respectively.

Ozone gas, electrolyzed water production and berry treatment

Ozone gas was produced using an ozone generator (Model C32-AG, Industrie De Nora SpA, MI, Italy) that was fed by an oxygen concentrator and had a nominal production capacity of 32 g O_3/h. The ozone gas was released into a sealed chamber. The ozone concentration in the chamber was adjusted to 30 µL/L and continuously monitored by the recirculation of ozone-enriched air from the chamber through a BMT 964 UV-photometric ozone analyzer (BMT Messtechnik Gmbh, DE) that controlled the ozone generator output. The environmental conditions of the chamber were continuously recorded during the berry treatments using temperature and relative humidity data loggers (HOBO H8 RH/Temp, Onset Computer Corporation, Bourne, MA, USA). The sample boxes were introduced into the chamber, and the berries were exposed to ozone for 24 hours at a relative humidity of 57 ± 3% and a temperature of 20 ± 1 °C. Air exposed berries subjected to the same environmental conditions were used as control samples.

EW was produced from diluted salt (KCl) in tap water using an Eva System® 100 (Industrie De Nora SpA). The system produced EW of approximately 4 g/L free chlorine, pH 9 and 1% residual KCl. For the berry treatment, the original EW was diluted to 400 mg/L free chlorine using deionized water. Free chlorine concentration and pH were confirmed by iodometric titration (APHA, 1992) and potentiometry with an InoLab 730 pHmeter, respectively. Each berry was singularly immersed into 10 mL of EW for 10 minutes. The control samples were identically treated using deionized water (DW) rather than electrolyzed water (EW).

Berry skin mechanical parameters

A Universal Testing Machine (UTM) TA.XTplus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) equipped with an HDP/90 platform and a 5-kg load cell was used for the berry skin texture analyses. Skin hardness was evaluated with a puncture test using an SMS P/2N needle probe (Stable Micro Systems), a test speed of 1 mm/s and a penetration depth of 3 mm (Letaief *et al.* 2008). Each berry (before and after treatment) was individually punctured in the lateral face, and the skin break energy (mJ, as W_{sk}) was measured. The use of a needle probe allowed for the independent estimation of skin hardness by minimizing possible interference from the pulp firmness. All data were acquired at 500 points per second, and the skin mechanical properties were calculated from force-distance curves using the Texture Exponent software package (Stable Micro Systems).

The berries were singularly evaluated before and 4 hours after the treatment. To allow for the estimation of the storage effects after treatment, the table grapes samples were also analyzed 7 days after treatment. Additionally, the wine grape berries that were treated with electrolyzed/deionized water were also analyzed 24 hours after treatment. This last evaluation was not considered appropriate for the ozone/air treatment samples of the wine grapes because they are typically processed shortly after harvest, and the postharvest treatment itself already accounted for

24 hours. The storage conditions were a relative humidity of 57 ± 3% and a temperature of 20 ± 1 °C.

The results were then expressed as the means of the differences between the berry skin break energy measurements after and before treatment, which were calculated individually for each berry and after-treatment measurement.

Statistical analyses

The statistical analyses were performed using the SPSS Statistics software package (IBM Corporation, Armonk, NY, USA). The Tukey-b test was used to establish significant differences at $p < 0.05$ following one-way analysis of variance (ANOVA).

Results and Discussion

Technological ripeness parameters

The technological ripeness parameters of the Italia, Muscat Hamburg, Merlot and Barbera grapes sorted according to density are shown in Table 1. For each cultivar, the SSC values increased significantly with increased berry density. The pH and TA values were not significantly different according to berry density, with the exception of the Merlot berries. For this cultivar, significant decreasing trends in the concentrations of tartaric, malic and citric acids were observed with increasing berry density, and these trends agreed with the TA values. In the table grapes, the SSC/TA ratios also increased significantly with increasing berry density. In the wine grapes, this ratio was not relevant. The changes in these chemical parameters with berry density agreed with those observed in previous studies of various table grape (Río Segade *et al*. 2013a,b, ; Rolle *et al*. 2015) and wine grape (Rolle *et al*. 2012b; Zouid *et al*. 2013) cultivars.

Berry skin mechanical parameters

In this study, berry skin hardness was evaluated as skin break energy (W_{sk}). The Italia and Muscat Hamburg table grape and the Merlot and Barbera wine grape cultivars were selected according to the W_{sk} values of the untreated berries (0.578 ± 0.256, 0.868 ± 0.292, 1.041 ± 0.242 and 1.189 ± 0.350 mJ, respectively). These values were within the usual ranges for these cultivars given the variations maturity, climate, season, soil and viticulture (Giacosa *et al*. 2013; Río Segade *et al*. 2013b; Rolle *et al*. 2015).

A preliminary test that involved puncturing the berry skin in different equatorial places (5 punctures for each of 10 berries) was performed and revealed that for the studied cultivars, variations in W_{sk} (ΔW_{sk}) of approximately ±0.08 mJ due to puncture position were observed. Letaief *et al.* (2008) previously reported that the effect of the berry puncture position (i.e., bottom, side and top) on the W_{sk} value is variety dependent. With the aim of considering only treatment effects and avoiding the variations inherent to the mechanical method, ΔW_{sk} values between -0.08 and +0.08 mJ were not taken into account.

TABLE 1. TECHNOLOGICAL RIPENESS PARAMETERS OF ANALYZED GRAPE SAMPLES SORTED ACCORDING TO DENSITY.

Cultivar	Density class	SSC (ºBrix)	pH	TA (g/L tartaric acid)	SSC/TA ratio	Glucose/Fructose ratio	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)
Italia	A	16.6 ± 0.1a	3.62 ± 0.01	3.21 ± 0.01	51.02 ± 0.01a	0.948 ± 0.001a	0.16 ± 0.01	6.04 ± 0.01b	1.75 ± 0.01
	B	18.1 ± 0.1b	3.61 ± 0.05	3.19 ± 0.26	56.49 ± 4.18a	0.985 ± 0.001c	0.20 ± 0.01	5.72 ± 0.14b	1.52 ± 0.10
	C	19.4 ± 0.1c	3.72 ± 0.03	2.70 ± 0.08	71.99 ± 2.86b	0.978 ± 0.001b	0.15 ± 0.01	5.25 ± 0.02a	1.55 ± 0.04
	Sign^a	***	ns	ns	*	***	ns	**	ns
Muscat Hamburg	B	17.7 ± 0.1a	3.62 ± 0.07	4.09 ± 0.27	44.21 ± 3.01a	0.999 ± 0.009	0.33 ± 0.01	3.58 ± 0.10a	2.88 ± 0.09b
	C	18.8 ± 0.1b	3.59 ± 0.02	4.16 ± 0.16	46.46 ± 1.75a	0.996 ± 0.008	0.32 ± 0.03	3.84 ± 0.01a	2.59 ± 0.15b
	D	21.1 ± 0.1c	3.71 ± 0.08	3.92 ± 0.03	55.38 ± 0.26b	0.969 ± 0.005	0.29 ± 0.01	4.46 ± 0.15b	1.83 ± 0.04a
	Sign^a	***	ns	ns	*	ns	ns	**	**
Merlot	D	20.2 ± 0.1a	3.44 ± 0.02	6.30 ± 0.11b	-	1.029 ± 0.001	0.18 ± 0.01b	5.91 ± 0.05b	2.47 ± 0.03b
	E	21.5 ± 0.2b	3.43 ± 0.04	6.09 ± 0.24ab	-	1.031 ± 0.006	0.17 ± 0.01ab	5.91 ± 0.05b	2.16 ± 0.15b
	F	23.5 ± 0.1c	3.47 ± 0.02	5.59 ± 0.05a	-	1.019 ± 0.002	0.11 ± 0.02a	5.59 ± 0.06a	1.73 ± 0.07a
	Sign^a	***	ns	*		ns	*	*	*
Barbera	G	24.6 ± 0.1a	3.13 ± 0.02	9.49 ± 0.05	-	1.010 ± 0.004b	0.34 ± 0.01c	7.87 ± 0.49	4.09 ± 0.09c
	H	25.5 ± 0.1b	3.13 ± 0.04	9.13 ± 0.40	-	1.007 ± 0.001b	0.30 ± 0.01b	7.46 ± 0.54	3.64 ± 0.02b
	I	28.7 ± 0.1c	3.04 ± 0.01	9.71 ± 0.05	-	0.994 ± 0.001a	0.26 ± 0.01a	7.83 ± 0.01	3.30 ± 0.01a
	Sign^a	***	ns	ns		*	**	ns	**

All data are expressed as average value ± standard deviation (n = 2). Different Latin letters within the same column indicate significant differences (^{a}) among the three density classes for each cultivar (Tukey-b test; $p < 0.05$). Sign^a: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively. A = 1069 kg/m^3; B = 1075 kg/m^3; C = 1081 kg/m^3; D = 1088 kg/m^3; E = 1094 kg/m^3; F = 1100 kg/m^3; G = 1107 kg/m^3; H = 1115 kg/m^3; I = 1119 kg/m^3. SSC = total soluble solids concentration; TA = titratable acidity; SSC/TA = SSC expressed as g/L and TA expressed as g/L tartaric acid.

Tables 2 and 3 illustrate the variations between the determinations of this mechanical parameter before and after the berry treatments in densimetrically sorted table and wine grapes, respectively. For all cultivars and treatments, no significant differences were observed in the variations in skin hardness among the berries of the three studied density classes with the exception of the Barbera wine grapes at 24 h after EW treatment. Previously published studies have shown that W_{sk} values are not influenced by berry density in table grape (Río Segade et al., 2013a,b) or wine grape (Rolle *et al.* 2012b) cultivars.

With a few exceptions following the EW and DW treatments, the ΔW_{sk} values were positive, which indicated increased skin hardness after each treatment. Furthermore, these increases were significantly greater at 7 days after all treatments of the Italia and Muscat Hamburg table grapes and at 24 h after treatments of the Barbera wine grapes with EW and DW compared to the measurements taken 4 h after the treatments. This skin hardening has occurred during the postharvest storage of the fruit without significant berry weight loss. Increases in skin hardness are also observed during the on-vine dehydration process of wine grapes under uncontrolled environmental conditions (Rolle *et al.* 2010), and increases in the skin resistance to puncturing during postharvest dehydration have even been reported at 20 ºC and 45% RH (Muganu *et al.* 2011).

When the effects of the different examined berry treatments on skin hardness were compared among the table grape cultivars, the highest ΔW_{sk} values were observed in the ozone-treated berries of any density class at 4 h and 7 days after treatment. The differences in the ΔW_{sk} values were not significant between the berries that were exposed to ozone and air or between the berries that were treated with EW or DW (the berries that were exposed to air and treated with DW were used as the control samples as mentioned above in the materials and methods section). Nevertheless, regarding the Italia grapes belonging to density classes B and C, the increases in skin hardness were significantly greater among the ozone-treated berries than among the EW-treated berries. The percentages of berries with significantly harder skins ($\Delta W_{sk} > 0.08$ mJ) varied between 35.6% and 73.3% (following EW treatment, the density classes B and C exhibited values of 35.6% and 36.7%, respectively, and following ozone treatment, these values were 70.0% and 59.3%, respectively) at 4 h after berry treatment, whereas these values ranged from 73.3% to 94.4% (following EW treatment, the density classes B and C exhibited values of 78.0% and 73.3%, respectively, and following ozone treatment, these values were 91.7% and 89.8%, respectively) after 7 days. For the Muscat Hamburg grapes, significant differences in the ΔW_{sk} values were also observed between berries that were treated with ozone and those that were treated with EW at 4 h after treatment in the density class D and at 7 days after treatment in the density classes C and D. In these cases, the proportions of berries with significantly harder skins varied between 30.0% and 70.0% (following EW treatment, the density class D exhibited a value of 35.6% for EW, and this value was 70.0% following ozone treatment) at 4 h after berry treatment, whereas these values ranged from 71.1% to 96.7% (following EW treatment, density classes C and D exhibited values of: 74.6% and 71.1%, respectively, and these values following ozone treatment were 88.3% and 96.7%, respectively) after 7 days.

TABLE 2. SKIN HARDNESS OF ITALIA AND MUSCAT HAMBURG TABLE GRAPES SORTED ACCORDING TO DENSITY.

Cultivar	Treatment	Density class	ΔW_{sk} after 4 hours (mJ)	ΔW_{sk} after 7 days (mJ)	Sign[c]
Italia	EW	A	0.088 ± 0.375αβ	0.361 ± 0.306	**
		B	-0.007 ± 0.276α	0.278 ± 0.342α	***
		C	0.008 ± 0.271α	0.258 ± 0.272α	***
	Sign[a]		ns	ns	
	DW	A	-0.023 ± 0.288α	0.408 ± 0.380	***
		B	0.013 ± 0.272αβ	0.272 ± 0.300α	***
		C	0.066 ± 0.323αβ	0.298 ± 0.333αβ	***
	Sign[a]		ns	ns	
	O_3	A	0.220 ± 0.303β	0.543 ± 0.318	***
		B	0.206 ± 0.282β	0.495 ± 0.281β	***
		C	0.153 ± 0.269β	0.494 ± 0.351β	***
	Sign[a]		ns	ns	
	Air	A	0.110 ± 0.198αβ	0.511 ± 0.242	***
		B	0.101 ± 0.328αβ	0.441 ± 0.324αβ	***
		C	0.038 ± 0.313αβ	0.366 ± 0.315αβ	***
	Sign[a]		ns	ns	
Sign[b]			*, ***, *	ns, ***, ***	
Muscat Hamburg	EW	B	-0.039 ± 0.310	0.349 ± 0.390	***
		C	0.020 ± 0.309	0.273 ± 0.338α	***
		D	-0.051 ± 0.302α	0.280 ± 0.400α	***
	Sign[a]		ns	ns	
	DW	B	0.008 ± 0.354	0.331 ± 0.448	***
		C	0.060 ± 0.319	0.341 ± 0.415αβ	***
		D	-0.017 ± 0.281α	0.360 ± 0.370αβ	***
	Sign[a]		ns	ns	
	O_3	B	0.076 ± 0.342	0.432 ± 0.438	***
		C	0.108 ± 0.285	0.454 ± 0.334β	***
		D	0.234 ± 0.415β	0.548 ± 0.316β	**
	Sign[a]		ns	ns	
	Air	B	0.072 ± 0.352	0.401 ± 0.413	***
		C	0.085 ± 0.324	0.361 ± 0.386αβ	***
		D	0.105 ± 0.314αβ	0.353 ± 0.243αβ	**
	Sign[a]		ns	ns	
Sign[b]			ns, ns, **	ns, *, **	

All data are expressed as average value ± standard deviation (n = 60). Sign[a,b,c]: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, ([a]) among density classes, ([b]) among treatments and ([c]) among times after treatment. Different Greek letters within the same column indicate significant differences ([b]) among treatments for each density class and cultivar (Tukey-b test; $p < 0.05$). A = 1069 kg/m^3; B = 1075 kg/m^3; C = 1081 kg/m^3; D = 1088 kg/m^3. EW = electrolyzed water; DW = deionized water; O_3 = ozone gas; ΔW_{sk} = variation between skin break energy determinations performed before and after berry treatment.

TABLE 3. SKIN HARDNESS OF MERLOT AND BARBERA WINE GRAPES SORTED ACCORDING TO DENSITY.

Cultivar	Treatment	Density class	ΔW_{sk} after 4 hours (mJ)	ΔW_{sk} after 24 hours (mJ)	Sign^c
Merlot	EW	D	0.056 ± 0.314	0.068 ± 0.282	ns
		E	-0.018 ± 0.288α	0.058 ± 0.327	ns
		F	0.043 ± 0.312	0.028 ± 0.357	ns
	Sign^a		ns	ns	
	DW	D	0.072 ± 0.282	0.093 ± 0.306	ns
		E	-0.007 ± 0.316α	0.108 ± 0.298	*
		F	0.060 ± 0.314	0.034 ± 0.310	ns
	Sign^a		ns	ns	
	O_3	D	0.054 ± 0.299	-	
		E	0.141 ± 0.328β	-	
		F	0.108 ± 0.338	-	
	Sign^a		ns		
Sign^b			ns, *, ns	ns, ns, ns	
Barbera	EW	G	0.082 ± 0.385	0.231 ± 0.424ab	*
		H	0.046 ± 0.384	0.403 ± 0.456b	***
		I	-0.039 ± 0.282α	0.111 ± 0.379a	*
	Sign^a		ns	***	
	DW	G	0.075 ± 0.431	0.231 ± 0.334	*
		H	0.013 ± 0.297	0.200 ± 0.423	**
		I	-0.032 ± 0.410α	0.222 ± 0.355	**
	Sign^a		ns	ns	
	O_3	G	0.234 ± 0.412	-	
		H	0.110 ± 0.432	-	
		I	0.249 ± 0.585β	-	
	Sign^a		ns		
Sign^b			ns, ns, **	ns, ns, ns	

All data are expressed as average value ± standard deviation (n = 60). Sign^{a,b,c}: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, (^{a}) among density classes, (^{b}) among treatments and (^{c}) among times after treatment. Different Latin letters within the same column indicate significant differences (^{a}) among density classes for each treatment and cultivar (Tukey-b test; $p < 0.05$). Different Greek letters within the same column indicate significant differences (^{b}) among treatments for each density class and cultivar (Tukey-b test; $p < 0.05$). D = 1088 kg/m^3; E = 1094 kg/m^3; F = 1100 kg/m^3; G = 1107 kg/m^3; H =1115 kg/m^3; I = 1119 kg/m^3. EW = electrolyzed water; DW = deionized water; O_3 = ozone gas; ΔW_{sk} = variation between skin break energy determinations performed before and after berry treatment.

Rodoni *et al*. (2010) reported decreased pectin solubilization, polyuronide depolymerization and pectin methyl esterase activity in ozone-treated fruits. Reduced fruit softening following exposure to ozone might be related to reduced disassembly of cell wall pectic polysaccharides. Furthermore, the cuticle may also be a determinant factor of changes in the mechanical properties of fruit (Lara *et al.* 2014). Probably, this protective barrier reduces water loss following ozone treatment and could therefore increase cell turgor compared with other treatments, which would also explain the greater required skin rupture energy (De Belie *et al*. 1999). From the practical perspective, ozone-treated table grapes can be more easily handled postharvest due to their greater skin hardness, and they can exhibit longer shelf lives due to postharvest microbial control (Horvitz and Cantalejo 2014; Karaca and Velioglu 2007).

Regarding the wine grape cultivars, the ozone-treated berries of all of the density classes also exhibited the greater skin hardening at 4 h after treatment compared with the DW- and EW-treated berries, with the exception of density class D Merlot grapes. However, significantly higher ΔW_{sk} values were observed only in the ozone-treated density class E Merlot berries and the density class I Barbera berries. For all of the wine grape cultivars of all density classes, the differences in the ΔW_{sk} values were not significant between the berries that were treated with EW and DW after 4 or 24 h. The percentages of Merlot berries with significantly harder skins ($\Delta W_{sk} > 0.08$ mJ) varied between 35.0% and 58.3% (the density class E berries exhibited values of 35.0% and 40.7% for the DW and EW, respectively, and 58.3% for ozone) at 4 h after berry treatment, whereas these values ranged from 31.8% to 65.0% among the Barbera berries (the density class I berries exhibited values of 31.8% and 32.8% for the DW and EW, respectively, and 61.7% for ozone). After 24 h, the proportions of Merlot berries that experienced skin hardening after the DW and EW treatments varied between 40.7% and 48.3%, whereas these values were 53.4% to 71.7% for the Barbera berries. Among all of the studied cultivars, the changes in skin hardness during storage were lowest for the Merlot berries, and the ΔW_{sk} values typically corroborated this finding at 4 and 24 h after DW and EW treatment.

Regarding the Merlot and Barbera wine grapes, the increases in skin hardness during exposure to ozone might have resulted in greater advantages in terms of the extractability of phenolic compounds compared with the DW and EW treatments. Higher skin hardness values promotes greater yields and slow extraction kinetics of anthocyanins from the skin (Rolle *et al*. 2008). This latter aspect is particularly favorable for varieties with high proportions of disubstituted anthocyanins. These compounds are diffused during maceration faster than trisubstituted anthocyanins, but the former compounds are more easily lost during winemaking (González-Neves *et al*. 2008). Increased skin hardness can also favor the extractability of other phenolic compounds, such as flavanols (Rolle *et al*. 2011). The improved extractions of skin anthocyanins and flavanols would likely result in wines with higher color intensity, smoother taste and lower astringency.

Conclusion

In the food industry, the growing demand to minimize human health and safety issues has promoted the study of ozone and electrolyzed water as alternative sanitizing agents. Berry treatments with ozone gas and electrolyzed water typically induce increases in skin hardness, but exposure to ozone promotes significantly greater increases than immersion in electrolyzed water for berries with certain density values. However, within the same treatments, skin hardening was independent of berry density, and the magnitude of this effect was cultivar-dependent. This study showed the potential of ozone gas to extend the shelf lives of table grapes and to potentially facilitate the extraction of anthocyanins and flavanols from wine grapes during maceration due to increased skin hardness. Nevertheless, further studies are necessary to thoroughly evaluate other quality aspects of the obtained grapes and wines, such as chemical compositions, chromatic characteristics and sensory properties.

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CONCLUSÃO GERAL

O ozônio foi mais efetivo que o SO_2 em reduzir as populações de bactérias láticas, *in vitro*, sem afetar as populações de leveduras fermentativas, mesmo na presença dos componentes da uva e em condições de vinificação. As bactérias acéticas também foram reduzidas pelo ozônio, porém, a produção de biofilme pode ter afetado os resultados.

Quando aplicado diretamente ao mosto o ozônio não afetou as leveduras, bactérias láticas ou acéticas. Os vinhos ozonizados adquiriram características de vinho envelhecido, em termos de cor. Não houve significativas alterações no conteúdo fenólico, na atividade antioxidante e na maioria dos compostos voláteis analisados. Porém todos os vinhos produzidos com ozônio apresentaram acetaldeído em quantidades que podem ser percebidas sensorialmente e em alguns deles a concentração de metanol encontrada estava acima do permitido pela legislação brasileira.

Apesar de não terem sido observadas mudanças significativas causadas pela aplicação do ozônio sobre maioria dos parâmetros estudados, os compostos voláteis que sofreram alterações são de grande importância em termos de qualidade sensorial e segurança dos vinhos. Deste modo, tais efeitos devem ser considerados antes de se empregar o ozônio como um substituto dos sulfitos na produção de vinho, dando-se preferência ao uso do ozônio em menores concentrações e por tempos de aplicação mais curtos.

Com base nos resultados e nas limitações encontradas na execução deste trabalho são sugeridos como temas para estudos futuros:

- Eliminação da interferência do biofilme na avaliação da eficácia do ozônio no controle de bactérias acéticas;

- Avaliação do efeito do ozônio sobre mostos com elevada carga microbiana, levando em conta o seu efeito oxidativo sobre componentes de qualidade do vinho;
- Elucidação das reações do ozônio com componentes do vinho, como compostos fenólicos, assim avaliação da toxicidade dos possíveis produtos formados.