The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine

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Abstract Careful control of spoilage microflora inside wine containers is a key issue during winemaking. To date, attention has been paid to the development of an effective protocol for the eradication of spoilage agents, especially *Brettanomyces*, from barrels. Few studies have taken into account the modifications caused by sanitation treatments in wine and wood barrels. In the present study the effects of two sanitation treatments (ozone and sodium hydroxide) on barrel spoilage microflora and the composition of the wine stored inside them were evaluated. The phenols of wine (38 compounds) were characterised using a UHPLC–MS during the first 3 months of wine ageing, to see possible alterations in composition due to the chemical exchange from wood to wine in presence of sanitising agents. With the same scope, a panel of 13 judges carried out sensorial analysis of wines. The results showed that the tested treatments had little effect on the organoleptic characteristics of wines, but underline the different performance of the sanitation treatments in terms of eradicating microorganisms.

Keywords Wine · Spoilage microflora · Ozone · Barrel · Simple phenols

Introduction

Wood has always been used in the past, in oenology to make instruments and wine containers. Today the use of wood is limited to barrels, irreplaceable tools for the production of high quality wines. Wood provides a unique environment for wine ageing, because it acts as a semi-permeable barrier between wine and the environment, allowing a tailored exchange of gases, such as oxygen (Schmidtke et al. 2011), and the release of valuable compounds in wine (Chira and Teissedre 2015; Garde Cerdan and Ancin-Azpilicueta 2006; Singleton 1995). The combination of these two factors sparks off essential processes in wine ageing involving phenolic compounds. Wine colour stabilisation and the reduction of bitterness due to non-polymerized tannins are two of the most well-known examples of reactions occurring in wines during their permanence in barrels (Oberholster et al. 2015; Marginean et al. 2011).

Unfortunately, the use of barrels is accompanied by some problems. Wood is a material difficult to sanitise due to its porosity and chemical-physical inertness, which reduces the effectiveness of most of the sanitising agents (Guzzon et al. 2011; Stanga 2010). While the wine is present in barrels, the presence of organic matter due to fermentative microorganisms, and close contact between the wood and microbiota mediated by the liquid matrix allow the penetration of microorganisms into the wood. This phenomena is particular dangerous in the case of contamination of wine by spoilage microorganisms, such as *Brettanomyces* spp. The species, associated to the wine environment, belonging from this genera are two: *Dekkera/Brettanomyces bruxellensis* and *Brettanomyces anomalus*, with the majority of the strains being to the first one (Oelofse et al. 2008). These microorganisms are associated

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at the organoleptic depreciation of wine, due to the accumulation of volatile phenols, acetic acid and other off-flavours. Also, some detrimental modifications in wine colour and taste are observed in the case of proliferation of *Brettanomyces* (Oelofse et al. 2008). It has been shown that *Brettanomyces* spp. is able to penetrate deeply into the wood, up to a depth of 8 mm below the surface of the staves (Suarez et al. 2007). Contact between wood and wine containing *Brettanomyces* for a period of two weeks is sufficient to cause significant contamination of barrels. If the contact between wine and wood is prolonged for 10 weeks, spoilage yeasts can reach a depth of more than 1 cm (Swaffield et al. 1997).

To reduce the risks of wine alteration during barrel ageing, preventive strategies are recommended but not always practicable. Immediately after alcoholic fermentation, there are technological limits in terms of the quantification of wine spoilage agents (OIV 2015; Guzzon et al. 2011) and it is not always possible to employ sanitization treatments, such as the addition of sulphur dioxide or filtration (Renouf et al. 2007; Ribereau-Gayon et al. 2006). Careful sanitation of barrels, therefore, plays a crucial role in avoiding cross-contamination between different wines and the settlement of an alternative microflora in the barrels. Traditionally, barrel sanitation was achieved using chemical agents such as organic acid or derivate of chlorine, sodium or potassium. There are some doubts about both the effectiveness and ecological sustainability of these approaches, due to the risk of residues remaining. Aqueous steam is widely used in wineries, but has not shown good efficacy because wood isolates microorganisms from high temperatures (Costantini et al. 2015; Guzzon et al. 2013). Other treatments, such as UV, high-power ultrasonics or ionizing radiation, have been proposed but have encountered difficulties in terms of widespread application due to operational limits or lack of efficacy (Guzzon et al. 2011; Schmid et al. 2011). Ozone has been proposed as a promising alternative for efficient and safe sanitation in the winery. The widespread application of this molecule in the agri-food industry (Erickson and Ortega 2006; Jin-Gab et al. 2003; Khadre et al. 2001; Foegeding 1985), and previous experience in winemaking (Guzzon et al. 2013; Guillen et al. 2010; Hester 2006; Coggan 2003), suggest that ozone could help to eliminate the problem of microbiological contamination of winemaking containers and equipment. However, to encourage the broad application of ozone to winemaking, doubts must be dispelled about residues in the winery environment that could lead to undesirable changes to wine.

In this work we compared ozone treatment with a traditional sanitation system based on chemical agents, for the purpose of eradicating spoilage microflora in wine barrels. The evolution of spoilage microflora was monitored by

plate counts using a specifically tailored sampling approach and differential media, according to previous experiences of the authors (Guzzon et al. 2011). After sanitation, the barrels were filled with red wine and the evolution of a wide range of simple phenols was monitored during the first 3 months of wine ageing by an original UHPLC–MS method (Barnaba et al. 2015). This analytical dataset, accompanied by sensorial analysis of wines, provided information about the effectiveness of ozone as a sanitising agent of wine barrels, and about its interaction with the most valuable phenols characterising wood wine containers.

Materials and methods

Reagents and solutions

LC-MS grade acetonitrile (ACN, 99.9%), LC-MS grade methanol (MeOH, 99.9%), MS grade formic acid (98%) and DL-dithiothreitol (threo-1,4-dimercapto-2,3-butane-diol; 99.5%) were purchased from Fluka (St. Louis, MO, USA), while L-glutathione reduced 99% and *p*-nitrophenol 99% were purchased from Sigma Aldrich (St. Louis, MO, USA). The target phenolic compound suppliers are summarised in Table 1. Water–methanol standard stock solutions were prepared with organic solvent content ranging from 15 to 55%, according to compound solubility.

Experimental plan

15 barrels (225 L each) were chosen in the cooperative winery of Giralan (Cornaiano, I), because contaminated by *Brettanomyces* at a concentration of over 3 log units. The barrels were made by the following suppliers: Fassbinderei Stockinger GmbH (A), Tonnellerie Boutes (F), Tonnellerie Taransaud (F), Pauscha Fassbinderei GmbH (A), and Tonnellerie Berthomieu (F). The barrels were washed with pressurised cold water (1×10^5 Pa, 5 min) and then filled with 50 litres of cold water, previously sterilised using a 0.45 µm filter. The water remained inside the barrels for 24 h, with periodic shaking, and was then sampled for microbiological analysis (1 L for each barrel). The barrels were emptied, dried for 24 h at 20 °C and randomly divided into 3 groups of 5 barrels. The 1st group (not treated, NT) was washed with cold water for 30 min. The 2nd group (chemical treatment, CT) was treated with the *TM Recond* system (Thonhauser GmbH, A), which provides for a barrel cleaning process in 4 steps: 4 min, 70 °C, 1% v/v of aqueous solution of *TM Recond AC* detergent (25% v/v aqueous solution of potassium hydroxide); 4 min, 70 °C, 1% of aqueous solution of *TM Recond pH*; 4 min, 70 °C, hot water; 2 min, cold water. The 3rd group of 5 barrels

Table 1 Phenolic compound characteristics and performance parameters for the chromatographic method

Compounds	Supplier	[M–H] (m/z)	MS/MS fragments	RT (min)	Linearity range (µg/L)	R ²	LOQ (µg/L)	Recovery (%)
Gallic acid	b	169.0142	125.0244	5.60	0.10–8802	0.999	0.10	83
Protocatechuic acid	a	153.0193	109.0294	5.76	0.10–5030	0.999	0.10	94
Gentisic acid	a	153.0193	109.0295, 108.0217	6.21	0.11–5300	0.999	0.11	94
Hydroxytyrosol	c	153.0557	123.0437, 95.0487	6.28	0.52–5150	0.990	0.52	83
Vanillic acid	a	167.0350	152.0114, 123.0452	6.42	0.10–3036	0.977	0.10	91
Syringic acid	a	197.0455	182.0216, 166.9984	6.57	0.11–4256	0.989	0.11	95
Caffeic acid	a	179.0350	135.0452	6.60	0.11–5330	0.996	0.11	76
Homovanillic acid	b	181.0506	137.0617, 122.0373	6.79	0.99–2970	0.988	0.99	105
Tyrosol	b	137.0608	119.0502, 106.0426	6.79	0.11–3150	0.984	0.11	95
Pyrocatechol	b	109.0295	108.0202, 91.0176	7.28	0.50–8946	0.992	0.50	95
<i>p</i> -coumaric acid	b	163.0401	119.0502, 93.1266	7.37	0.10–5200	0.993	0.10	88
Salicylic acid	d	137.0244	93.0346, 122.0374	7.72	0.10–8854	0.999	0.10	103
Catechin	a	289.0717	245.0805, 221.0812	7.89	5.1–5120	0.999	5.12	100
Ferulic acid	a	193.0506	178.0268, 149.0608	8.17	0.12–6210	0.997	0.12	94
Aesculetin	b	177.0193	133.0296, 105.0345	8.48	0.11–9720	0.998	0.11	91
Sinapinic acid	a	223.0611	208.0373, 179.0714	8.54	0.5–4990	0.999	0.50	89
Homovanillic alcohol	b	167.0714	152.0477, 122.0375	8.78	5.1–5100	0.995	5.10	92
Epicatechin	b	289.0718	245.0805, 221.0812	9.67	0.10–9018	0.992	0.10	94
Vanillin	b	151.0401	136.0152, 108.0202	9.86	0.11–5360	0.998	0.11	96
Coniferyl alcohol	b	179.0714	164.0478, 121.0296	10.11	10.7–5350	0.998	10.7	97
Syringaldehyde	e	181.0506	166.0269, 151.0035	10.42	0.75–13,500	0.999	0.75	89
Isopropiovanillone	b	179.0714	164.0477, 121.0295	10.55	5.4–5400	0.996	5.40	93
Scopoletin	a	191.0350	176.0112, 148.0166	10.66	1.0–9108	0.993	1.01	83
Acetovanillone	b	165.0557	150.0321, 122.0371	10.69	0.10–5120	0.998	0.10	89
Isoacetovanillone	b	165.0557	150.0321, 122.0371	10.79	0.10–5120	0.998	0.10	90
Isopropiosiringone	f	209.0819	194.0581, 179.0348	10.81	1.1–4392	0.997	1.10	94
Acetosyringone	b	195.0662	180.0426, 165.0190	11.00	0.10–5190	0.999	0.10	88
Isoacetosyringone	f	195.0662	180.0426, 165.0190	11.24	1.1–9720	0.998	1.08	91
Syringol	b	153.0557	138.0321, 123.0087	11.32	12.9–6460	0.996	12.9	90
Sinapinaldehyde	b	207.0663	192.0427, 177.0193	11.64	1.0–5040	0.997	1.01	77
Tryptophol	a	160.0767	142.0659, 130.0660	11.87	110–5510	0.997	110	95
<i>O</i> -vanillina	b	151.0401	136.0152, 123.0083	12.09	1.0–4980	0.999	1.00	89
Methyl vanillate	e	181.0506	166.0268, 151.0036	12.13	0.52–9270	0.995	0.52	93
4-ethylcatechol	c	137.0608	122.0374	12.30	0.0005–9.25	0.993	0.005	93
Guaiacol	b	123.0451	108.0215, 105.0346	12.85	11.0–9882	0.999	11.0	98
Ethyl vanillate	b	195.0662	180.0415, 130.9911	13.69	0.55–9900	0.995	0.55	100
4-ethylphenol	a	121.0658	106.0423, 83.9854	14.22	0.1022–5.11	0.999	0.1022	114
4-ethylguaiacol	d	151.0764	136.0529, 121.0293	14.58	0.0009–8.57	0.999	0.009	113

Supplier: a = Fluka (St. Louis, MO, USA); b = Sigma Aldrich (St. Louis, MO, USA); c = CHEMOS GmbH (Regenstauf, Germany); d = Aldrich (St. Louis, MO, USA); e = SAFC (St. Louis, MO, USA); f = TransMIT (Gießen, Germany); RT retention time; R² = coefficient of determination; LOQ limit of quantitation

(ozone treatment, OT) was treated with gaseous ozone generated by a cold plasma generator (Moving Fluid, I) with a nominal capacity of 32 g/h of ozone, equipped with an ozone detector B&C Electronics Srl (I); gas flow was set at 10 L/h with a treatment time of 30 min. After treatment,

the barrels were again filled with sterile water, which was sampled as previously described. Finally, the barrels were filled with red wine (*Lagrein* cv., ethanol 13.1% vol., total acidity 5.70 g/L tartaric acid, acetic acid 0.45 g/L, malic acid 0.82 g/L, lactic acid 2.26 g/L, free SO₂ 12.5 mg/L,

total SO₂ 20.5 mg/L). The wine was aged in the barrels for 97 days, carrying out periodic sampling for microbiological and chemical analysis. Sensorial analysis was performed on the wine after barrel ageing.

Microbiological analysis

All microbiological analysis was performed as reported by the International Vine and Wine Organization (OIV 2015), using the following synthetic media: WL Agar (Oxoid, UK) for the enumeration of yeasts and acetic bacteria; Lysine Agar (Oxoid) for the quantification of non-*Saccharomyces* yeasts; DBDM (Morneau et al. 2011.) and WL Agar + 0.1% v/v of Cicloeximide solution (1% v/v aqueous solution, Oxoid) for the enumeration of *Brettanomyces*; MRS Agar for the quantification of lactic acid bacteria. All media were incubated at 25 °C for a time of between 4 and 10 days; MRS Agar was incubated under anaerobic conditions (Anaerogen KIT, Oxoid).

Simple phenolic characterisation of wine

Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, CA, USA) equipped with an on-line purification SPE system, adapting the method proposed by Barnaba et al. (2015). Mass analysis was performed with a Q-ExactiveTM Hybrid Quadrupole-Orbitrap Mass Spectrometer (HQ-OMS, Thermo Scientific) equipped with heated electrospray ionization (HESI-II) and operating in negative ion mode. A HyperSepTM Retain PEP spe cartridge (3.0 mm × 10 mm, 40–60 µm, Thermo Scientific) was used for on-line sample purification and Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 µm particle size; Waters, MA, USA) as the analytical column. Chromatographic separation was performed with H₂O-ACN, by managing the ACN concentration as follows: from 4.0 to 5.5 min eluent B at 5%, from 5.5 to 17 min a linear increase to 60%, from 17.0 to 18.5 min a linear increase to 100%, then column equilibration from 18.5 to 22.0 min at 5%. During the first 4 min online SPE purification took place. During sample purification the flow rate was set to 0.250 mL/min, while during chromatographic separation the flow rate was set at 0.400 mL/min. The sample inject volume was 2 µL. Mass spectra were acquired in profile mode through full MS-data dependent MS/MS analysis (full MS–dd MS/MS). Full mass spectra were recorded at a mass resolving power of 140,000 full width at half-maximum (FWHM), while data-dependent mass spectra were recorded at a mass resolving power of 17,500 FWHM. The mass spectrometer operated with the following parameters: spray voltage, 2.80 kV; sheath gas flow rate at 30 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; capillary temperature at 310 °C; capillary gas heater temperature, 280 °C. Full mass spectral

data were used for identification and quantification of analytes, while data-dependent mass spectral results were used to confirm the presence of analytes in real matrices. Table 1 summarises the exact masses and characteristic fragments used respectively for quantification and confirmation of the target compounds. Limits of detection (LODs) and of quantitation (LOQs) were established according to Mol et al. (2011) and Eurachem (1993) respectively. Method accuracy was estimated in terms of mean relative recovery by spiking 8 natural samples with 1000 µg/L of each phenol. Before analysis the sample was filtered using 0.45 µm PTFE filter cartridges (Sartorius AG, D), diluted 10 times, and added to the internal standard (*p*-nitrophenol, final concentration 500 µg/L) and formic acid (aqueous solution, 0.1% v/v).

Sensorial analysis of the wine

Wine from each individual barrel (15 samples) was tasted blindly following 97 days of ageing in the treated barriques. The panel consisted of 13 judges, previously trained wine sensory analysis at the laboratories of Laimburg Research Centre for Agriculture and Forestry (BZ, Italy). Judges were divided into two groups to which the wine were served in a different order to minimize systematic errors. Sensory evaluation was carried out using unstructured rating scales (Stone et al., 2008) that express each parameter on a linear scale from a minimum and a maximum. The evaluation scheme contained 12 parameters for both flavour (fruitiness, ink and leather, *Brett* smell/horse sweat, cleanness of the aroma, complexity, varietal typicality, reduced or oxidative character) and the taste (quantity of tannins, quality of tannins, balance, astringency and overall quality). 3 wine samples were replicated twice to check the sensitivity and reliability of each judge, using the method proposed by Kobler (1996).

Statistical analysis

All statistical analysis was performed using Statistica 9.1 (StatSoft, CA, USA). No detectable data were fixed at a value equal to half the LOQ determined for each parameter. Analytical compounds detectable in less than 10% of samples were not considered during statistical elaboration. Data not normally distributed (gentisic acid, syringaldehyde, sinapaldehyde, vanillin; Kolmogorov–Smirnov test, $p < 0.05$) were normalised by applying Box-Cox transformation. Honestly significant difference (HSD) Tukey test ($p < 0.05$) was applied in order to identify differences between treatments or sampling times both from microbiological and chemical determination. Principal Component Analysis was applied to phenols found to be significantly different due to ageing and treatment.

Results and discussion

Survey of the microbiological state of barrels before sanitation

Preliminary analysis of the wine stored in the barrels (data not shown) showed presence of *Brettanomyces* spp. at a concentration of up to 2 log units, an exceptionally higher value because it can lead to immediate alterations in wine (Renouf et al. 2007). Despite the diverse features of barrels (type of wood, degree of roasting, age) the profile of the microbiota found inside them, before sanitization treatments, was similar (Table 2) and related to the microbiological contamination of the wines previously stored in the barrels. This evidence led to consider the presence of spoilage microorganisms in winery equipment as a stable and widespread phenomenon that must be properly counteracted by a microbiological surveillance and an effective sanitization plan. The recovery onto petri plate containing a non-selective media (WL) was 1 log unit below the results of plate counts performed by DBDM and WLd, two media specifically tailored for *Brettanomyces* genera (Table 2). Comparison between the results of plate counts on WL Agar and Lysine Agar (Table 2) indicated that the yeast population was mainly made up of non-Saccharomyces yeasts. This observation agreed with previous works on the ecology of wine after alcoholic fermentation, revealing the presence of a complex yeast consortium during wine ageing (Perez-Martin et al. 2014; Sangorrin et al. 2008; Shinohara et al. 2000). The concentration of *Brettanomyces* genera, and of other yeasts grown onto the same selective media, was similar to that observed in wines earlier stored in barrels, about the 2–3 log units. Acetic bacteria were widely present in barrels, indeed lactic acid bacteria were not found in the samples, probably due to the standard practice of this winery of performing malolactic and alcoholic fermentation simultaneously, in the stainless-steel

fermentation tanks. The differences in the recovery of analysis performed onto diverse synthetic media suggested that the microbiological monitoring of spoilage agents have consider different approaches, tailored for the microbial groups of interest in relation to the wine features (Zuehlke et al. 2011; Rodrigues et al. 2001).

Effectiveness of sanitation treatments

Barrels were treated using the 3 different protocols described in “Experimental plan” section. The first treatment was performed without a specific sanitising agent, using water to remove residues of wine from the internal surface of barrels. In the other cases (CT and OT) an aqueous solution of potassium hydroxide and gaseous ozone respectively were used as sanitising agents. Table 2 gives the results of the treatments, expressed as the microbial contamination found inside barrels after sanitisation. The first conclusion was that the sanitising agents did not result in complete removal of the microbes from the internal surface of barrels; this agreed with previous experience and confirmed the complexity of microbial control inside wood containers (Oelofse et al. 2008; Hester 2006; Du Toit and Pretorius 2000). In the case of washing with water (NT barrels), the decrease in cell concentration observed for certain groups of microorganism (Table 2) was probably related to mechanical removal of cells from the wood surface. In this context, one example was represented by acetic bacteria, whose aerobic nature and tendency to grow on the surface of the substrate (in this case wood) facilitated mechanical removal during washing with water. On the other hand, in the event of a more vigorous microorganism, *i.e.* yeasts, an increase in contamination was observed. A reasonable explanation for this behaviour would involve taking into account the increase in water activity inside the wood due to the water treatment, which is thus counterproductive.

Table 2 Results of microbiological tests in barrels before and after sanitation treatments

Sanitation treatment	Sampling	Total yeasts ($\times 10^1$)	Non-Saccharomyces yeasts ($\times 10^1$)	Acetic bacteria ($\times 10^2$)	<i>Brettanomyces</i> spp. ($\times 10^2$)	
		WL Agar (4 days)	LYS Agar (4 days)	WL Agar (7 days)	DBDM (10 days)	WLd (10 days)
Chemical sanitizer (Sodium hydroxide)	BT	6.2 ± 3.7^A	7.3 ± 5.4^A	3.4 ± 2.7^A	4.9 ± 2.5^A	8.0 ± 4.1^A
	AT	2.2 ± 1.1^A	2.3 ± 0.6^A	2.2 ± 1.6^A	1.3 ± 0.7^A	1.1 ± 1.6^B
Cold water washing	BT	3.2 ± 1.6^A	3.2 ± 1.4^A	7.1 ± 1.8^A	7.1 ± 3.7^A	9.6 ± 0.8^A
	AT	3.6 ± 2.4^A	1.9 ± 1.0^A	1.6 ± 0.4^B	6.0 ± 2.2^A	8.1 ± 0.8^A
Gaseous ozone	BT	4.6 ± 1.6^A	4.8 ± 3.1^A	4.0 ± 2.3^A	6.4 ± 0.7^A	6.8 ± 0.9^A
	AT	0.3 ± 0.4^B	0.8 ± 0.1^A	0.6 ± 0.1^B	nd. ^B	nd. ^B

The concentration is expressed in CFU/mL, referring to the microbial contamination of 50 litres of sterile water kept inside the barrels for 24 h with shaking. BT samples before sanitization treatment, AT samples after sanitization treatment; nd not detectable (<5 ufc/mL). Number of barrels with the same treatment (n) = 5, statistical analysis was performed comparing the microbial load before and after each treatment

In chemically treated barrels (CT) the residual microbial population was almost 30% of the initial level, for both yeasts and acetic bacteria, while it decreased to 15% in the case of *Brettanomyces*. More effective results were achieved in barrels subjected to the OT treatment, which reduced yeast contamination below 10% of the initial concentration and eliminated *Brettanomyces*: its concentration was negligible in sampling, after sanitations. The differences observed between CT and OT treatments were due to diverse mechanisms of action of the sanitising agents. CT was based on massive production of carbon dioxide due to the reaction of the active ingredients (potassium hydroxide, potassium carbonate and tetrapotassium pyrophosphate) with water, therefore mainly caused mechanical removal of the dirt. In contrast, OT acts by triggering radical reactions that target the double bonds typical of some molecules essential for cell life. It is therefore referred that the action of ozone was more strongly directed against living microorganisms, in comparison to CT. The various classes of microorganisms showed different sensitivity to OT. This behaviour could be explained by considering the different aerobic characteristics of oenological microorganisms and, consequently, different resistance to the oxidative stress exerted by ozone (Guzzon et al. 2013). *Brettanomyces* spp., residual concentration was $1.3 \pm 7 \times 10^2$ and $6.0 \pm 2 \times 10^2$ CFU/mL (Table 2) respectively in NT and CT barrels exposed wines to a significant risk of wine spoilage, considering that volatile phenols are generally produced when *Brettanomyces* exceeds 2 logarithmic units (Oelofse et al. 2008; Chatonnet et al. 1995). Conversely, in OT barrels the *Brettanomyces* concentration was close to the plate count detection limits and of no technological relevance.

Evolution of the phenolic content of wine stored in barrels subjected to different sanitation treatments over time

Almost all the target phenolic compounds were found in wines stored in barrels previously subjected to sanitation treatments at a concentration higher than their relative limits of quantitation. Two compounds differed from the general rule: sinapaldehyde and 4-ethylguaiaicol. As regards phenols of microbiological origin, responsible for the “Brett character” in wine (Shinohara et al. 2000; Chatonnet et al. 1995), at the end of the 3rd month (97 days) of ageing 4-ethylphenol content ranged between 8.17 and 62.5 µg/L, with a median of 42.8 µg/L in the OT samples, between 4.9 and 45.2 µg/L, with a median of 31.9 µg/L in the CT samples, and between 29.4 and 46.7 µg/L, with a median of 34.9 µg/L in the NT samples. A similar situation was observed in the case of 4-ethylcatechol, which showed a median value of 30.2 µg/L in the

OT samples (range $28.2 \div 31.9$ µg/L), 28.6 µg/L in the CT samples (range $26.7 \div 30.4$ µg/L), and 29.4 µg/L in the NT samples (range $27.5 \div 31.5$ µg/L). Considering the sensorial threshold, generally above 420 µg/L as the sum of 4-ethylphenol and 4-ethylguaiaicol (Knapp et al. 2010), all the wines were unaffected by “Brett character”.

The proposed sanitation treatments did not directly involve the wine, but rather the wood of the barrels, so chemical characterisation therefore focused on the phenol profile (35 compounds), supposing that the variations of their concentrations should depend mainly on the interaction between wood and wine. As regards the content of wines subjected to the different sanitation treatments, significant differences (Tukey Test, $p < 0.05$) were observed between the wines sampled after 37 and 97 days of ageing, as reported in Fig. 1 for five compounds having different, and characteristics, trends. Minimum, median and maximum phenol content, and significant differences between the median concentration measured following 1, 2 and 3 months’ ageing are summarised in Table 3. Acetovanillone increased by about 12% in wines aged in ozone-treated barrels (OT) over time, and also increased in wine from chemically treated barrels (+8%), albeit not significantly, but decreased in wine from untreated barrels (−3%). Gentisic acid increased in OT (+44%) and CT wines (+66%), and also in NT wines (+56%), albeit not significantly. Interestingly for wine aroma perception, vanillin and homovanillic acid increased significantly with ozone treatment (+72% and +12% respectively), but not significantly with the other treatments (+30% and +13% for CT; +13% and +10% for NT respectively). Isoacetovanillone and o-vanillin increased in OT (+20% and +26% respectively) and CT wines (+13 and +29% respectively), but did not significantly change in NT wines (−8 and +10% respectively). Catechin, which did not change in NT (+4%) and CT (+2%) wines, decreased in OT samples (−4%). Isoacetosyringone decreased over time both in NT (−27%) and OT (−36%) wines, but did not change in CT wine (−1%). Isopropiovanillone decreased significantly in OT wines (−36%), but not in NT (−15%) and CT wines (−18%). Epicatechin decreased in NT (−6%) and CT wines (−6%), albeit not significantly, but did not change in OT wines (+1%). Caffeic and salicylic acids decreased significantly in NT (−5 and −23% respectively), but not in CT (−3 and −15% respectively) and OT wines (−5 and −12% respectively). Gallic acid decreased significantly in NT (−35%) and CT samples (−28%), and also noticeably in OT (−28%) wine. Guaiaicol increased significantly in NT (+27%) and noticeably in CT (+25%), but not in OT samples (−3%). Hydroxytyrosol increased in NT (+3%) and OT (+5%), but only significantly in CT wines (+4%). Methyl vanillate and protocatechuic acid decreased significantly in NT (−28%

Fig. 1 Box plot of simple phenolic content after 3 months of ageing (97 days) in differently treated barrels (NT: non-treated barrels; CT: barrels treated with a chemical sanitising agent; OT: barrels treated with ozone). A single compound, of those found to be significantly

different (Tukey Test, $p < 0.05$) for the treatments, is reported for each trend described. Vanillin, the only not significantly different compound, is given as an example of wood's impact on barrel-aged products

Fig. 2 Score plot (two first principal components: PC1 = 25%, PC2 = 20%) for wines aged for 37, 67 and 97 days in barrels distinguished on the basis of the three tested treatments (NT: non-treated barrels; CT: barrels treated with a chemical sanitising agent; OT: barrels treated with ozone)

and 29% respectively) and CT samples (−25 and −20% respectively), and also noticeably in OT (−19 and −13% respectively).

Impact of sanitation treatments on the wine phenolic profile following 97 days ageing

Statistical analysis of phenols for 97-day-aged samples had eight compounds with significant difference (Tukey Test, $p < 0.05$) between treatments were acetovanillone, isoacetovanillone, gallic acid, methyl vanillate, salicylic acid, protocatechuic acid, isoacetosyringone and vanillic acid. Aceto- and isoacetovanillone content was statistically higher for OT wines. Gallic acid, methyl vanillate, salicylic and protocatechuic acid were higher for OT than for NT. Isoacetosyringone content was lower for OT than for CT. Finally, vanillic acid was higher for OT than for CT. Figure 1 shows the distribution of these phenols after 3-month ageing for the different treatments. The vanillin content was very important due to the possible impact on wine aroma and although not significantly different for the barrel

Table 3 Original phenolic content of the test wine (not treated)

Compounds	Not treated mean (N = 3)	Treatment	After 37 days			After 67 days			After 97 days		
			Min	Median	Max	Min	Median	Max	Min	Median	Max
Acetovanillone	23.8	NT	28.7	29.3	30.4	27.3	28.3	35.3	27.8	28.4	29.6
		CT	26.2	27.4 ^B	28.7	31.0	31.7 ^A	34.2	26.7	29.6 ^{AB}	32.3
		OT	27.7	29.0 ^B	30.1	28.6	30.7 ^B	31.5	31.6	32.6 ^A	33.7
Caffeic acid	28,380	NT	31,752	32,521 ^A	33,771	30,662	32,450 ^{AB}	33,180	29,614	31,032 ^B	31,525
		CT	30,509	31,910	32,320	30,508	31,580	32,261	29,682	30,820	31,879
		OT	29,854	32,250	34,521	30,524	30,855	31,513	30,278	30,665	31,464
Catechin	26,800	NT	25,049	25,825	26,857	26,333	27,128	31,763	24,905	26,834	27,553
		CT	23,776	25,484 ^B	27,068	27,541	28,643 ^A	29,199	23,226	26,081 ^B	26,632
		OT	24,821	26,042 ^A	28,875	25,679	26,532 ^A	27,443	22,332	25,009 ^B	25,216
Coniferyl alcohol	799	NT	634	748	804	563	674	712	627	665	677
		CT	576	690 ^A	818	508	539 ^B	638	595	617 ^{AB}	728
		OT	679	729	762	457	492	630	549	644	811
Epicatechin	13,100	NT	12,051	12,631 ^A	13,233	11,477	12,016 ^B	12,301	11,534	11,858 ^B	12,062
		CT	12,054	12,480	13,157	11,465	12,085	12,431	11,294	11,737	13,042
		OT	11,583	12,182 ^A	13,080	10,962	11,200 ^B	11,497	11,912	12,299 ^A	12,932
Gallic acid	13,200	NT	16,258	16,879 ^A	18,831	12,662	14,265 ^B	15,601	10,852	11,022 ^C	12,053
		CT	15,059	16,322 ^A	19,064	14,285	14,589 ^A	15,398	11,485	11,770 ^B	14,473
		OT	17,161	19,067	21,756	14,677	18,279	22,265	12,014	13,661	19,477
Gentisic acid	54.8	NT	60.0	71.0	92.0	51.0	76.0	146	91.0	111	141
		CT	53.3	70.6 ^B	81.6	101	109 ^A	129	79.0	117 ^A	146
		OT	68.1	76.5 ^B	78.1	104	119 ^A	137	94.0	110 ^A	134
Guaiacol	198	NT	214	229 ^B	244	224	243 ^B	270	248	291 ^A	296
		CT	212	217	256	191	265	275	190	271	293
		OT	210	239	246	195	228	289	206	231	269
Homovanillic acid	173	NT	188	199	246	219	224	263	191	219	236
		CT	170	202	234	210	258	270	208	229	237
		OT	178	210 ^B	225	212	216 ^{AB}	238	217	236 ^A	257
Hydroxytyrosol	1260	NT	1283	1361	1388	1256	1404	1444	1277	1403	1420
		CT	1302	1330 ^B	1335	1313	1351 ^B	1367	1389	1401 ^A	1441
		OT	1261	1324	1401	1245	1324	1346	1269	1382	1415
Isoacetosyringone	5.9	NT	13.1	14.3 ^A	17.4	10.1	11.9 ^{AB}	14.9	9.07	10.4 ^B	11.6
		CT	11.4	14.1	16.2	11.9	13.4	15.9	8.5	13.9	17.0
		OT	12.4	14.6 ^A	21.2	8.65	10.1 ^{AB}	15.5	7.71	9.37 ^B	11.4

Table 3 continued

Compounds	Not treated mean (N = 3)	Treatment	After 37 days			After 67 days			After 97 days		
			Min	Median	Max	Min	Median	Max	Min	Median	Max
Isoacetovanillone	12.1	NT	13.9	14.6	15.5	12.5	12.9	16.9	13.3	13.5	14.1
		CT	12.4	12.5 ^B	13.9	14.8	15.1 ^A	16.4	12.7	14.1 ^{AB}	15.7
		OT	12.2	13.2 ^C	13.7	13.6	14.7 ^B	15.0	15.4	15.9 ^A	16.4
Isopropiovanillone	14.0	NT	12.0	13.3	16.0	10.5	17.6	22.6	10.7	11.3	13.3
		CT	11.8	14.7	23.0	12.6	14.2	15.5	9.35	12.0	15.4
		OT	14.7	19.0 ^A	19.7	10.6	14.4 ^B	17.1	11.0	12.1 ^B	15.0
Methyl vanillate	1080	NT	1548	1613 ^A	1730	1346	1468 ^B	1514	1094	1159 ^C	1193
		CT	1452	1691 ^A	1781	1385	1457 ^A	1592	1191	1275 ^B	1381
		OT	1729	1791	2571	1448	1747	2395	1220	1456	2113
<i>o</i> -Vanillin	116	NT	115	119	180	112	136	146	119	131	163
		CT	95.0	100 ^B	123	104	112 ^{AB}	142	125	129 ^A	155
		OT	93.3	124 ^B	127	102	119 ^B	139	142	156 ^A	166
Protocatechuic acid	1850	NT	2566	2587 ^A	2723	2007	2286 ^B	2503	1755	1827 ^C	1919
		CT	2370	2519 ^A	2748	2185	2336 ^A	2496	1785	2022 ^B	2119
		OT	2444	2555	3422	2228	2638	3305	1944	2224	3016
Salicylic acid	206	NT	280	298 ^A	305	251	282 ^A	322	220	229 ^B	256
		CT	261	295 ^{AB}	303	294	316 ^A	321	246	252 ^B	295
		OT	289	313	349	261	306	377	256	274	347
Scopoletin	5.6	NT	5.81	6.21	7.63	5.85	7.34	9.05	6.39	6.82	8.03
		CT	4.99	6.06 ^B	6.60	6.56	7.07 ^A	7.86	5.50	6.36 ^{AB}	7.03
		OT	6.45	7.32	8.34	6.45	6.94	7.58	5.98	7.33	9.58
Tryptophol	1490	NT	1519	1613 ^B	1659	1679	1696 ^A	1863	1596	1655 ^{AB}	1694
		CT	1570	1584	1782	1673	1715	1774	1609	1659	1705
		OT	1576	1650 ^{AB}	1789	1653	1722 ^A	1782	1526	1619 ^B	1645
Vanillin	15.6	NT	15.6	19.3	37.0	14.3	17.7	53.5	16.7	21.8	71.0
		CT	15.6	16.6	22.0	15.2	16.8	23.8	20.1	21.6	41.2
		OT	17.7	23.6 ^B	28.6	18.8	27.3 ^{AB}	31.4	26.6	40.6 ^A	44.1

Minimum, median and maximum content (µg/L) of phenolic compounds shown to be significantly different (Tukey Test, $p < 0.05$) for at least one barrel treatment in wines aged for one month (37 days), two months (67 days) and three months (97 days). Median superscript letters on the same row indicate significant differences between ageing times for the same treatment

treatments, it had a median content notably higher (almost double) for OT wines as observed in a previous work by Guzzon et al. (2013). All the variations described for hydroxybenzochetonic derivatives (methyl vanillate, isoacetosyringone, aceto- and isoacetovanillone), and hydroxybenzoic acids (gallic, salicylic, protocatechuic and vanillic acid) were consistent with earlier results for red wines (Barnaba et al. 2015). However, compounds with a possible impact on wine aroma, being vanillin derivatives and mostly related to wood transfer in barrel-aged products, such as aceto-, isoacetovanillone, methyl vanillate, vanillic acid and vanillin itself, were generally higher in wines aged in OT barrels as compared to those from differently treated barrels.

Figure 2 presents a graphic PCA description of data related to the phenols found to be significantly different in terms of ageing and treatment. *O*-vanillin, gentisic acid and guayacol were the most significant components with positive eigenvalues (0.41, 0.36 and 0.33 respectively), while protocatechuic acid, gallic acid and methyl vanillate had negative eigenvalues (−0.91, −0.91 and −0.90 respectively) for the first function (Fatt. 1), which explained 25% of total variability. Acetovanillone, isoacetovanillone and gentisic acid were the most significant components with positive eigenvalues (0.84, 0.80 and 0.76 respectively), while coniferyl alcohol, isoacetosyringone and isopropiovanillone had negative eigenvalues (−0.50, −0.41 and −0.35 respectively) for the second function (Fatt. 2), which explained 20% of total variability. Barrel treatment seems to have affected the wine phenolic profile in a more moderate way than the duration of ageing, and as it is

reasonable to expect, the main compositional differences can be observed between 37-day and 97-day aged samples. These results provided further confirmation that OT, as well as being effective in eradicating spoilage microorganisms, did not effect the aroma profile of wines.

Sensorial analysis of wine

Sensorial analysis showed no significant differences between the different sanitation treatments for the descriptors considered (Table 4). According to the chemical assay, the flavour descriptors associated with aroma alterations due to the *Brettanomyces bruxellensis* metabolism (ink and leather, Brett smell/horse sweat, cleanness in the aroma) were detected at low levels. These results underlined that in the experimental conditions adopted, *Brettanomyces* was not able to cause alterations to the sensorial impression of wines. The main factor that limited *Brettanomyces* activity was probably the short ageing time (3 months), thus defined because it was specifically the standard procedure for the production of Lagrein wine. In future, additional experiments involving wines suitable for prolonged storage in barrels will be needed to better clarify this aspect. Other parameters in the sensorial tests (i.e. fruitiness, complexity, varietal typicality, reduced/oxidative character, quantity of tannins, quality of tannins and balance) were more related to wine features, deriving for example from the varietal contribution, rather than to winemaking variables such as the ageing time in barrels. Consequently, for the purpose of this work, these descriptors have the greatest relevance. The non-significant

Table 4 Sensory analysis (descriptive test) of Lagrein wine, 2014 vintage, after 97 days of barrel ageing

Parameter	Significance	NT	CT	OT
Fruitiness	n.s.	5.10 ± 0.66	5.23 ± 0.72	5.61 ± 1.00
Ink	n.s.	0.86 ± 0.31	0.90 ± 0.34	0.88 ± 0.19
Horse sweat	n.s.	0.92 ± 0.15	0.91 ± 0.31	0.81 ± 0.21
Cleanness in the aroma	n.s.	6.26 ± 0.50	6.34 ± 0.62	6.36 ± 0.39
Complexity	n.s.	5.81 ± .021	5.65 ± 0.42	5.86 ± 0.67
Varietal typicality	n.s.	5.98 ± 0.53	5.94 ± 0.35	5.96 ± 0.61
Reduced/oxidative character	n.s.	−1.73 ± 0.43	−1.78 ± 0.50	−1.48 ± 1.07
Quantity of tannins	n.s.	0.34 ± 0.72	0.22 ± 0.47	0.47 ± 0.24
Quality of tannins	n.s.	4.21 ± 0.36	4.52 ± 0.42	4.53 ± 0.17
Balance	n.s.	4.11 ± 0.65	4.43 ± 0.64	4.59 ± 0.63
Astringency	n.s.	3.83 ± 0.55	3.59 ± 0.30	3.43 ± 0.39
Overall quality	n.s.	4.97 ± 0.60	5.15 ± 0.48	5.03 ± 0.58

All data are expressed as the average of the five replicates ± standard deviation. NT non-treated barrels; CT barrels treated with a chemical sanitising agent; OT barrels treated with ozone. Statistical analysis: one-factor ANOVA and Tukey-B test. Different letters indicate statistically significant differences at $p < 0.05$. n.s. not significantly different. *Values are significantly different at $p < 0.05$. **Values are significantly different at $p < 0.01$. *** Values are significantly different at $p < 0.001$

n.s. not significantly different

differences detected for all of these descriptors proved that the treatments considered had no negative effects on the wine's organoleptic characteristics. While this was already known for the chemical treatment described, it is described for the first time, as far as we know, for sanitation treatment with ozone.

Conclusion

This work verified the applicability of ozone as a sanitising agent for barrels used to age wines in real winemaking conditions, maintaining accuracy and statistical robustness similar to laboratory tests in the experimental plan and analysis of results. Ozone proved to be an effective sanitising agent, against the main wine spoilage organisms, such as *Brettanomyces*. The extended range of chemical analysis performed on wines during the 3-months' ageing in barrels allowed the evolution of the phenolic profile of wines to be studied, both in terms of the kinetics of exchange between wine and wood, and the quantitative and qualitative composition of the finished wines. Finally, sensory analysis confirmed the results of chemical tests, excluding any depreciation of the wine due to treatment of the barrels with ozone. In conclusion, ozone proved to be an interesting alternative to traditional sanitising agents used in the winery, capable of guaranteeing effective and safe sanitation during winemaking.

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