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Antioxidant activity and phenolic composition of wine spirit resulting from an alternative ageing technology using micro-oxygenation: a preliminary study

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ABSTRACT

Aim: Alternative technologies for the aging of wine spirit, as for other spirit beverages, have been developed over the past decade in order to meet consumer and industry expectations of differentiation and sustainability. Physicochemical characterisation of the resulting products has been carried out, but no previous research has evaluated their antioxidant activity. This preliminary study examined the effect of an alternative ageing technology (micro-oxygenation combined with wood staves from chestnut or Limousin oak in 1000 L stainless steel tanks) in comparison with traditional technology (250 L new barrels) on the antioxidant activity and related phenolic composition of the wine spirit.
Methods and results: The wine spirits resulting from both technologies were sampled after 8, 15, 30, 180 and 365 days of ageing. Samples taken were analysed using the DPPH method, HPLC and total phenolic index. Significantly higher antioxidant activity (50.43 % *vs* 36.42 % DPPH inhibition), together with greater enrichment in wood-derived compounds (51.79 *vs* 27.72 total phenolic index), was achieved in wine spirits aged using the alternative technology than in new barrels. Moreover, chestnut wood stood out from Limousin oak wood with higher promoted antioxidant activity (62.69 % *vs* 21.35 % DPPH inhibition), and higher phenolic index (49.03 *vs* 25.67). Significant correlations between the wine spirits' antioxidant activity, total phenolic index, ellagic acid, gallic acid, vanillin and syringaldehyde concentrations were observed, particularly in those aged using the alternative technology.
Conclusion: Micro-oxygenation combined with staves resulted in higher accumulation of bioactive compounds and antioxidant activity, thus increasing wine spirit quality and adding value to the product.
Significance of the study: These preliminary results show that the characteristics acquired by the wine spirit, coupled with those revealed by previous research and the promotion of faster and cheaper ageing, make the alternative technology a promising option for the industry.

KEYWORDS

wine spirit, ageing technologies, micro-oxygenation, chestnut wood, Limousin oak wood, antioxidant activity, phenolics

INTRODUCTION

Increasing attention has been given by the scientific community to the antioxidant activities of foods and beverages owing to their health benefits (Santana-Gálvez *et al.*, 2019). As regards alcoholic beverages, research has mainly focused on wine (Li and Sun, 2017; Comert and Gokmen, 2018), and only a limited number of studies on spirits has been published, particularly on those aged in wooden barrels, such as wine spirit (Canas *et al.*, 2008; Ziyatdinova *et al.*, 2014), brandy (Schwarz *et al.*, 2009; Ziyatdinova *et al.*, 2014), whisky (Koga *et al.*, 2007), mescal (Ávila-Reyes *et al.*, 2010) and rum (Regalado *et al.*, 2019). Their antioxidant activities have been assigned to phenolic compounds extracted from the wood. Indeed, the corresponding distillates are characterised by high ethanol content and richness of volatile compounds, but are devoid of phenolic compounds other than the volatile ones (Lea and Piggott, 2012). On the contrary, the wood used is rich in phenolic compounds derived from lignin and tannins (Martínez-Gil *et al.*, 2018a), which are released into the distillate during the ageing process (Canas, 2017). Therefore, ageing is a key stage in the beverage production process, specifically for wine spirit, contributing not only to the chemical characteristics and related sensory properties imparted, such as colour, aroma and taste (Caldeira *et al.*, 2017; Canas, 2017), but also to the enrichment in bioactive valuable compounds. This last feature is of great importance in spirits, since the ingestion of such compounds, with moderate consumption of a drink, can have a positive health effect (Duriez *et al.*, 2001; Umar *et al.*, 2003), as opposed to ethanol-induced damage (WHO, 2018). In this context, the aged spirit can be seen as a contributor to a phenolic-rich diet. Several epidemiological studies evidenced that people following a phenolic-rich diet are at low risk of a range of chronic diseases, including obesity, diabetes, cancer, cardiovascular and neurodegenerative diseases as a consequence of the wide set of activities it promotes; among them, the free radical scavenging activity against the oxidative stress occurring in the initial stage and progression of such diseases is of utmost relevance (Rasouli *et al.*, 2017; Niki, 2018).

Similar to wine (Clodoveo *et al.*, 2016) and other spirit beverages (Schwarz *et al.*, 2014; Rodríguez-Solana *et al.*, 2017), alternative technologies for the ageing of wine spirit have been developed over the past decade to meet consumer and industry expectations of differentiation and sustainability. The most widespread technology consists in adding pieces of wood to the beverage stored in stainless steel tanks (Canas *et al.*, 2016). Recently, replicating the oxygen transfer occurring in the barrel, the micro-oxygenation (MOX) technique developed by Ducournau and Laplace (Gómez-Plaza and Bautista-Ortín, 2019) was applied to optimise this novel ageing technology. Early phenolic and colour responses (Canas *et al.*, 2019) attained within the first six months of ageing have shown greater accumulation of wood-derived phenolic compounds and higher colour evolution in the wine spirits aged using MOX combined with staves in stainless steel tanks than those aged using traditional technology in new wooden barrels. These promising outcomes boosted the development of a comprehensive approach - including several aspects underlying the quality acquired over ageing time and of the final product - to the full characterisation of this novel technology. In addition, as far as we know, no previous research on spirit beverages has examined the impact of different ageing technologies - including MOX combined with wooden staves - on their antioxidant activity.

Considering its pivotal role in the ageing result, obtained by either the traditional (Canas, 2017) or alternative (Canas *et al.*, 2019) technology, it is imperative to continue studying the interaction of the botanical species of the wood with MOX, and the effects on antioxidant activity and related phenolic composition of the wine spirit.

This preliminary work aimed to ascertain if the novel technology would result in a comprehensive enhancement of the quality of the wine spirit. This was achieved by assessing *in vitro* antioxidant activity (free radical scavenging activity), using the DPPH method, and its relationship with the phenolic composition (total phenolic index and low molecular weight compounds analysed by HPLC) of the same wine spirit after one year of ageing using different technologies (MOX combined with wood staves in 1000 L stainless steel tanks *vs* 250 L new barrels) and two different kinds of wood (Limousin oak and chestnut). The evolution of antioxidant activity over ageing time was also examined.

MATERIALS AND METHODS

1. Chemicals

1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich (Steinheim, Germany). The solutions were freshly prepared prior to use with methanol analytical grade (Merck, Darmstadt, Germany). Ellagic acid dehydrate (Ellag), gallic acid monohydrate (Gall), syringic acid (Syrg), vanillic acid (Van) and vanillin (Vanil) were purchased from Fluka (Buchs, Switzerland). Coniferaldehyde (Cofde), sinapaldehyde (Sipde), syringaldehyde (Syrde) and 4-hydroxybenzaldehyde were purchased from Aldrich (Steinheim, Germany). All of them were used as standards (purity>97 %) without further purification. The standard solutions were freshly prepared prior to use with ethanol/water (75:25 v/v). All solvents used in the chromatographic analysis were HPLC gradient grade purchased from Merck (Darmstadt, Germany).

2. Experimental design and wine spirit sampling

A two way factorial design (two ageing technologies x two kinds of wood) was established. The same wine distillate (alcohol strength, 77.4 v/v; pH, 5.44; total acidity, as acetic acid, 0.13 g/hL of absolute ethanol; volatile acidity, as acetic acid, 0.11 g/hL of absolute ethanol) produced by the Adega Cooperativa da Lourinhã, Portugal, was aged using the alternative technology in 1000 L stainless steel tanks with MOX and wood staves, and using the traditional technology in 250 L new barrels. The staves and the barrels were made from two different kinds of wood: Portuguese chestnut (*Castanea sativa* Mill.) and Limousin oak (*Quercus robur* L.). Two replicates were used for the stainless steel tanks and three for the barrels, which were all placed in the cellar of Adega Cooperativa de Lourinhã in the same environmental conditions. The latter requirement was essential to ensure that the influence of the cellar conditions on all barrels was alike, ensuring that the kind of wood was primarily responsible for the differences chestnut and Limousin oak barrels. Indeed, environmental conditions, especially relative humidity, greatly influence several physicochemical phenomena underlying the ageing process (de Adana *et al.*, 2005; Canas, 2017), determining the wetting status of the barrel and, consequently, the oxygen transfer rate through the wood into the liquid inside (del Álamo-Sanza and Nevares, 2014). Specific behaviour of the two kinds of barrels was expected, given that wood anatomy and gas content affect the diffusion of oxygen through the wood (Sorz and Hietz, 2006; del Álamo-Sanza and Nevares, 2014).

The pieces of wood and barrels were manufactured by J. M. Gonçalves cooperage (Palaçoulo, Portugal) with medium plus toasting level; i.e., 90 min at an average temperature of 240 °C. The staves (91 cm length x 5 cm width x 1.8 cm thickness) were heated in an industrial oven, and the barrels (with staves of 95 cm length x 8 cm width x 2.7 cm thickness) were heated over a fire of wood offcuts; the temperature of the wood was rigorously controlled to guarantee the same toasting level.

The quantity of staves to be inserted into the stainless steel tanks was calculated in order to reproduce the surface area to volume ratio of a 250 L barrel (85 cm^2/L). The staves were positioned around the internal wall of the tanks.

During the ageing period, MOX was applied to the wine spirit kept in stainless steel tanks. Pure oxygen (X50S Food, Gasin, Portugal) was supplied at a flow rate of 2 mL/L/month, using a multiple diffuser micro-oxygenator (VISIO 6, Vivelys, France) with ceramic diffusers. The flow rate was selected based on the literature and to ensure an adequate supply of oxygen (Canas *et al.*, 2019).

The ten aged wine spirits (two from stainless steel tanks with chestnut wood staves and MOX; two from stainless steel tanks with Limousin oak wood staves and MOX; three from chestnut barrels; three from Limousin oak barrels) were sampled at middle height of the tank or barrel after 8, 15, 30, 180 and 365 days of ageing; a total of 50 samples were taken and analysed. Samples taken after 365 days of ageing were used to determine the antioxidant activity and the related phenolic composition; all the samples were used to examine the evolution of antioxidant activity over the ageing time, as well as the correlations between antioxidant activity and phenolic composition. The collection of samples from a height corresponding to the middle of the tanks and barrels allowed good and reliable results with an acceptable variability to be obtained during the course of the trial.

3. *In vitro* antioxidant activity analysis

This preliminary study of the *in vitro* antioxidant activity (free radical scavenging activity) of wine spirits aged through different technologies was based on the DPPH method, adapted from Ziyatdinova *et al.* (2014). The DPPH method was chosen based on the following criteria: despite results depending on the experimental conditions (Apak, 2019), DPPH is a remarkably stable free radical (Molyneux, 2003), which has an electronic configuration similar to that of peroxyl radicals, the kind of radicals most found in biological systems (Amorati and Valgimigli, 2015; Comert and Gokmen, 2018); the method can be applied to solid and liquid matrices, regardless of the sample complexity (Giovanelli, 2005), and is useful for screening the antioxidant content of natural extracts and foods (Amorati and Valgimigli, 2015); it has been widely used in the study of the antioxidant activity of spirit drinks (Da Porto *et al.*, 2000; Koga *et al.*, 2007; Canas *et al.*, 2008; Ávila-Reyes *et al.*, 2010; Ziyatdinova *et al.*, 2014), enabling the comparison of results.

Briefly, 10 µL of methanol was added to 3 mL of a 8.5 x 10^{-5} M $DPPH^{\bullet}$ methanolic solution and the absorbance was measured (A_i). Next, 10 µl of aged wine spirit was added to 3 mL of a 8.5 x 10^{-5} M $DPPH^{\bullet}$ methanolic solution kept in a test tube wrapped with aluminium foil. The tube was shaken, using a vortex, every 10 min for 60 min. Absorbance was then measured (A_f). Determinations were performed at 515 nm using a Varian Cary 100 Bio spectrophotometer (Santa Clara, USA) and a 10-mm path quartz cell. In both steps, pure methanol was used as a reference. To assure the repeatability of the method, the $DPPH^{\bullet}$ solution, the wine spirit samples and the test tubes were kept in a bath at 30 ºC. The analysis was performed in triplicate.

The inhibition percentage of the $DPPH^{\bullet}$ solution was calculated according to Equation 1.

$$\% \text{ DPPH inhibition} = (A_i - A_f)/A_i \times 100 \qquad (1)$$

The method was tested for linearity and repeatability in order to assure the reliability of the results. The study of linearity was performed with the analysis of six solutions of Trolox (a standard antioxidant), in duplicate. Calculations were carried out using the least-squares method. The linear regression (y=6.188x+1.5939, where y is the % DPPH inhibition and x is the Trolox concentration; r=0.99936) was the best model for establishing a relationship between the percentage of DPPH inhibition and the Trolox concentration, based on the following statistical criteria (ISO 8466/1, 1990): a slope significantly different from zero; an intercept not significantly different from zero; a correlation coefficient higher than 0.999; a residual variance due to the adjustment error (lack-of-fit) not significant (P = 99.5 %). The repeatability was calculated from ten replicates of two quite different wine spirits analysed under constant operating conditions (laboratory, equipment, operator and method) over a short period of time (ISO 5725/2, 1994). The satisfactory relative standard deviation obtained, ranging between 4.89 % and 6.01 %, attested the high precision of the method.

4. Analysis of total phenolic index

The total phenolic index (TPI) of the aged wine spirits was determined as described by Cetó *et al.* (2012). Briefly, the wine spirits were diluted with ethanol/water 77:23 v/v, and the absorbance measurement was performed at 280 nm using a Varian Cary 100 Bio spectrophotometer (Santa Clara, California, USA) and a 10-mm quartz cell. The analysis was performed in duplicate. The total phenolic index was calculated by multiplying the measured absorbance by the dilution factor.

5. Analysis of low molecular weight phenolic compounds

sweight phenolic compounds of the aged wine spirits were quantified according to the method of Canas *et al.* (2003). Chromatography was performed using a HPLC Lachrom Merck Hitachi system (Merck, Darmstadt, Germany) equipped with a quaternary pump L-7100, a column oven L-7350, a UV-Vis detector L-7400, and an autosampler L-7250, coupled with HSM D-7000 software (Merck, Darmstadt, Germany) for management, acquisition and treatment of data. A 250 mm × 4 mm i.d. LiChrospher RP 18 (5 µm) column (Merck, Darmstadt, Germany) was used as a stationary phase. Detection was made at 280 nm for phenolic acids (gallic acid, vanillic acid, syringic acid and ellagic acid), and at 320 nm for phenolic aldehydes (vanillin, syringaldehyde, coniferaldehyde and sinapal-dehyde). Samples were spiked with an internal standard (20 mg/L of 4-hydroxybenzaldehyde), filtered through 0.45 µm membrane (Titan, Scientific Resources Ltd., Gloucester, UK) and analysed by direct injection of 20 µL. The identification of chromatographic peaks was carried out using both retention time and UV-Vis spectra matching with standards. The chromatographic purity of the peaks and the UV-Vis spectra (200–400 nm) were assessed using a Waters system equipped with a photodiode-array detector (Waters 996), with the same chromatographic conditions, managed by 'Millennium 2010' software (Waters, Milford, USA). The analysis was performed in duplicate. Quantification of each compound was carried out through calibration curve made with the corresponding commercial standard.

6. Statistical analysis

A two-way analysis of variance (ANOVA) was carried out to examine the effects of the ageing technology (two levels: stainless steel tanks with MOX and wood staves; new barrels) and the kind of wood (two levels: chestnut; Limousin oak), as fixed factors, on the antioxidant activity and phenolic composition of the wine spirits. Fisher's Least Significant Difference (LSD) test was used to compare the averages when a significant difference ($p<0.05$) was detected. A correlation analysis between the antioxidant activity and the phenolic composition was also performed. All the calculations were made using Statistica vs '98 edition (Statsoft Inc., Tulsa, USA).

RESULTS AND DISCUSSION

1. Effect of the ageing technology and kind of wood on the antioxidant activity and phenolic content

Table 1 shows the two-way ANOVA results for the antioxidant activity, expressed as percentage of DPPH inhibition, and for the total phenolic index of the wine spirits after one year of ageing. The average values are arranged according to the ageing technology and the wood used. The significance of the interaction between these two ageing factors is also presented to evidence whether or not the effect of the ageing technology depends on the kind of wood. Since the interaction was not significant for both antioxidant activity and total phenolic index, the discussion only covers the effect of each factor separately.

Regardless of the kind of wood, after one year of ageing, the antioxidant activity was significantly higher in the wine spirits resulting from the alternative technology (MOX + staves) than from the traditional one (new barrels), despite the variability observed; the percentage of DPPH inhibition was about 1.5-fold higher in the former. Similar average values for wine spirits aged in new barrels (made from Limousin oak and chestnut wood) were obtained in a previous study (Canas *et al.*, 2008). As previously mentioned, no previous research on spirit beverages has examined the impact of different ageing technologies on their antioxidant activity. Regarding red wine, the ageing process using MOX combined with wood pieces has often been studied, but there is no information available in the literature about its influence on the antioxidant activity. For these reasons, while it would be interesting to compare the results obtained with those of other similar alcoholic beverages, it is not possible to do so.

Correspondingly, the total phenolic index was significantly influenced by the ageing technology, being about two-fold higher in the wine spirits aged with MOX and staves than in those aged in barrels. Indeed, the alternative technology

TABLE 1. Average values of antioxidant activity and total phenolic index in one-year aged wine spirits according to ageing technology and kind of wood.

	% DPPH inhibition	TPI
Technology	0.0021***	0.0000***
MOX + staves	50.43 ± 23.11 b	51.79 ± 16.11 b
New barrels	36.42 ± 23.59 a	27.72 ± 11.27 a
Wood	0.0000***	0.0000***
Chestnut	62.69 ± 8.46 b	49.03 ± 15.32 b
Limousin oak	21.35 ± 8.62 a	25.67 ± 11.20 a
Interaction technology * wood	0.6380	0.0518

Results expressed as mean ± standard deviation. For each factor, p values and their significance are given, *** significance at $p<0.001$. For each factor, means within the same column followed by different letters are significantly different ($p<0.05$). TPI – total phenolic index.

enhanced the extraction of wood-derived phenolic compounds, reinforcing the performance noticed in the first six months of ageing (Canas *et al.*, 2019). This behaviour has also been observed in several studies on red wine aged with pieces of wood (chips and staves among others) and in barrels, and has been assigned to a greater absorption of the beverage and a faster release of phenolic compounds from the pieces of wood than from the barrel (Laqui-Estaña *et al.*, 2018). According to Jourdes *et al.* (2011), extraction from pieces of wood is favoured, because the liquid can easily access the primary and secondary xylem vessels, which are not so easily reached in the barrel staves, on which they are oriented perpendicularly to liquid permeation.

The slightly higher difference between wine spirits resulting from different ageing technologies in total phenolic index than in antioxidant activity suggests that both the concentration and qualitative aspects (synergistic and/or antagonistic phenomena between phenolic compounds) have determined this property (Karvela *et al.*, 2008).

Regarding the wood used, irrespective of the ageing technology, the results highlight the highest antioxidant activity induced by the chestnut wood, which surpassed by 41% that promoted by Limousin oak in the one-year aged wine spirits. This outcome was expected given the noticeable antioxidant activity that has been detected in the chestnut wood itself by Alañón *et al.* (2011). Furthermore, Canas *et al.* (2008) have pointed out the greater antioxidant activity conferred by chestnut wood than by Limousin oak to wine spirits aged in new barrels. Interestingly, Alañón *et al.* (2011) have reported the highest antioxidant activity of *Castanea sativa* wood and *Quercus robur* wood compared with that of *Quercus pyrenaica*, *Quercus petraea* and *Prunus avium* from Spanish forests, but they have also found higher average values for *Quercus robur* than for *Castanea sativa,* although without significant differences. The discrepancy between our results and the latter can be ascribed to the geographical origin of the wood (Martínez-Gil *et al.*, 2018a), the influence of the heat treatment on the phenolic composition, and the phenomena involving phenolic compounds during ageing (Canas, 2017).

The same effect was noted for the total phenolic index; that is, greater enrichment (about double) of the wine spirits afforded by the chestnut wood. This result is in agreement with those of previous work on the traditional ageing of wine spirit (Canas *et al.*, 2008) and red wine (Gambuti *et al.*, 2010). This reflects the higher pool of extractable compounds in chestnut wood than in Limousin oak wood (Canas *et al.*, 2018), and the likely influence of the former's higher porosity (Carvalho, 1998) in both extraction and oxidation reactions involving the wood-derived phenolic compounds, whether in barrels or in stainless steel tanks (Canas *et al.*, 2018; Canas *et al.*, 2019). Despite the aforementioned non-significant interaction ageing technology x wood, the results emphasise the importance of exploring other kinds of wood besides oak for their features, to attain the best ageing performance, especially with alternative technology.

To examine the related phenolic composition, the two-way ANOVA results for low molecular weight phenolic compounds concentrations were also arranged according to the ageing technology and the kind of wood (Table 2). Their concentrations were higher than the limit of quantification, except for vanillic acid (LOQ=1.01 mg/L) (Canas *et al.*, 2003). Therefore, the results obtained for this last compound will not be discussed.

As for the total phenolic index, significant differences in the concentrations of wood-derived phenolic compounds were observed depending on the ageing technology; higher levels resulted from MOX and staves, except for gallic acid. Similar behaviour identified in the first six months of ageing (Canas *et al.*, 2019) indicates that the extraction/oxidation pattern in each ageing technology remained until the end of the first year.

Regarding syringic acid and phenolic aldehydes, they can be directly released from the wood, in which they exist, as a result of either the thermal degradation of lignin during the heat treatment of the staves and the barrel (Martínez-Gil *et al.*, 2018b), or of the hydroalcoholysis of lignin during ageing (Canas *et al.*, 2013). In addition, oxidation reactions occurring in the liquid medium are responsible for the transformation of cinnamic aldehydes (coniferaldehyde and sinapaldehyde) into benzoic aldehydes (vanillin and syringal-dehyde), and from aldehydes into the corresponding phenolic acids (Canas *et al.*, 2013). The balance between such chemical pathways determines phenolic concentrations in aged wine spirits. Since oxygen plays a key role in these reactions (Cernîsev, 2017), its direct supply combined with staves, may have favoured the

TABLE 2. Average values of low molecular weight phenolic compounds concentrations (mg/L) of the wine spirits after one year of ageing depending on the ageing techn

	Gallic acid	Vanillic acid	Syringic acid	Ellagic acid	Vanillin	Syringaldehyde	Coniferaldehyde	Sinapaldehyde
Technology	0.0137*	0.1433	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***
MOX + staves	36.51 ± 37.27 a	0.69 ± 0.23	11.17 ± 0.87 b	18.42 ± 7.53 b	7.73 ± 1.11 b	22.32 ± 0.83 b	16.79 ± 3.27 b	63.20 ± 9.45 b
New barrels	60.49 ± 60.36 b	1.35 ± 1.22	2.24 ±0.40 a	9.25 ± 4.03 a	3.14 ± 0.56 a	7.54 ± 1.03 a	6.74 ± 0.59 a	19.02 ± 2.27 a
Wood	0.0000***	0.0401*	0.0034**	0.0000***	0.0000***	0.1942	0.0002***	0.0023**
Chestnut	96.23 ± 28.33 b	1.69 ± 1.08 b	6.23 ± 5.20 b	17.67 ± 6.67 b	5.64 ± 2.78 b	13.91 ± 8.05	9.78 ± 3.84 a	34.36 ± 19.11 a
Limousin oak	5.57 ± 1.26 a	0.49 ± 0.30 a	5.39 ± 4.60 a	8.16 ± 3.49 a	4.30 ± 2.26 a	13.00 ± 8.22	11.74 ± 7.20 b	39.02 ± 29.42 b
Interaction technology * wood	0.0202*	0.0817	0.0338*	0.0032**	0.0073**	0.8649	0.0000***	0.0003***
MOX + staves * chestnut	68.76 ± 2.74 b	0.79 ±0.01	11.92 ± 0.06 c	24.89 ± 1.26 c	8.68 ± 0.02 d	22.71 ±0.96	13.97 ± 0.17 b	55.18 ± 3.34 b
MOX + staves * Limousin oak	4.26 ± 0.49 a	0.58 ±0.33	10.42 ± 0.10 b	11.94 ± 0.88 b	6.77 ± 0.09 c	21.94 ± 0.73	19.61 ± 0.41 c	71.22 ± 0.14 c
New barrels * chestnut	114.54 ± 18.55 c	2.29 ±0.99	2.44 ± 0.31 a	12.86 ± 1.16 b	3.61 ± 0.22 b	8.04 ±0.16	7.00 ± 0.57 a	20.48 ± 1.48 a
New barrels * Limousin oak	6.45 ± 0.45 a	0.42 ±0.33	2.04 ± 0.44 a	5.64 ± 0.34 a	2.66 ± 0.23 a	7.04 ± 1.37	6.49 ± 0.58 a	17.56 ± 2.07 a

Results expressed as mean ± standard deviation. For each factor and its interaction, p values and their significance are presented, * significance at p<0.05, ** significance at p<0.01; *** significance at p<0.001. For each factor and its interaction, means within the same column followed by different letters are significantly different (p<0.05).

accumulation of lignin derivatives in the wine spirits produced by the alternative technology.

Ellagic acid provided by the wood is closely related to the thermal degradation of ellagitannins during the heat treatment in cooperage (Gagić *et al.*, 2019). Oxidation and hydrolysis of ellagitannins in the liquid medium (Karvela *et al.*, 2008; García-Estévez *et al.*, 2017) can also give rise to ellagic acid. Taking into account the literature (Jourdes *et al.*, 2011; García-Estévez *et al.*, 2017), faster extraction and higher rate of oxidation reactions involving ellagitannins are expected under conditions created by MOX and staves, especially during the ageing of spirits (Karvela *et al.*, 2008). This may explain the higher level of ellagic acid found in the wine spirits aged by the alternative technology.

Gallic acid can also be directly extracted from the wood and/or derived from gallotannins (Martínez-Gil *et al.*, 2018b). Lower levels in wine spirits aged with MOX and staves can be explained by a more intense degradation and/or involvement of this acid in chemical reactions under the direct supply of oxygen. Indeed, gallic acid is prone to oxidation with the formation of hydrogen peroxide, quinones and semiquinones (Eslami *et al.*, 2010) as has been observed in the chemical oxidation of wine (del Álamo-Sanza and Nevares, 2018).

The kind of wood used also had a significant impact on the content of low molecular weight compounds in wine spirits that have aged for one year. The chestnut wood induced higher content, as a result of higher levels of syringic acid, ellagic acid, vanillin, and especially gallic acid. Conversely, Limousin oak only promoted higher levels of coniferaldehyde and sinapaldehyde. These differences express the features of each wood (Martínez-Gil *et al.*, 2018a), and are also in accordance with studies on traditional ageing (Canas, 2017). When comparing the results with those obtained in the first six months of ageing (Canas *et al.*, 2019), similar effects were attained, but in the beginning of ageing coniferaldehyde and sinapaldehyde contents were not significantly influenced by the wood. Hence, it is worth noting the greater differentiation of the wine spirits obtained at the end of the first year.

In addition, significant interactions between the ageing technology and the kind of wood on levels of gallic acid, syringic acid, ellagic acid, vanillin, coniferaldehyde and sinapaldehyde were found.

The results show that MOX combined with staves potentiated the effect of each kind of wood, except for that on gallic acid, which exhibited higher contents in the wine spirits aged in barrels. The highest levels of syringic acid, ellagic acid and vanillin were observed in the wine spirits aged by the alternative technology with chestnut wood, whereas the highest levels of coniferaldehyde and sinapaldehyde were observed in the wine spirits aged by the alternative technology with Limousin oak wood.

Regarding the overall effect on the antioxidant activity, total phenolic index and low molecular weight compounds contents of the one-year wine spirits, the best performance was attained when applying the alternative technology especially with chestnut wood staves. Although there is less information available on chestnut wood for ageing purposes than on oak wood, particularly on the oxygen transfer rate, the former seemed to promote more intense extraction/oxidation reactions involving wood-derived compounds as a result of its physical and chemical characteristics.

2. Changes in the antioxidant activity over the ageing time

A two-way ANOVA was applied to the data of each sampling time (8, 15, 30, 180 and 365 days) to examine the influence of the ageing technology and the kind of wood on the antioxidant activity over ageing time. The results shown in Figure 1 reveal that MOX combined with staves promoted higher antioxidant activity than the new barrel throughout the ageing period. Significant and consistent differences were observed between traditional and alternative technology involving chestnut wood from the 8th day (4.06 % *vs* 8.51 %) until the end of the first year (15 days – 6.81 % *vs* 10.93 %; 30 days – 9.32 % *vs* 18.05 %; 180 days – 33.38 % *vs* 49.29 %; 365 days – 57.63 % *vs* 70.29 %). Nevertheless, significant differences between the Limousin oak modalities were only observed after 180 days (9.78 % *vs* 17.21 %) to 365 days (15.20 % *vs* 30.58 %). Regardless of the kind of wood, the antioxidant activity increased progressively over ageing time, but the change was more marked from 180 days onwards. Similar results have been reported for other spirit beverages aged in barrels: Koga *et al.* (2007) found a positive correlation between the antioxidant activity and the ageing time of commercial Scottish and Japanese whiskies; Schwarz *et al.* (2009) described an increasing antioxidant activity of "Brandy de Jerez" with time spent in wooden barrels irrespective of the ageing system used (dynamic *vs* static) and the kind of brandy (experimental *vs* commercial); Ávila-Reyes *et al.* (2010) observed an increase of mescal's antioxidant activity over seven months of ageing in oak wooden barrels; Ziyatdinova *et al.* (2014) asserted that the antioxidant activity of commercial wine spirits and brandies from Russia, Armenia, Ukraine and Azerbaijan increased with beverage age.

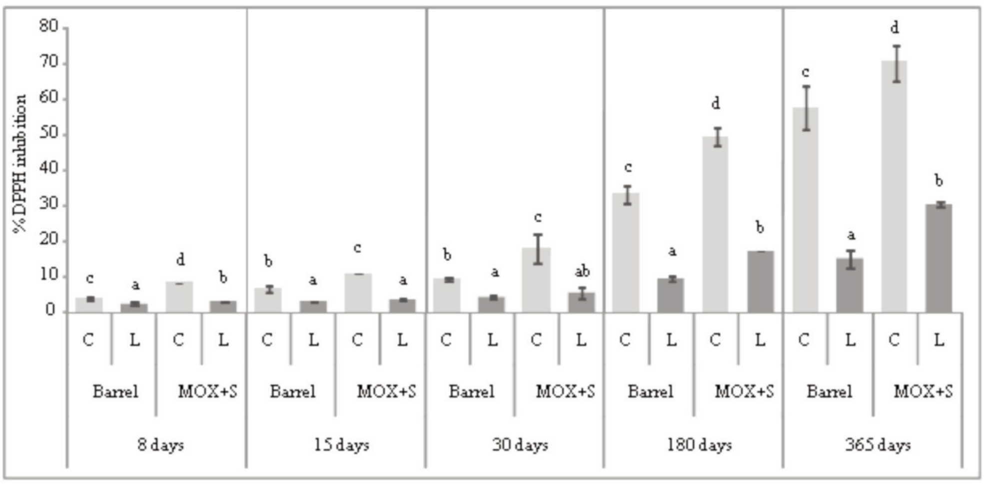

FIGURE 1. Average values of antioxidant activity of the wine spirits over ageing time according to ageing technology and kind of wood.

For each sampling time, columns with different letters are significantly different ($p<0.05$). C : chestnut wood; L: Limousin oak wood; Barrel – New barrel; MOX + S – micro-oxygenation combined with staves.

TABLE 3. Correlation coefficients between antioxidant activity, total phenolic index and low molecular weight phenolic compounds of the wine spirits.

	Both technologies	MOX + Staves	Barrels
Total phenolic index	0.9540*	0.9819*	0.9790*
Gallic acid	0.8752*	0.9249*	0.9784*
Syringic acid	0.7254*	0.8872*	0.8295*
Ellagic acid	0.9539*	0.9845*	0.9715*
Vanillic acid	0.8450*	0.9060*	0.8750*
Syringaldehyde	0.7663*	0.8381*	0.8074*
Coniferaldehyde	0.6953*	0.6955*	0.7813*
Sinapaldehyde	0.6938*	0.7414*	0.8073*

*n=50 for both technologies; n=20 for MOX + staves; n=30 for new barrels; *significant correlation ($p<0.05$).

It is noteworthy that the antioxidant activity reached at the end of the first year by the wine spirits aged in new barrels was achieved within 1-6 months by those aged in stainless steel tanks with MOX and staves, regardless of the kind of wood.

The results of the evolution of antioxidant activity over time were coherent with those of total phenolic index found for the same aged wine spirits by Canas *et al.* (2019).

3. Correlation between the antioxidant activity and the phenolic composition

Based on the results of antioxidant activity and phenolic composition of all samples taken during the ageing period, three correlation analyses were performed taking into consideration both ageing technologies together and each ageing technology separately. The results obtained are shown in Table 3. A positive and significant correlation between the antioxidant activity and the total phenolic index of the aged wine spirits was found, especially when associated with MOX combined with staves (r=0.9819). This latter reflects the remarkable enrichment of the wine spirits in wood-derived compounds resulting from this alternative technology, as mentioned above, and their contribution to antioxidant activity. This finding is of paramount importance since several studies have reported the free radical-scavenging activity of phenolic compounds and their crucial role in the human body in preventing damage to biological molecules that trigger numerous diseases (Rasouli *et al.*, 2017; Niki, 2018).

Specifically, despite the positive and significant correlations observed for all the low molecular weight phenolic compounds, those of ellagic acid, gallic acid, vanillin and syringaldehyde should be highlighted. Indeed, these compounds stood out, because the strongest relationship was found between their concentrations and the antioxidant activity of the wine spirits under study. Furthermore, the highest correlations were related to the alternative technology, except for gallic acid. Such behaviour was expected, given that higher levels of most of the individual phenolic compounds were observed in the wine spirits aged in stainless steel tanks with MOX and staves, while gallic acid level was higher in those aged in new barrels.

These results are in line with the literature: ellagic acid and gallic acid have been widely recognised as potent free radical scavengers based on *in vitro* and *in vivo* studies (Martins *et al.*, 2016), and as major contributors to the antioxidant activity of alcoholic beverages aged in wooden barrels (Schwarz *et al.*, 2009; Ziyatdinova *et al.*, 2014). Although vanillin and syringaldehyde are less commonly referred to as antioxidants, their free radical scavenging activity has been reported (Bountagkidou *et al.*, 2010; Ibrahim *et al.*, 2012).

CONCLUSION

Under the experimental conditions of this preliminary essay, the *in vitro* antioxidant activity (free radical scavenging activity) of the aged wine spirits was significantly influenced by the ageing technology and by the kind of wood used. Higher antioxidant activity, together with greater enrichment in wood-derived phenolic compounds, was achieved in the wine spirits aged in stainless steel tanks with MOX and staves than in new barrels. Furthermore, chestnut wood stood out from Limousin oak for higher antioxidant activity and phenolic content induced in the aged wine spirits. This behaviour was consistently observed over the first year of ageing, but more markedly so from six months onwards. Despite less information being available on chestnut wood for ageing purposes than on oak wood, particularly on oxygen transfer rate, the former seemed to favour the extraction/oxidation reactions involving wood-derived compounds as a result of its physical and chemical characteristics.

In addition, positive and significant correlations between the wine spirits' antioxidant activity, total phenolic index and concentrations of ellagic acid, gallic acid, vanillin and syringaldehyde were found, particularly in those obtained through the alternative technology.

Therefore, while the ageing process enhanced the antioxidant activity of the wine spirits, MOX combined with staves afforded higher accumulation of bioactive compounds, thus increasing wine spirit quality and adding value to the product. As a result, the novel technology is a promising option for the industry, alongside the promotion of faster and cheaper ageing processes..

Further research using other assays for estimating antioxidant activity will be performed in order to gain a comprehensive insight into this feature of wine spirits using the alternative technology. More detailed experiments will also be required to elucidate the role of hydrolysable tannins in the antioxidant activity of aged wine spirits. Moreover, it seems relevant to apply a sensory analytical approach to the wine spirits aged through the novel technology in further studies.

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