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Antioxidant profile of red-single variety wines microoxygenated before malolactic fermentation

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ABSTRACT

The controlled addition of oxygen before malolactic fermentation involves stability of wine colour and an improvement in their organoleptic quality. This study has examined its effect on the "in vitro" antioxidant profile of a variety of Spanish red-single variety wines of different ages. Total antioxidant capacity scavenger activity and the biomarkers of oxidative stress were all analysed. Neither antioxidant capacity, nor scavenger activity were influenced by the microoxygenation. However, the biomarkers of oxidative stress showed some effect of this technique. The controlled addition of small amounts of oxygen produced an increase in the prevention of DNA-damage and seems to decrease the capacity to inhibit lipid peroxidation. The effect on the capacity to protect DNA-damages was statistically significant in the groups of young and one year old wines, where microoxygenated wines showed higher values than their respective control ones. Furthermore, the effect on the prevention of lipid peroxidation was only qualitative, any statistical significant difference was found. A varietal effect was observed in analyzing the results, being Tinta del País wines the most influenced by this technique.

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1. Introduction

Moderate consumption of wine appears associated with a decrease in certain disorders due to oxidative stress, such as cardiovascular and neurodegenerative disease and cancer. The antioxidant potential explains this effect and it can be evaluated by "in vitro" and "in vivo" methods (Agewall et al., 2000; Pérez et al., 2002).

"In vitro" methods are the first approximation to the beneficial effect of wine. They can be chemical methods that evaluate antioxidant capacity (AOC) as ABTS, DPPH, DMPD, FRAP and ORAC or methods that evaluate the scavenger activity towards reactive oxygen species (ROS), as hydroxyl radical (HRSA) or superoxide radical (SRSA). On the other hand, there are also "in vitro" methods used as biomarkers of oxidative stress that measure the damage to biomolecules such as the damage to lipids (ABAP-LP) or damage to DNA (DNA-damage).

The antioxidant effect of wine, has been evidenced by numerous experimental assays (Roussis, Lambropoulos, & Soulti, 2005; Tang, 2005; Kar, Laight, Shaw, & Cummings, 2006; Villaño, Fernández-Pachón, Trocoso, & García-Parrilla et al., 2006) and epidemiological studies (Lagrue-Lak-Hal & Andriantsitohaina, 2006; Quincozes-Santos et al., 2007; Rajdl, Racek, Trefil, & Siala, 2007). This activity has been attributable to phenolic compounds present in the wine, mainly due to flavonoid compounds (anthocyanins, flavonols and flavanols) among others. It should be considered that the phenolic composition of wine undergoes continuous changes during wine-making and ageing processes (Fernández-Pachón, Bakkali, Villaño, Troncoso, & García-Parrilla, 2006; Gómez-Cordovés & González-Sanjosé, 1995; Gómez-Plaza, Miñano, & López-Roca, 2006; Netzel et al., 2003; Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2006; Villaño et al., 2006).

New wine-making techniques as microoxygenation and the use of wood substitutes or thermo-vinification, are implemented with the objective of achieving quality wines, reducing costs and improving their organoleptic properties.

Microoxygenation is a technique developed in 1991 in Marinan (France) as a result of the experiences of Ducornau and Lemaire with the French company Oenov. Since then it has expanded enormously over many wine regions. This technique allows the addition of small, continuous and controlled amounts of oxygen into wines through a porous diffuser (Moutonet, Mazauric, Ducournau, & Lemaire, 2001), to reproduce, and even accelerate the aging processes in barrels. Nowadays, the microoxygenation technique is an oenological practice widely used in wineries. In fact, some works have been focused on their application to improve wine quality.

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Some of them relied on the effect of microoxygenation on fermentation development (Heux, Sablayrolles, Cachon, & Dequin, 2006; Poole, Barros-Lopes, & Jiranek, 2002), others on the effect on aging process (Binder, 2003; Kelly & Wollan, 2003). Some papers have been focused on phenolic composition and colour of red wines (Cano-López, Pardo-Minguez, López-Roca, & Gómez-Plaza, 2006; Gerbi, Caudana, Zeppa, & Cagnasso, 2006; Pérez-Magariño, Sanchez-Iglesias, Ortega-Heras, González-Huerta, & González-Sanjosé, 2007; Toit, Lisjak, Marais, & Toit, 2006), while others studied microoxygenation effects on the volatile composition (Ortega-Herás, Rivero-Pérez, Pérez-Magariño, González-Huerta, & González-Sanjosé, 2008), or on the sensorial properties (Otto, 2003; Pour-Nikfardjam & Dykes, 2002; Pour-Nikfardjam & Dykes, 2003; Weiand, 2005).

Oxygen plays an important role in the different processes that take place during winemaking process and the aging of wine. Besides, oxygen has an influence on the phenolic composition and, indirectly, also has an effect on some sensorial characteristics, such as colour, aroma and astringency, all of which determine wine quality (Atanasova, Fulcrand, Cheynier, & Moutonet, 2002; Ortega-Heras, Gonzãlez-Huerta, & Gonzãlez-Sanjosé, 2007). Oxidation, condensation and polymerization reactions in which different compounds are involved (mainly phenolic compounds) are oxygen dependent. These reactions lead to the formation of new pigments and polymeric compounds that can stabilize wine colour, as pyranoanthocyanins and ethyl-bridged adducts.

Undoubtedly, the antioxidant capacity of phenolic compounds depends on their chemical structure, because polyphenols can donate and hydrogen or an electron from their hydroxyl groups and stabilize the phenoxy radical formed by delocalisation of the unpaired electron within the aromatic structure (Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005).

In this sense, no paper has been published with regard to the effect of microoxygenation on the antioxidant profile of wine. This study will enable to determine if the positive effects on the quality of wine, also has a positive effect on their antioxidant properties. It is considered interesting to know whether the structural and conformational changes of phenolic compounds, produced by microoxygenation treatment, could modify the antioxidant potential of wine.

For this reason, the principal aim of this work was to evaluate the possible influence of the microoxygenation on the antioxidant profile of Spanish red wines. The methods used to measure AOC were ABTS, DPPH, DMPD, ORAC and FRAP assays. The scavenging activities of hydroxyl and superoxide radicals, together with two assays of biomarkers of oxidative stress (lipid peroxidation and DNA-damage inhibition) were also evaluated.

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH·), *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), β-phycoerythrin (PE), 2,2′-diazobis-(2-aminodinopropane)-dihydrochloride (ABAP), 6-hidroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (TROLOX), 2,4,6-*Tris* (2-pyridyl)-*S*-triazine) (TPTZ), 2-deoxy-D-ribose, phenazin methosulfate (PMS) and calf thymus DNA from Sigma–Aldrich Co (St. Louis, MO, USA). Potassium persulphate ($K_2O_8S_2$), ferric (III) chloride acid ($FeCl_3$), ferrous (II) sulphate ($FeSO_4$), hydrogen peroxide (H_2O_2), L-ascorbic acid and trichloroacetic acid (TCA) were obtained from Panreac (Barcelona, Spain). Thiobarbituric acid (TBA) was purchased from Merck (Darmastadt, Germany). Bovine serum albumin (BSA), nicotinamide adenine dinucleotide disodium salt (NADH) and 4-nitroblue tetrazolium chloride (NBT) from Roche (In, USA). TRIS and EDTA tretrasodium salt, from Amresco (Ohio, USA).

2.2. Samples

A total of 162 Spanish red wines of different ages were studied, which were elaborated from *Vitis vinifera* grapes from the four most characteristic red grape varieties cultivated in the various winemaking regions of Castilla y Leon with the following Apellations of Origin: Tinta de Toro (TT), Mencía (M), Tempranillo (T) and Tinta del País (TP). Wines from grape of tree consecutives vintages, 2002–2004, were elaborated at the experimental winery of the Enological Station of Castilla y Leon. Series of control and microoxygenated wines were prepared each year. The wines were made by duplicate, according to the following process: around 5000 kg of grapes of each variety and replique were de-stemmed, crushed and 50 mg/kg of SO_2 was added. Alcoholic fermentation took place in stainless steel tanks without yeast inoculation. Temperature was controlled not to rise above 25 °C. Once alcoholic fermentation finished (sugar level under 2 g/L), each wine was strained off from the tank and transferred to two different tanks. One of them, 2000 L capacity and 2.5 m high, was supplied with small and controlled amounts of oxygen, microoxygenated wine (MO), while the other tank (1.500 L capacity) was non-microoxygenated, i.e. the control wine (C). Pure oxygen was applied using a microoxygenation equipment supplied by Oenodev (France). The doses of oxygen applied were set up according to the initial characteristics of the wine (structure, astringency, presence of green tannins and vegetal and reductive aromas), and they ranged from 35 to 47 mL/L/month. Doses and duration of treatment were established mainly by sensory analysis, although volatile acidity and quantity of oxygen dissolved in the wine were also controlled (Pérez-Magariño et al., 2007).

Microoxygenated and control wines carried out malolactic fermentation spontaneously, without the inoculation of lactic acid bacteria. Once malolactic fermentation was finished (malic acid $\leqslant$0.1 g/L) were racked off to new American oak barrels where they were aged for 4–24 months.

Young wines (20), and wines kept for 4 (26), 12 (36) and 24 (32) months in barrels were studied, as well as wines that had spent 12 months in barrels and 6 months in the bottle (16) and 12 months in barrels and 12 months in the bottle (32). So, this work was carried out with a large number of samples of different varieties and ages and always taking control wines that have undergone no microoxygenation.

2.3. Analytical methods

2.3.1. 2.3.1.ABTS·+ method

This assay is based on decoloration that occurs when the radical cation ABTS·+ is reduced to ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (Re, Pellegrini, Proteggente, Pannala, & Yang, 1999). The radical was generated by reaction of a 7 mM solution of ABTS in water with 2.45 mM potassium persulphate (1:1). The assay was made up with 980 μL of ABTS·+ solutions and 20 μL of the sample (at a dilution of 1:50 in water) (12). Absorbance measurements at 734 nm were made after 15 min of reaction time. The results were expressed in mM of Trolox, using the relevant calibration curve.

2.3.2. DPPH· method

This method is based on the reduction of the free radical DPPH· (2,2-diphenyl-1-picrylhydrazyl) (Brand-Williams, Cuvelier, & Berset, 1995). The reaction takes place when 980 μL of DPPH· (60 μM) was mixed with 20 μL of the sample (at a dilution of

1:50 in water). After a reaction time of two hours absorbance values at 517 nm were measured. Results were expressed in mM of Trolox using the dose–response curve described by this substance.

2.3.3. DMPD$^{\cdot+}$ method

The method described by (Fogliano, Verde, Randazzo, & Ritieni, 1999) was applied. 1 mL of DMPD solution (200 mM) and 0.4 mL of ferric chloride (III) (0.05 M) were mixed with sodium acetate buffer solution (0.1 M) until final volume of 100 mL and pH was adjusted to 5.25. The reaction took place when 50 µL of the sample (a dilution of 1:10 in water) were added to 950 µL of the DMPD$^{\cdot+}$ solution. Absorbance at 505 nm was measured after 10 min of continuous stirring. The results were quantified in mM of Trolox on the relevant calibration curve.

2.3.4. FRAP "ferric reducing/antioxidant power"

This method was used to measure the reductive power of a sample (Benzie & Strain, 1996). It is based on increased absorbance at 593 nm due to formation of tripyridyl-*s*-triazine complexes with ferrous (II) (TPTZ–Fe(II)) in the presence of a reductive agent. The reactive mixture was prepared by mixing 25 mL of sodium acetate buffer solution (0.3 M, pH 3.6), 2.5 mL of TPTZ (10 mM), 2.5 mL of $FeCl_3$ (20 mM) and 3 mL of water. 30 µL of the sample (diluted in water at 1:50) was added to 970 µL of the latter reactive mixture and is incubated at 37 °C for 30 min. The results were expressed as mM of Fe (II), using linear calibration obtained with different concentrations of $FeSO_4$.**

2.3.5. ORAC "Oxygen radical absorbance capacity assay"

This method is based on the fluorescent qualities of ß-phycoerythrin, which is a pigment extracted from algae (Cao, Alessio, & Cutler, 1993). The presence of an oxidant (ABAP) leads to the formation of peroxyl radicals that directly attack the ß-phycoerythrin protein molecule and reduce its fluorescence. The action of an antioxidant halts the decay of the molecule and its fluorescence is therefore not completely reduced. The reaction was prompted by mixing 553 µL of ß-phycoerythrin (16.7 nM) with 20 µL of the sample (diluted at 1:10 with water) and 1177 µL of potassium phosphate buffer solution 75 mM (pH 7.4). After 15 min of incubation at 37 °C 750 µL of ABAP were added (32 mM of the final concentration) and fluorescence was measured. The results were calculated by measuring the difference of net areas under the curve (AUC net) taken from the spectrofluorimeter between the white and the sample colours, expressed as mM of Trolox on an appropriate calibration curve. The fluorescence decay curve was measured for 80 min, using emission and excitation wavelengths of 584 nm and 544 nm, respectively.

2.3.6. HRSA

Desoxyribose (2-desoxy-D-ribose) decays when exposed to hydroxyl radicals generated by the Fenton reaction (Halliwell, Gutteridge, & Aruoma, 1987). The hydroxyl radicals ($HO^{\cdot}$) were generated through the following system: 10 µL of $FeCl_3$ (0.1 mM), 10 µL of ascorbic acid (0.1 mM), 10 µL of H_2O_2 (1 mM) and 10 µL of EDTA (0.1 mM). 15 µL of the sample (diluted at 1:50 in water) were incubated at 37 °C for 1 h, with 20 µL of desoxyribose (1 mM final concentration) in the presence of $FeCl_3$, ascorbic acid, H_2O_2 and EDTA in the cited amounts. 1.5 mL of TCA (28% w/v) and 1 mL of TBA (1% w/v, NaOH 0.05 M) was added to 1 mL of the sample under incubation and held for 15 min at 100 °C after which it was left to cool to room temperature. The MDA (malondialdehyde) formed from the decay of the desoxyribose is evaluated in reaction with TBA and espectrofluorimetrically measured at 532 nm. The results were expressed as an inhibition % in relation to a control test (without the sample).

2.3.7. SRSA

The superoxide radical which reacts with NBT generates a coloured compound with absorbance at 560 nm (Liu, Ooi, & Chang, 1997). The antioxidant scavenging superoxide radical leading the decolorization. The reactive was made up with 50 µL NADH (77 µM), 50 µL NBT (50 µM), 5 µL of PMS (3.3 µM) in a medium of Tris–HCl (16 mM, pH 8) and 10 µL of the sample (diluted at 1:5). The results were expressed as an inhibition % in relation to a control test (without the sample).

2.3.8. ABAP-LP

The microsomes from the liver of a 250–300 g male Wistar rat were extracted in line with the method of Kessler, Ubeaud, and Jung (2003). Total microsomal protein content is determined using the Bradford method (Bradford, 1976). A microsomal fraction (1 mg/mL of protein) was incubated with a solution of ABAP (10 mM), prepared in a Tris–HCl buffer solution immediately prior to use, in the presence of 50 µL of the sample (diluted at 1:50). The incubation temperature was set at 37 °C for a period of 90 min. Lipid peroxidation was evaluated with the TBA assay to quantify MDA. Absorbance measured at 532 nm was proportional to the quantity of peroxyl radicals generated and the results were expressed as an inhibition % in relation to a control test (without the sample).

2.3.9. Damage to DNA

2.3.9.1. Incubation of DNA calf thymus. The DNA calf thymus (200 µg) in the absence and the presence of 200 µL of undiluted wine was exposed to the action of hydroxyl radicals generated by the mixture of 100 µL of ascorbic acid (1 mM final concentration) and 10 µL of copper sulphate (II) (100 µM final concentration). The mixture was incubated at 37 °C for 1 h after which the fragments were separated by electrophoresis.

2.3.9.2. Agarose gel electrophoresis. Electrophoresis was carried out with 1% agarose at room temperature using a BIO-RAD power–Pac 1000 (Hercules, CA, USA) electrophoresis system. Subsequently, the gel containing 15 µL of ethidium bromide (10 mg/mL) was observed under ultraviolet light, using a transilluminator, and photographed. The results were expressed as DNA bp fragments, calculated using the standard molecular weight markers such as the 100 bp DNA Ladder of 100–1500 bp and the λ DNA/Hind III of 125 bp to 23.1 Kb, both from Invitrogen (CA, USA).

ABTS, DPPH, DMPD and HRSA methods were applied to all of the wines, whereas FRAP, ORAC, SRSA, ABAP-LP and DNA-damage assays were only applied to the young wines and to those, which were aged 12 and 24 months in barrels. This selection was due to logistical reasons, mainly referred to analysis cost, especially cost in time, but it was also based in previous work, which showed that antioxidant capacities of bottled wines changed slower than those of aged in barrels (Rivero-Pérez, González-Sanjosé, Muñiz, Ortega-Heras, & Pérez-Magariño, 2006).

All of the cited assays were carried out by quadruplicate.

2.4. Statistical analysis

The statistical analysis of the data was carried out by analysis of the variance (ANOVA) and the LSD test (least significant difference) which showed the values statistically different. A significance level of $\alpha \leqslant 0.05$ was used. Statgraphics Plus software programme (Manugistic Inc., 1999) was used.

3. Results and discussion

The microoxygenated and control wines analyzed did not show significant differences in the antioxidant capacity or scavenger

activity values, but in the evaluation of biomarkers of oxidative stress differences were detected (Fig. 1). In this sense, the transformations in the wine after the microoxygenation treatment seem to induce substantial changes in biomarkers methods. Thus, the controlled application of oxygen, increased the protection of damage to DNA, while it reduced the prevention of lipid peroxidation.

Numerous studies have revealed the chemical changes occurring in the wine after the microoxygenation treatment, implying a decline of phenolic compounds, particularly acute in the levels of total anthocyanins. The detailed analysis has detected significant increases in modified and polymeric anthocyanins, while decreasing free anthocyanins (Bosso, Guaita, Vaudano, & Stefano, 2000; Castel, Morland, Pujol, Peyron, & Naudin, 2001; Pérez-Magariño et al., 2007). Furthermore, condensations produced under anaerobic conditions, direct condensations of anthocyanins and procyanidins and those induced by acetaldehyde do not affect to the number of the hydroxyl groups of the involved polyphenols (Roginsky et al., 2006). This fact explains that polymerisation reactions do not always produce a substantial modification of the antioxidant capacity (Tubaro, Rapuzzi, & Ursini, 1999; Ursini & Sevanian, 2002; Zafrilla et al., 2003), such it was also observed in this paper (results of antioxidant capacities evaluated by chemical methods).

The results of biomarker methods, which showed differences between microoxygenated and control wines, it is associated with the fact that different methodologies used to evaluate antioxidant capacities gives different information (Huang, Ou, & Prior, 2005), and therefore they usually give different results, fact also observed by Santos-Buelga and Scalbert (2000).

The protection to DNA-damage results are consistent with those of Lodovici, Guglielmi, Meoni, & Dolora, 2001, which observed greater involvement of polymeric phenols, with respect to monomers in the prevention of DNA damage. ABAP-LP results are easy understood after considering the difficulty to incorporate an antioxidant into the microsomal membranes (De Beer, Joubert, Gelderblom, & Manley, 2005; Rice-Evans, Miller, & Paganga, 1996); which is especially difficult to polymers of high molecular weight, as polyphenol polymers which can be only absorbed after being extensively metabolised (Levrat et al., 1993; Santos-Buelga & Scalbert, 2000). Moreover, the values of ABAP-LP coincide with the fact that the planar structural conformation of simple phenolic compounds promotes incorporation of these molecules into the membrane, whereas a twisted conformation formed for various substituted rings and present in polymers can obstruct this incorporation (De Beer et al., 2005).

Due to the heterogeneity of wines analyzed it is possible that other factors such as the variety and the age interfere in the potential impact of the microoxygenation in the wine, therefore it was decided to evaluate the results grouped by variety and age.

In the separate analysis of the varieties, some differences were found with respect to those described for the global indexes (Fig. 2). The difference between microoxygenated and control wines to protect biomolecules was varietal dependent. Thus, microoxygenated (M), (T) and (TP) wines had higher values of DNA protection than their respective controls. Moreover, differences were only found between the control and microoxygenated T wines. Besides, some differences were found between control and microoxygenated wines for the chemical methods of antioxidant capacity. Thus, microoxygenated (TT) wines showed higher values of DPPH and lower values of DMPD and ORAC. Moreover, microoxygenated TP wines showed lower values of DPPH.

The particular behaviour of TT wines can be associated with events previously described (Pérez-Magariño et al., 2007), which have described the wines of this variety as susceptible to changes of phenolic composition to be subjected to microoxygenation. Moreover, the most DPPH values may be due to the higher content of total flavonols, which have shown great activity against DPPH radical (Furusawa et al., 2005; Wang et al., 2006).

The different effect of microoxygenation on the single variety wines studied justifies and explains the fact that more statistically significant differences were detected between microoxygenated wines than between control wines.

Previous works have also described that the differences arising from microoxygenation are reduced during the ageing process (Ortega-Herás et al., 2007). Accordingly, to detect the possible interference of the age of wine, it was decided to analyze separately data from young wines (without aging), and data from wines aged in barrel during 12 and 24 months (Fig. 3). The results found indicated that the age of the wines does not interfere significantly in the overall results initially commented. So, it was confirmed again that young microoxygenated wines had greater ability to protect the DNA than their respective control wines. The significant difference between MO wines and control ones was also detected in the group of wines aged 12 months in barrels, but it was not detected in the group of wines of 24 months of aging, although also in this cases qualitatively higher values were obtained. Only important qualitative differences were detected between the values of ABAP-LP of microoxygenated and control wines after 12 months of aging.

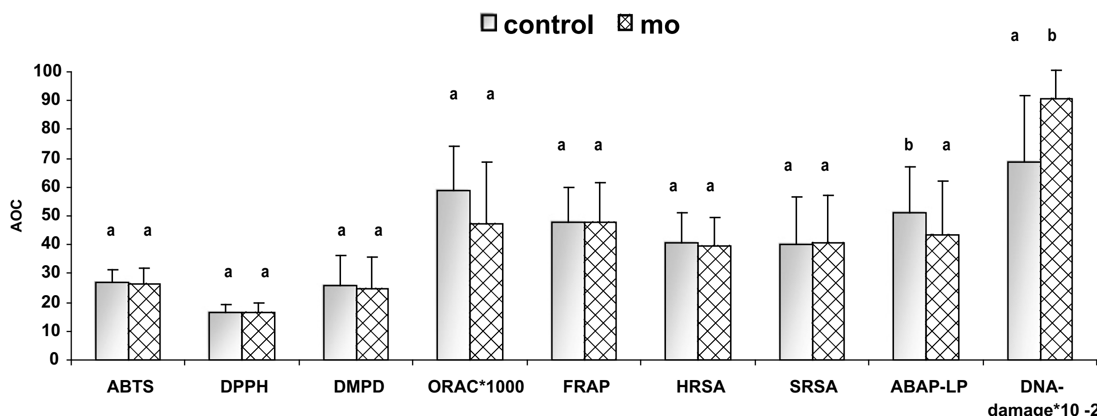

Fig. 1. Antioxidant profile of non-microoxygenated (control) and microoxygenated (mo) Spanish red wines. Averages values are showed ($n = 648$; 162 wines × 4 repliques). Values with different letters are significantly different (LSD test, $p = 0.05$).

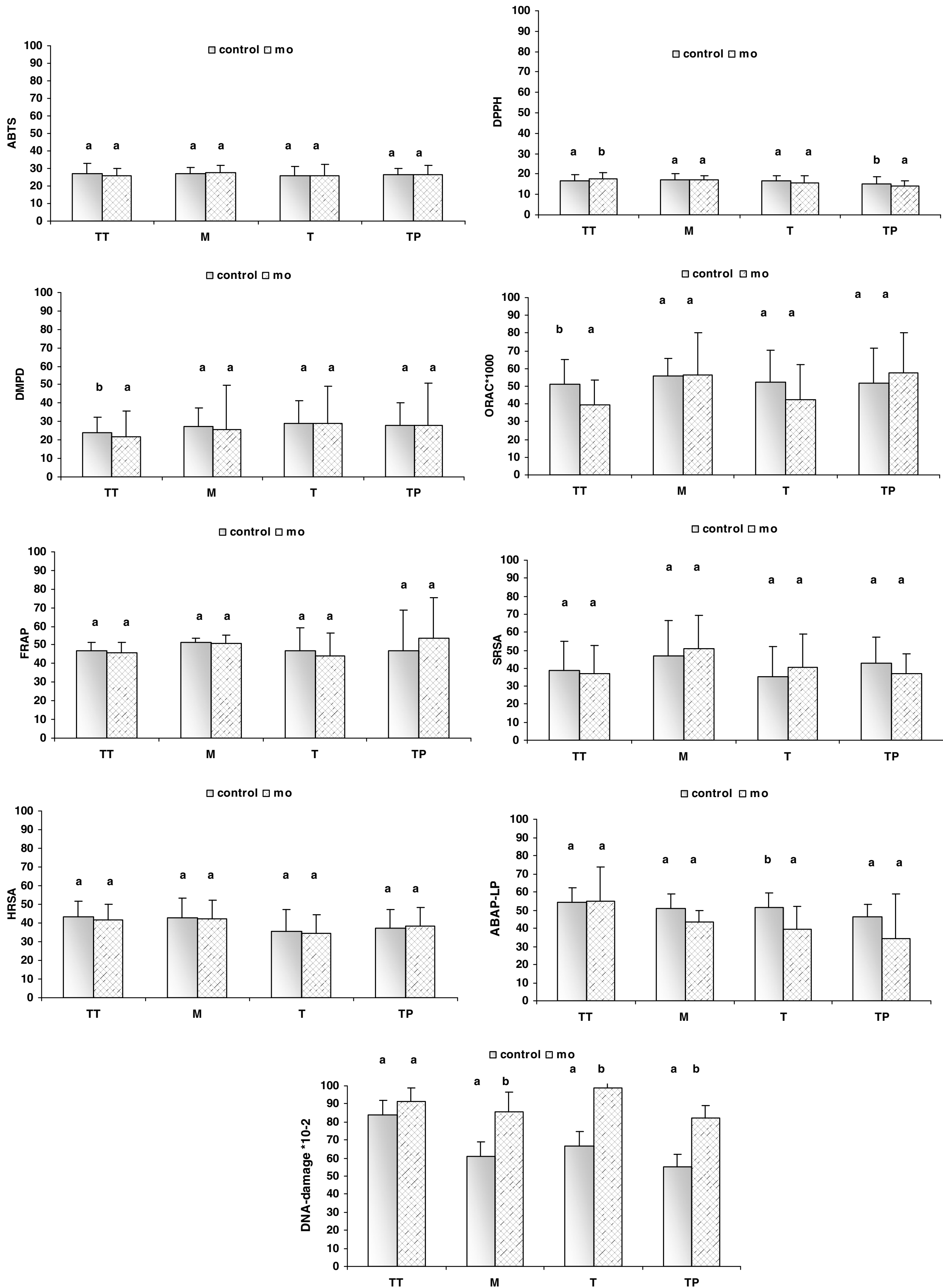

Fig. 2. Antioxidant profile of non-microoxygenated (control) and microoxygenated (mo) Spanish red wines of different varieties. TT (Tinta de Toro); M (Mencía); T (Tempranillo); TP (Tinta del País). Averages values are showed ($n = 648$; 162 wines × 4 repliques). Values with different letters are statistically different (LSD test, $p = 0.05$).

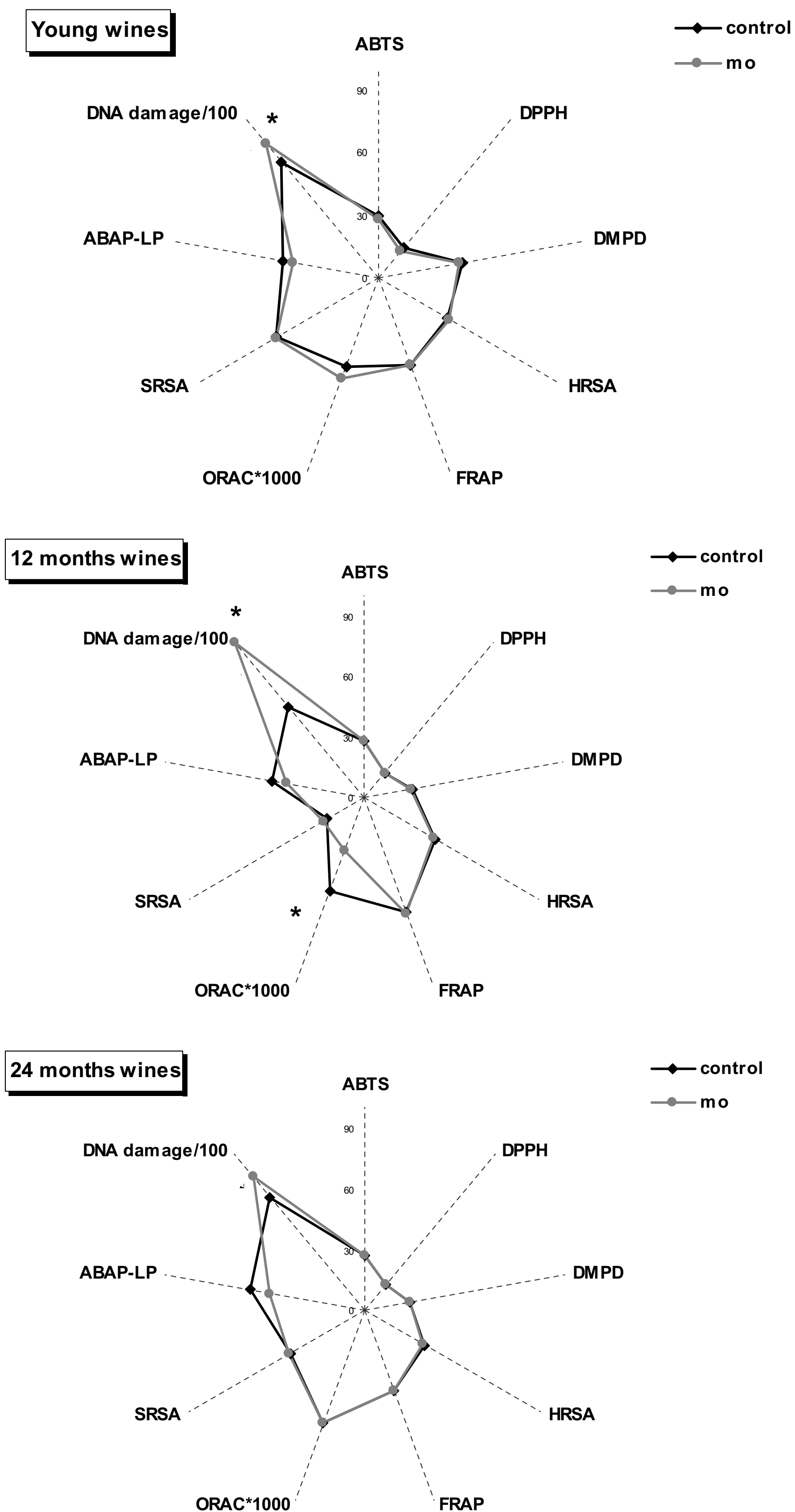

Fig. 3. Antioxidant profile of non-microoxygenated (control) and microoxygenated (mo) Spanish red wines of different ages. Averages values are showed ($n = 648$; 162 wines × 4 bottles). *This symbol indicate values statistically differents between control and microoxygenated wine.

4. Conclusions

The results presented and commented show that the controlled addition of oxygen into well-structured wines, do not lead to significant changes in the antioxidant profiles. No differences were found between the control and the microoxygenated wines for AOC, or scavenger activity. Differences were only found for biomarkers methods. Inhibitory capacity of DNA-damage increases from the microoxygenated wines, while inhibition of lipid peroxidation decreases.

Overall, the effect of microoxygenation on the antioxidant profile of red wine is variety dependent, and is more intense in young wines, remaining even in wines that have been up to 12 months in barrel.

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