Oxygen consumption rates by different oenological tannins in a model wine solution

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ABSTRACT

The kinetics of oxygen consumption by different oenological tannins were measured in a model wine solution using the non-invasive method based on luminiscence. The results indicate that the oxygen consumption rate follows second-order kinetics depending on tannin and oxygen concentrations. They also confirm that the oxygen consumption rate is influenced by temperature in accordance with Arrhenius law. The indications are that ellagitannins are the fastest oxygen consumers of the different oenological tannins, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins. This methodology can therefore be proposed as an index for determining the effectiveness of different commercial tannins in protecting wines against oxidation.

Keywords: Tannins, oxygen consumption rate, antioxidant capacity.

1. Introduction

Nowadays the use of oenological tannins is common practice in winemaking. However, their use is only authorized by the International Organization of Vine and Wine (OIV) to facilitate the clarification of wines and musts (International Oenological Codex, 2016). Nevertheless, it is unquestionable that they are also currently used for many other purposes. Indeed, the literature has attributed several other characteristics to oenological tannins, such as antioxidant activity (protection of wines against oxidation) (Ricci et al., 2016), direct consumption of dissolved oxygen (Navarro et al., 2016), ability to scavenge peroxyl radicals (Magalhães, Ramos, Reis & Segundo, 2014), ability to chelate iron (II), prevention of oxidative damage mediated by Fenton-based reactions (Pérez, Wei & Guo, 2009), antioxidasic activity (anti-laccase activity) (Obradovic, Schulz & Oatey, 2005), improvement of wine structure and mouthfeel (Vivas, 2001), color

improvement and stabilization of red wines (Canuti, Puccioni, Giovani, Salmi, Rosi & Bertuccioli 2012), copigmentation effect (Neves, Spranger, Zhao, Leandro & Sun, 2010), direct formation of new pigments (Versari, du Toit & Parpinello, 2013), elimination of reduction odours (Vivas, 2001) and even bacteriostatic effects (Lempereur, Blayteyron, Labarbe, Saucier, Klebek & Glories, 2002).

Obviously, to all these functions must be added the interactions between oenological tannins and proteins and the resulting ability to prevent protein haze (Ribéreau-Gayon, Glories, Maujean & Dubourdieu, 2006), help protein fining avoid gelatin over-fining (Mierczynska-Vasilev & Smith, 2015) and the effects on wine astringency and bitterness (Obreque-Slier, Peña-Neira & Lopez-Solis, 2012).

Although oenological tannins are commonly employed to these ends, there are questions about them that need to be clarified. Many commercial types of tannin of different plant origins and chemical compositions are available (Malacarne, Nardin, Bertoldi, Nicolini & Larcher, 2016; Obreque-Slíer, Pena-Neira, Lopez-Solis, Ramirez-Escudero & Zamora-Marin, 2009). The so-called oenological tannins on the market include ellagitannins from oak or chestnut, gallotannins from oak galls, and condensed tannins from grape seeds and skins and even other plant origins such as tara, quebracho and mimosa (Versari et al., 2013).

The chemical structure of ellagitannins consists of an open-chain glucose esterified at positions 4 and 6 by a hexahydroxydiphenoyl unit (HHDP) and a nonahydroxyterphenoyl unit (NHTP) esterified at positions 2, 3 and 5 with a C-glycosidic bond between the carbon of the glucose and position 2 of the trihydroxyphenoyl unit (Quideau et al., 2004; Takuo, Takashi, Tsutomu & Hideyuki, 2009). Several different ellagitannins have been described but castalagin and vescalagin are the most abundant in oak wood, accounting for between 40% and 60% of the total by weight (Fernández de Simón, Cadahía, Conde & García-Vallejo, 1999).

Gallotannins are formed by the esterification of gallic acid with the hydroxyl group of a polyol carbohydrate such as glucose (Hagerman, 1998). Gallotannin extracts, which are also commercially known as tannic acid, are mixtures of polygalloyl glucoses or polygalloyl quinic acid esters with a number of galloyl moieties per molecule ranging from 2 to 12 depending on the plant source used to extract the tannic acid (Sylla, Pouységu, Da Costa, Deffieux, Monti & Quideau, 2015).

The composition of condensed tannins depends on their plant origin. Thus grape-seed condensed tannins are procyanidins with a lower mean degree of polymerization (mDP) and a high level of galloylation (Santos-Buelga, Francis-Aricha & Escribano-Bailón, 1995), whereas grape-skin condensed tannins are a mixture of procyanidins and prodelphinidins with a higher mDP and a lower level of galloylation (Souquet, Cheynier, Brossaud & Moutounet, 1996). Condensed tannins from other plant sources also have different composition. Those from quebracho, for instance, are profisetinidins and not procyanidins because their acidic cleavage originates fisetinidin and not cyanidin (Celzard et al., 2015), while those from mimosa are prorobinetinidins (Celzard et al., 2015) because their acidic cleavage originates robinetinidin. Condensed tannins as a whole are called proanthocyanidins.

Of the different functions attributed to oenological tannins, their antioxidant capability is probably one of the main reasons why they are widely used in winemaking for preventing grape juice and wine oxidation. It is traditionally accepted that oenological tannins inhibit polyphenol oxidases, tyrosinase (EC 1.14.18.1) and laccase (EC 1.10.3.2), thus protecting wines against browning (Nichols-Orians, 1991; Versari et al. 2013), and that they directly consume oxygen, thereby protecting the other wine components from oxidation (Navarro et al., 2016; Vivas & Glories, 1996).

There are a number of references to the antioxidant properties of commercial tannins (Neves et al., 2010; Laghi, Parpinello, Del Rio, Calani, Mattioli & Versari et al., 2010; Magalhães et al., 2014) using different antioxidant assays (CUPRAC, DPPH, FRAP, ORAC, Folin-Ciocalteu, …). However, all these methods are indirect methods and none of them really measure the direct oxygen consumption. Moreover, the different antioxidant assays produce different and sometimes contradictory results (Magalhães et al., 2014). For these reasons, the aim of this paper is therefore to measure the kinetics of oxygen consumption by different commercial tannins in order to determine their real antioxidant capacities to protect wine against oxygen.

2. Materials and methods

2.1. Chemicals and equipment

All samples and standards were handled without exposure to light. ABTS: 2,2'azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%, manganese dioxide, AAPH: 2,2-azobis(2-methylpropionamidine) dihydrochloride, gallic acid, copper (II) sulfate pentahydrate, iron (III) chloride hexahydrate, Folin-Ciocalteu reagent and fluorescein sodium salt were purchased from Sigma® (St. Louis, MO, USA); DPPH: 2,2-diphenyl-1-picrylhydrazyl from Extrasynthèse (Genay, France); L(+)-tartaric acid, sodium hydroxide, potassium metabisulfite, ascorbic acid, ethanol and methanol HPLC grade were purchased from Panreac Química (Barcelona, Spain). Water was ultrapure Milli-Q quality (Millipore, Bedford, MA, USA).

2.2. Oenological tannins

Five different tannins were used in this study. Quebracho tannin (Tanin SR) from Enotecnia Cataluña S.L. (Vilafranca del Pènedes, Barcelona, Spain); seed tannin (Protan pépin), ellagitannin (Ellagitan Chêne) and gallotannin (Galovin) from AEB Iberica S.A. (Castellbisbal, Barcelona, Spain); and skin tannin (Tan Sutil) from Agrovin, (Alcazar de San Juan, Ciudad Real, Spain).

2.3. Experimental design

The experimental design previously described by Navarro et al. (2016) was used for oxygen consumption measurements. A model wine solution composed of ethanol (12% v/v) and tartaric acid (4 g/L) adjusted at pH = 3.5 with sodium hydroxide was used. This solution was enriched with 3 mg of Iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L in the form of copper (II) sulfate pentahydrate. We

have worked with this model wine solution and not with real wine because the naturally occurring phenolic compounds would compete with oenological tannins in oxygen consumption making it impossible to determine the kinetic constants. The different doses of oenological tannins, potassium metabisulfite or ascorbic acid were placed in clear glass bottles into which a pill had previously been inserted (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203-01_PSt3-0828-01, Regensburg, Germany) for the non-invasive measurement of dissolved oxygen by luminescence (Nomasense TM O2 Trace Oxigen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium). The bottles were completely filled with the model wine solution and closed immediately after with a crown cap and bidule so as to minimize the volume of headspace. The bottles were then gently shaken to dissolve the different antioxidants: ascorbic acid, sulfur dioxide (added as potassium metabisulfite) or the different oenological tannins. Oxygen (Diéval, Vidal, & Aagaard, 2011) was measured periodically to determine the oxygen consumption rate. All the experiments were performed in triplicate.

2.4. Estimation of the influence of sulfur dioxide, ascorbic acid and oenological tannin concentrations on the oxygen consumption rate

In this assay the oxygen concentration was fixed at saturation level, whereas the tannin concentration was variable and the temperature maintained at 20 ± 1 ºC. Three doses of the different oenological tannins (2, 4 and 6 g/L) were assayed. These doses were higher than those usually applied to musts and wines in order to minimize the measurement time required for the complete consumption of oxygen. Three doses (50, 100 and 150 mg/L) of sulfur dioxide (E220) and ascorbic acid (E300) were also studied to compare the oxygen consumption rate (OCR) of the different tannins with the OCR of the antioxidant additives most used in winemaking. The model wine solution was saturated with oxygen (8.0 mg/L) by bubbling with air for 10 min and then used immediately for the experiment. All assays were performed in triplicate, taking control bottles with the oxygen-saturated model wine solution without any addition as the control reference.

2.5. Estimation of the influence of temperature on the OCR

In this assay the oenological tannins (4 g/L) and the oxygen concentrations (saturation level; 8.0 mg/L) were fixed and the temperature was variable (8 ± 1, 20 ± 1 and 30 ± 1 ºC). All assays were performed in triplicate, taking control bottles with the oxygen-saturated model wine solution without any addition as the control reference.

2.6. Estimation of the influence of oxygen concentration on the OCR

In this assay the oenological tannin concentration was fixed (4 g/L), whereas the oxygen concentration was variable (2.5, 4.0 and 8.0 mg/L) and the temperature maintained at 20 ± 1 ºC. The model wine solution was initially saturated with oxygen by bubbling with air for 10 minutes and then used directly for the trials with 8 mg/L. The rest of the saturated solution was then bubbled with nitrogen to eliminate the excess oxygen

until the desired value was reached (2.5 or 4.0 mg/L). All assays were performed in triplicate, taking control bottles with the different oxygen concentrations without any addition as the control reference.

2.7. Antioxidant capacity

The antioxidant capacity of the different oenological tannins was measured using various methods: total concentration of phenolic substances by means of the Folin-Ciocalteu index (TPC, OIV, 2009), $ABTS^{+}$ and DPPH (Vallverdú-Queralt, Regueiro, Rinaldi de Alvarenga, Torrado & Lamuela-Raventos, 2014) and ORAC assays (Suarez, Romero, Ramo, Macìa & Motilva, 2009).

2.8. Statistics

All the chemical and physical data for the samples are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance tests ($p < 0.05$) were carried out with SPSS software (IBM, Barcelona, Spain), and multiple comparisons were performed using the Student–Newman–Keuls post-hoc test.

3. Results and discussion

Fig. 1 shows the oxygen consumption kinetics of ascorbic acid, sulfur dioxide and different oenological tannins at different concentrations in an oxygen-saturated model wine solution at 20 ºC. The oxygen consumption of the control model wine solution (without addition of any antioxidant) was very low and can be considered negligible (data not shown). In contrast, the supplementation with ascorbic acid, sulfur dioxide and all the oenological tannins resulted in an oxygen consumption that was clearly influenced by the nature and dosage of the added antioxidant. Ascorbic acid was clearly the most efficient antioxidant (Fig. 1B) because complete consumption of all oxygen took place in a matter of hours, whereas all the other antioxidants needed days to obtain the same effect. A comparison of the oxygen consumption kinetics of sulfur dioxide and the different oenological tannins seems to indicate the existence of differences in antioxidant effectiveness among all these antioxidant additives. However, the quantitative comparison of the oxygen consumption kinetics of all these compounds is not evident from looking at these graphics. A kinetic modelization is therefore necessary in order to better quantify the antioxidant effectiveness of the various additives.

Different mathematical regression approaches were tried (lineal, logarithmic, exponential …), but none of them provided satisfactory results (data not shown). After considering other possibilities, an acceptable modelization was obtained when the inverse of consumed oxygen was plotted versus the inverse of time. Fig. 2 shows the results obtained for sulfur dioxide (Fig. 2A), ascorbic acid (Fig. 2B) and seed tannins (Fig. 2C). It can be seen that satisfactory lineal regression coefficients were obtained and relatively low Mean Square Errors (MSE), thus confirming that this mathematical model works quite well. Similar results were

obtained for the other oenological tannins (Supplementary Figure 1), but they are not shown in this figure so as not to overcomplicate the graphic. Their OCRs are shown below.

According to this modelization, the following equation can be established: $1/[O_2] = A/t + B$. This equation describes the relationship between the consumed oxygen versus time and is shown in Fig. 2D, which also shows how the consumed oxygen can be cleared up, how the first derivative is obtained, and finally how the OCR at time zero ($OCRt_0$) can be determined. These correspond to the inverse of the slope of the initial equation.

Using this procedure it is possible to determine the relationship between the $OCRt_0$ and the antioxidant concentration for all the antioxidants studied. Figures 2.E, 2.F and 2.G show the representation of the $OCRt_0$ versus the concentration of sulfur dioxide, ascorbic acid and seed tannin respectively. These graphics clearly indicate that the $OCRt_0$ is lineally dependent on the antioxidant concentration, considering that the linear regression coefficients are reasonably good in all three examples (> 0.9700) and the MSE relatively low (< 0.02). Moreover, the slope of the straight lines obtained provides the real OCR in function of the antioxidant concentration. Similar behaviors were obtained for the other oenological tannins (Supplementary Figure 2), enabling calculation of the OCR of each antioxidant expressed as mg of oxygen per day and per gram of antioxidant.

Table 1 shows the antioxidant capacity of the different oenological tannins assessed by different analytical methods (TPC, DPPH, ABTS and ORAC) and also the OCR determined as described above. The aim of this comparison was to test whether or not the real OCR matches the most usual antioxidant capacity tests. Table 1 also shows the OCR of sulfur dioxide and ascorbic acid in order to compare the effectiveness of the different oenological tannins with the two most frequently used wine antioxidants.

In general the data confirm that the different antioxidant capacity tests obtained different and contradictory results. Briefly, the TPC test gave gallotannin the highest value followed by, in decreasing order, skin tannin, quebracho tannin, seed tannin and ellagitannin. The DPPH test also gave gallotannin the highest value but placed ellagitannin in second position followed by skin tannin, quebracho tannin and seed tannin. The results obtained with ABTS were quite similar to the DPPH test, the only difference being the position of quebracho tannin in last place just behind seed tannin. Finally, the ORAC test determined that the tannin with the highest antioxidant capacity was skin tannin followed by, in descending order, gallotannin, quebracho tannin, seed tannin and ellagitannin. Magalhães et al. (2014) obtained similar results using different antioxidant capacity tests on various oenological tannins, concluding that each antioxidant assay yields different information of a complementary nature. However, none of these assays provides useful information about the ability of each individual oenological tannin to protect wines against oxidation. In contrast, the method developed for OCR measurement determines the direct consumption of oxygen by the different oenological tannins in a model wine solution which provides a direct reference value for estimating the real capacity of the different oenological tannins to protect wines against oxidation. Moreover, the OCR obtained for the different oenological tannins can be compared with those of the most

frequently used antioxidants in wine, such as sulfur dioxide and ascorbic acid. However, to obtain a real comparison the OCR needs to refer to the usual dose of each of the antioxidants. In this regard we have considered 0.05 g/L for sulfur dioxide, 0.1 mg/L for ascorbic acid and the maximum authorized dose of 0.4 g/L for all oenological tannins. Using all these data, the percentage relative antioxidant capacity (RAC) of sulfur dioxide was calculated (Table 1). The RAC expresses the relative effectiveness as oxygen consumers of ascorbic acid and each of the oenological tannins compared to sulfur dioxide. The results indicate that ascorbic acid consumes oxygen 500 times faster than sulfur dioxide, confirming its reported high effectivity (Gibson, 2006). However, it must be taken into account that ascorbic acid generates hydrogen peroxide after consuming oxygen and its use in wine may therefore cause subsequent oxidations (Oliveira, Ferreira, De Freitas & Silva, 2011), which can affect the sensory quality of the wine. The RAC of different oenological tannins is also shown in Table 1. It can be seen that ellagitannins have a similar effectivity for oxygen consumption to sulfur dioxide, but that it is even higher (122%). This confirms that ellagitannins are very good antioxidants and suggests the possibility of their use as a complement (or even an alternative) for reducing (or even eliminating) the need for sulfur dioxide to protect wines against oxidation. Other tannins such as quebracho tannins and skin tannins have acceptable effectiveness (38% and 27% respectively). Finally, seed tannins (13%) and especially gallotannins (4%) showed lower values, indicating poor effectiveness in protecting wine against oxygen. It seems somewhat surprising to see the low RAC of gallotannins for oxygen, bearing in mind that their antioxidant capacity obtained via all the other procedures was the highest. Moreover, gallotannins are widely employed to prevent enzymatic browning in grape juice (Caillet, 2015; Crespy, 2002). A possible explanation for this could be that gallotannins can act as inhibitors of polyphenol oxidases (tyrosinase and laccase) (Nichols-Orians, 1991; Obradovic, 2006; Sugimoto et al., 2009), and therefore their protecting effect against oxidation would relate more to their polyphenol oxidase inhibitory effect than to their direct reaction with oxygen.

Figure 3 shows the influence of temperature on oxygen consumption by the different oenological tannins. As expected, the higher the temperature, the quicker the oxygen consumption. These data were used to determine the OCR for all the tannins at the different temperatures in accordance with what was described above. Considering the concentration of tannin used (0.4 g/L) and the OCR obtained, the kinetic constants (*K*) were calculated following the equation $OCR = K \bullet [Tannin]$. The Arrhenius plot (Ln *K* versus 1/Temperature) was then graphed using these data. Figure 3F shows the results obtained, which indicate that the OCRs of the different oenological tannins match reasonably well with the dependence of temperature described by Arrhenius law (Arrhenius, 1889).

Figure 4 shows the influence of oxygen concentration on oxygen consumption by the different oenological tannins. Tannin concentration was fixed at 0.4 g/L in all the cases. As expected, the higher the oxygen concentration, the faster the oxygen consumption. Since OCR is clearly influenced by tannin and oxygen concentration, it seems that oxygen consumption by all the oenological tannins is a second-order reaction from a kinetic point of view. In short, the oxygen consumption kinetics by oenological tannins can be described using the equation $OCR = K \bullet [Tannin] \bullet [O_2]$. Taking all the reported data into account it was possible to calculate the kinetic constant (*K*) for all the oenological tannins studied. This was done by

including all the calculated OCRs on a graph according to the product of the tannin and oxygen concentrations. The slope of the linear regression obtained should therefore be the kinetic constant (*K*). Table 2 shows these kinetic constants expressed as two different units (L.g of $tannin^{-1}$ day^{-1} and L.µg of $tannin^{-1}$ s^{-1}). These data clearly indicate that ellagitannins are the fastest oxygen consumers of the various oenological tannins, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins.

4. Conclusions

The measurement of the oxygen consumption rate (OCR) of different oenological tannins in a model wine solution using different concentrations of tannins and oxygen confirms that oxygen is consumed with a second-order kinetic in accordance with the equation ($OCR = K \bullet [Tannin] \bullet [O_2]$). These results also confirm that the OCR is influenced by temperature in line with Arrhenius law. Of the various oenological tannins, ellagitannins are the fastest oxygen consumers, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins. This methodology seems to be suitable for evaluating the oxygen consumption kinetics of the different oenological tannins and can therefore be proposed as an index to classify oenological tannins in terms of their effectiveness to consume oxygen.

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394

Table 1. Antioxidant capacity of oenological tannins assessed by different analytical methods compared with the oxygen consumption rate (OCR) in a model wine solution

Type of tannin	TPC (mg gallic acid/g)	DPPH (mmol Trolox/g)	ABTS (mmol Trolox/g)	ORAC (mmol Trolox/g)	OCR (mg O2/day.g)	RAC (%)
Sulfur dioxide	-	-	-	-	11.90 ± 0.63 E	100
Ascorbic acid	-	-	-	-	2,976 ± 625 F	50,000
Seed tannin	385 ± 17 B	4.91 ± 0.11 A	9.17 ± 0.48 A	2.26 ± 0.23 AB	0.19 ± 0.05 B	13
Skin tannin	484 ± 70 C	5.89 ± 0.44 B	9.41 ± 0.54 A	3.78 ± 0.26 C	0.40 ± 0.10 C	27
Quebracho tannin	434 ± 11 C	5.65 ± 0.48 B	8.92 ± 0.68 A	2.92 ± 0.74 B	0.57 ± 0.15 C	38
Gallotannin	780 ± 32 D	13.27 ± 0.73 D	16.00 ± 0.33 C	3.61 ± 0.25 C	0.06 ± 0.05 A	4.2
Ellagitannin	266 ± 7 A	7.10 ± 0.65 C	11.02 ± 0.49 B	1.86 ± 0.18 A	1.81 ± 0.15 D	122

All data is expressed as the arithmetic mean of three replicates with standard deviation. Different letters indicate statistically significant differences. TPC: total concentration of phenolic substances; DPPH: antiradical activity determined using 2,2-Diphenyl-1-picrylhydrazyl; ABTS: antiradical activity determined using 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); ORAC: oxygen radical absorbance capacity; OCR: Oxygen consumption rate; RAC: Relative Antioxidant Capacity referring to sulfur dioxide.

Table 2. Reaction rate constant ($OCR=K \bullet [Tannin] \bullet [O_2]$) for the different oenological tannins

Type of tannin	Reaction Rate Constant (K)		Significativity ($p < 0.05$)
	(L.g of tannin^{-1}.day^{-1})	(l.µg of tannin^{-1}.s^{-1})	
Seed Tannins	0.0234 ± 0.0021	6.46 ± 0.58	**B**
Skin Tannins	0.0397 ± 0.0039	10.95 ± 1.07	**C**
Quebracho Tannins	0.0572 ± 0.0047	15.78 ± 1.30	**D**
Gallotannins	0.0068 ± 0.0009	1.88 ± 0.25	**A**
Ellagitannins	0.2063 ± 0.0151	56.93 ± 4.18	**E**

All data is expressed as the arithmetic mean ± standard deviation. Different letters indicate statistically significant differences.

Figure(s)

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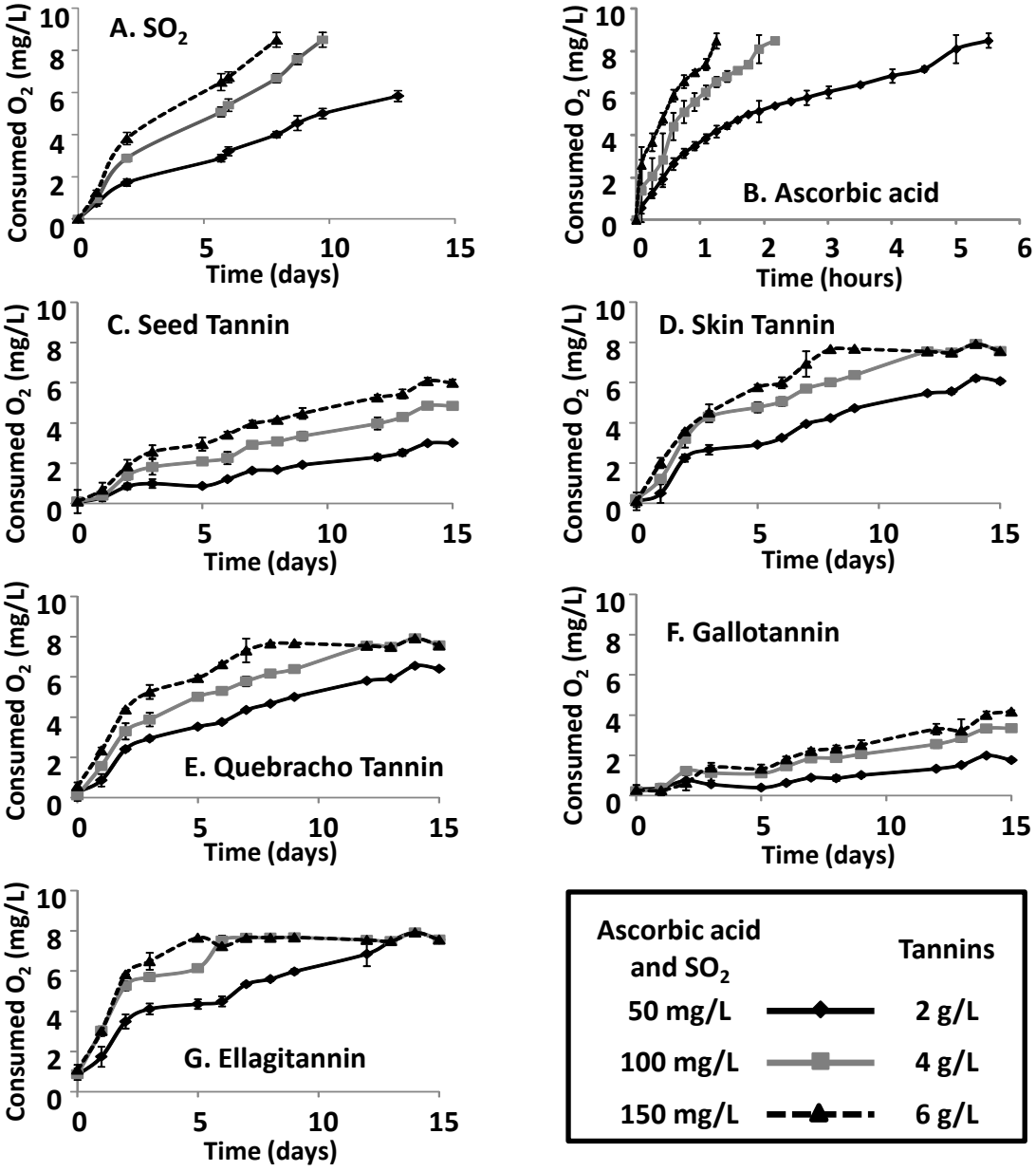
Figure 1. Influence of sulfur dioxide, ascorbic acid and different oenological tannin concentrations on oxygen consumption in an oxygen-saturated model wine solution at 20 ºC.



All data are expressed as the arithmetic mean of three replicates ± standard deviation

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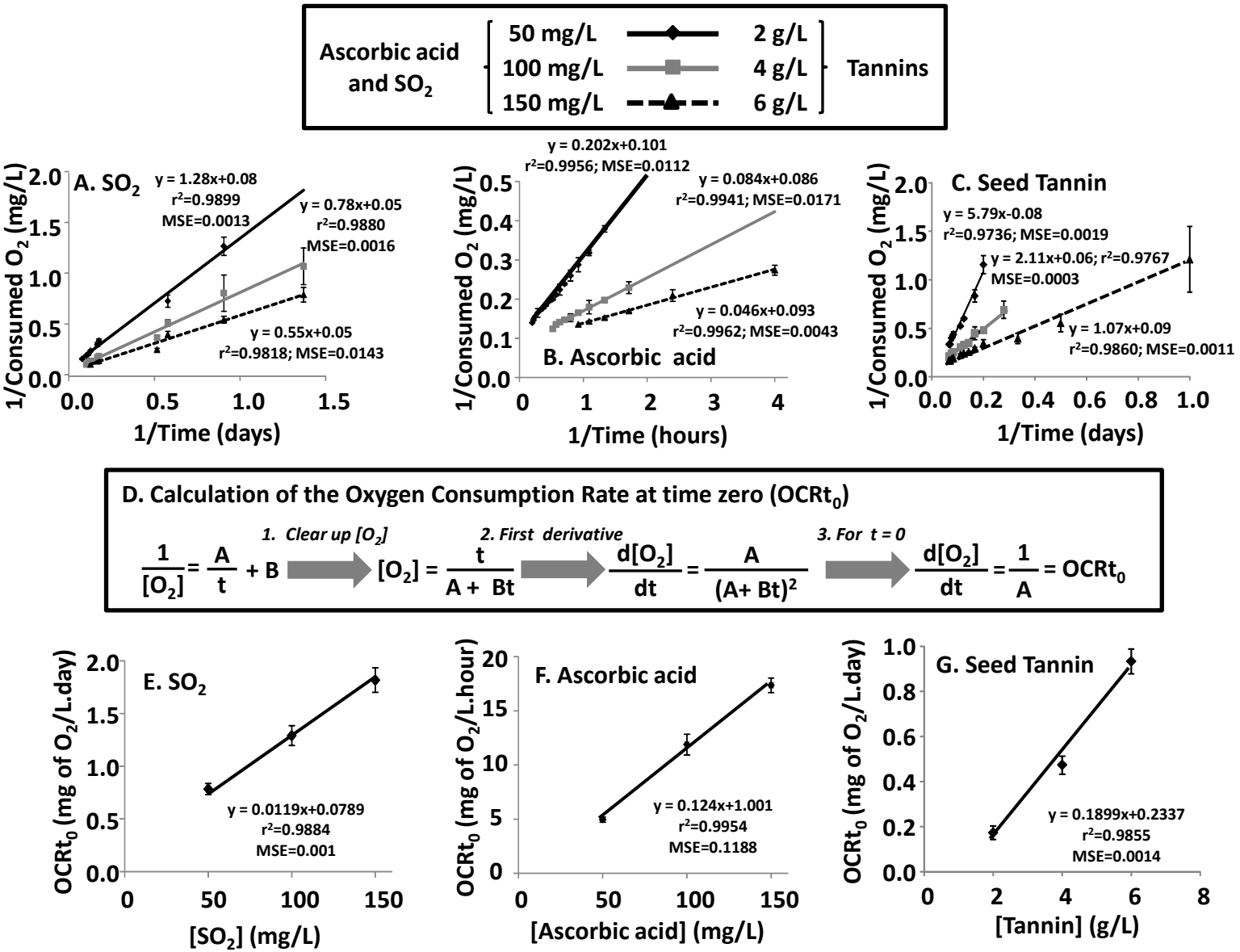
Figure 2. Influence of sulfur dioxide, ascorbic acid and seed tannin concentrations on the oxygen consumption rate (OCR) in an oxygen-saturated model wine solution at 20 ºC.



All data are expressed as the arithmetic mean of three replicates ± standard deviation

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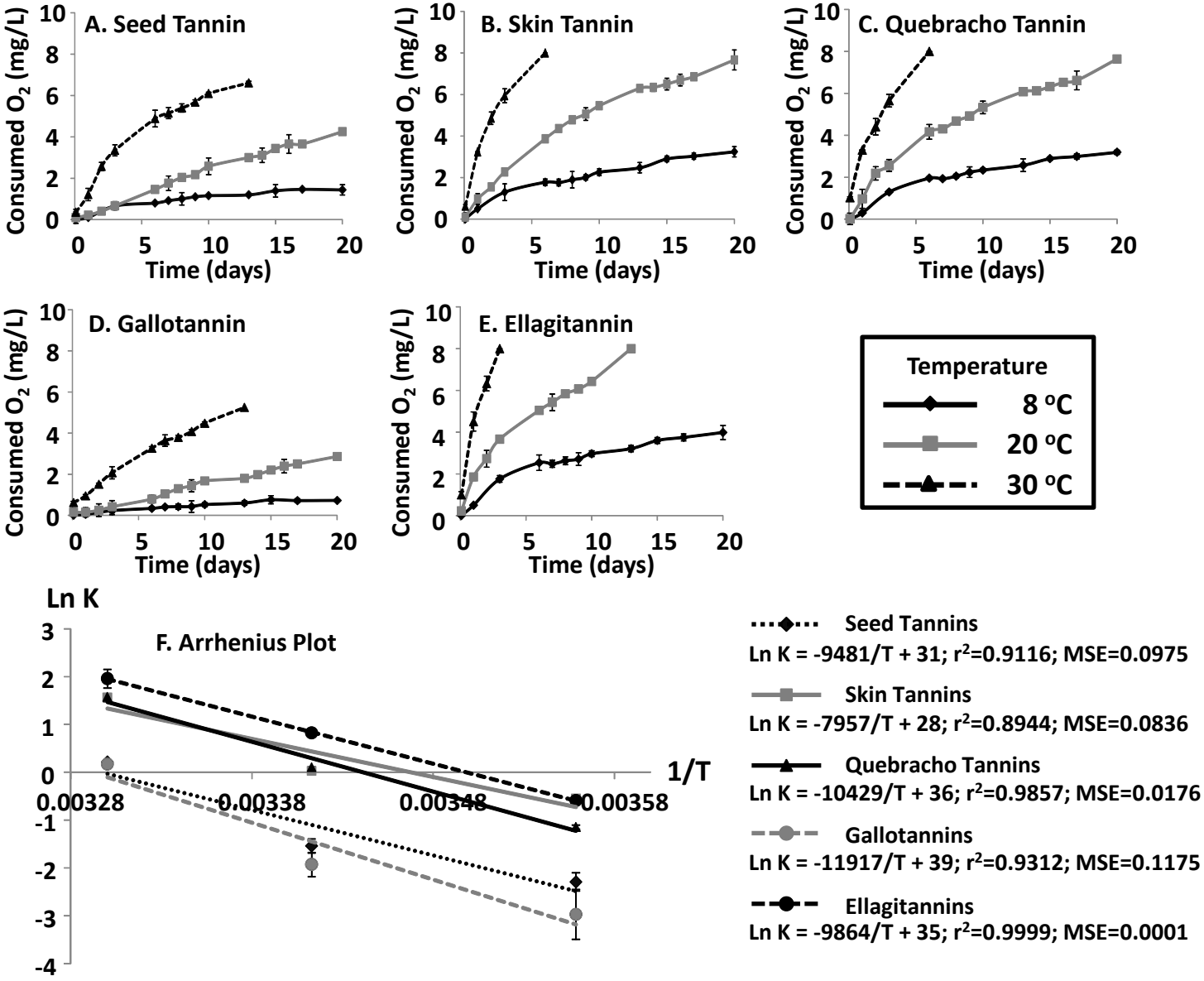
Figure 3. Influence of temperature on oxygen consumption by different oenological tannins in an oxygen-saturated model wine solution.



All data are expressed as the arithmetic mean of three replicates ± standard deviation

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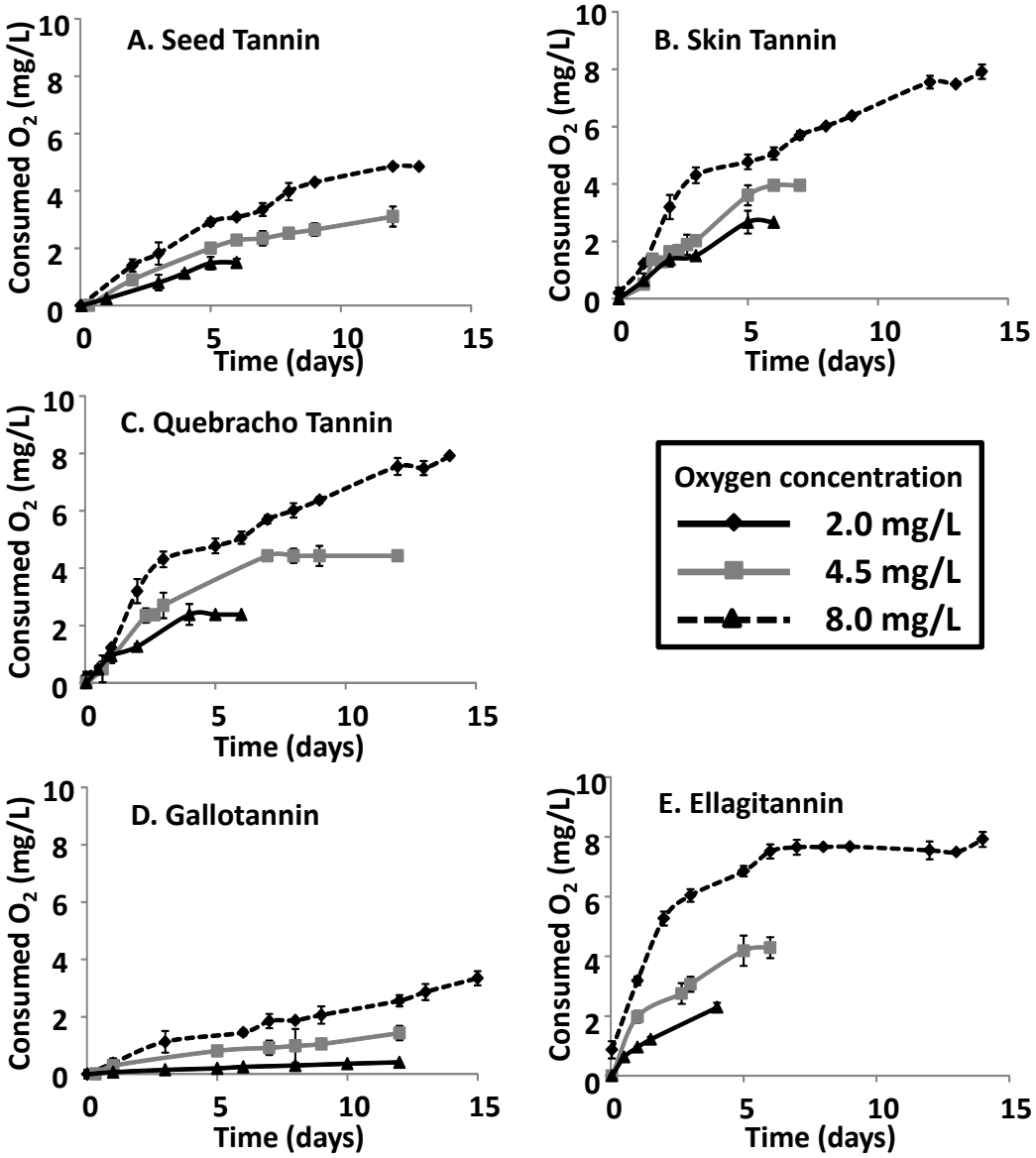
Figure 4. Influence of oxygen concentration on oxygen consumption by different oenological tannins in a model wine solution at 20 ºC.



All data are expressed as the arithmetic mean of three replicates ± standard deviation