The Impact of Different Tannin to Anthocyanin Ratios and of Oxygen on the Phenolic Polymerisation Over Time in a Wine-like Solution

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Colour and phenolic stability during ageing are influenced by the levels of distinct classes of phenolics in young red wines. The ratios between different classes of phenolic compounds also determine the colour and phenolic development of red wines. The present study evaluated the impact of forced oxidation on different anthocyanin/tannin (A/T) extracts and its consequent effect on the colour and phenolic evolution over time. The results showed that higher contents of seed tannins could enhance phenolic polymer formation, especially in the presence of oxygen. The addition of oxygen seemed to favour certain polymerisation reactions between tannins, leading to higher concentrations of monomeric anthocyanins in solution. A slower oxygen consumption was also observed as the phenolic composition of the wine-like extract evolved over time.

INTRODUCTION

Red wine quality can be directly influenced by phenolic compounds. Colour and phenolic stability of red wine are parameters that are related to consumer acceptance of the product. Grape phenolics are the main source of these secondary metabolites, which are progressively extracted during alcoholic fermentation as the ethanol concentration increases (Peyrot Des Gachons & Kennedy, 2003; González-Manzano *et al.*, 2004; Ribéreau-Gayon *et al.*, 2006). Grape anthocyanins and condensed tannins (proanthocyanidins) are the most important groups of phenolic compounds in red wines due to their involvement in determining the colour and sensory properties (Cheynier *et al.*, 2006; He *et al.*, 2012a, 2012b). Anthocyanins are normally exclusively extracted from the grape skins, whereas condensed tannins also originate from the grape skins, stems and seeds (Meyer & Hernandez, 1970; Pascual *et al.*, 2016). Differences in the chemical structure, polymer length and % galloylation of grape tannins can influence their extractability (González-Manzano *et al.*, 2006), reactivity and thereby their final impact on the sensory properties of wines (Peleg *et al.*, 1999). Grape tannins can be subdivided in two groups: procyanidins and prodelphinidins (Cheynier *et al.*, 2006; Kennedy *et al.*, 2006; Mattivi *et al.*, 2009). Prodelphinidins are only found in grape skins, whilst procyanidins are extracted from grape skins and seeds and consist of units of (+)-catechin and its epimer, (-)-epicatechin (Souquet *et al.*, 1996; Adams, 2006).

From crushing, anthocyanins and tannins are chemically modified and undergo continual evolution over time, leading to the formation of new and more stable compounds (Pérez-Magariño & González-SanJosé, 2004; Monagas *et al.*, 2006; He *et al.*, 2012b; Arapitsas *et al.*, 2014, 2016; Bimpilas *et al.*, 2015). Skin tannins, together with anthocyanins, follow a sigmoidal extraction, reaching a plateau in the early stages of the alcoholic fermentation (Mattivi *et al.*, 2009; Yacco *et al.*, 2016). However, longer maceration periods are required to reach better extraction of seed phenols due to a necessary hydration phase of the grape seeds (Casassa, 2017), leading to different levels of anthocyanins (A) and total tannins (T). The initial A/T ratio of wine must is thought to affect the subsequent polymerisation reactions in wine (Singleton & Trousdale, 1992; Sparrow *et al.*, 2015) and the interaction of phenolic compounds with other wine components (Bindon *et al.*, 2010; Springer *et al.*, 2016). Reactions such as polymerisation, condensation between tannins and anthocyanins, or the formation of complexes with proteins or polysaccharides depend on the different types of tannins and their concentrations in the wine.

Oxygen is involved in several of these reactions, such as phenolic polymerisation, which leads to the formation of more complex and stable phenolic compounds (Fulcrand *et al.*, 1996; Atanasova *et al.*, 2002). The oxidation of ethanol produces acetaldehyde, which enhances the formation of

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ethyl-bridged composite phenols (Timberlake & Bridle, 1977; Dallas *et al.*, 1996; Saucier *et al.*, 1997; Es-Safi *et al.*, 1999; Waterhouse & Laurie, 2006). At lower doses, the impact of oxygen can be beneficial, increasing colour stability and improving wine taste and structure (Waterhouse & Laurie, 2006; Gambuti *et al.*, 2013). However, the oxygen intake should be controlled to avoid excessive production of acetaldehyde and subsequent over-polymerisation and precipitation (Castellari, *et al.*, 2000; Du Toit, *et al.*, 2006a; Ribéreau-Gayon, *et al.*, 2006). The ageing potential of a wine seems to be influenced by the nature of the tannins and the relative A/T ratios (Singleton & Trousdale, 1992; Ribéreau-Gayon *et al.*, 2006; Pascual *et al.*, 2016; Picariello *et al.*, 2017), but this needs further clarification. Two recent publications have evaluated the oxygen consumption of several oenological tannins in a model wine solution, showing how the different nature of the tannins (model wine solutions rich in ellagitannins, gallotannins, skin tannins or seed tannins) influence the oxygen consumption rates (Pascual *et al.*, 2017; Vignault *et al.*, 2018). However, the impact of a continuous oxygen exposure and how the new phenolic polymers formed during ageing react towards it still remains unknown.

There are many unknowns in the continuous evolution of wine phenols during ageing. Current analytical methods can only provide limited information on the different phenolic polymer structures. The impact of oxygen on wine phenols in general has been examined extensively (Castellari *et al.*, 2000; Atanasova *et al.*, 2002; Wirth *et al.*, 2010; Arapitsas *et al.*, 2012; McRae *et al.*, 2015; Quaglieri *et al.*, 2017). However, only two recent publications have evaluated the effect of oxygen on wines with different A/T ratios (Picariello *et al.*, 2017; Carrascón *et al.*, 2018). In the study from Picariello *et al.* (2017), the commercial tannins used may contain additional non-tannin compounds, thereby not only altering tannin concentrations but also those of other wine components and complicating interpretation of their findings (Versari *et al.*, 2013). The present work aimed to investigate how oxygen affects phenolic polymerisation reactions at different A/T extracts in a wine-like (WL) system. To our knowledge this is the first study that focuses on proanthocyanidin polymerisation reactions as a function of the combination of these two variables (oxygen and A/T ratio). In addition, we also evaluate the different oxygen consumption patterns over time.

MATERIALS AND METHODS

Wine-like extracts

Shiraz grapes were harvested in 2015 from the Welgevallen experimental vineyard of the Department of Viticulture and Oenology at Stellenbosch University. The experimental design consisted of three different extracts, obtained by varying the amount of seeds used in the extraction and therefore the A/T ratio. The three extracts were obtained from 240 g grape skin without any grape seeds added (SK), in a normal seed (80 g) to skin ratio (SKSD) and in the presence of four times the normal seed (320 g) to skin ratio (SK4SD) found in the Shiraz grapes of the study. All extractions were carried out for nine days at 25ºC in 1 L hydroalcoholic solution (15% ethanol) at pH 3.4 and containing 6.0 g/L tartaric acid. To avoid the possibility of spontaneous fermentation during sample storage, 20 mg/L NaN_3 (Sigma-Aldrich, St. Louis, MO, USA) was added to the extracts. All extractions were manually shaken three times per day. A single extraction was performed per extract. After nine days, the skins and seeds from their corresponding extract were removed and softly pressed by hand in the presence of CO_2. The iron and copper concentrations were then adjusted to 5 mg/L and 0.3 mg/L, respectively, by adding the requisite amounts of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ (Sigma-Aldrich) according to Danilewicz (2007). The three final extracts (SK, SKSD, and SK4SD) were then centrifuged at 8000 rpm (5 min) to remove any residual grape skins.

The extracts were then divided into Control (C) and Oxygen treatments (Ox), transferred to vials (20 mL vials for C and 100 mL vials for Ox) and sealed hermetically, with the use of crimp caps. Ox samples were exposed to a forced oxidation before being transferred to the vials. The Ox samples were vigorously shaken by hand in a 500 mL volumetric flask for 2 minutes, allowing air to enter every 10 seconds to reach oxygen saturation. On the other hand, C treatments were directly transferred into the vials (previously filled with nitrogen), while blowing CO_2 into the vial. All vials were stored in the dark at 15ºC until the required analysis after 3 (3M), 6 (6M) and 9 months (9M) of storage. Once opened and analysed, C treatment vials were discarded, whereas in the case of Ox samples, 20 mL were drawn for the colour and phenolic analysis and the remainder of the extract was again saturated with oxygen before further storage. Glass beads were used to fill the headspace in the Ox vials at each of the sampling stages. In total, Ox samples were saturated with oxygen three times (at time 0 - 0 M, after 3 M and 6 M). The oxygen introduced ranged between 6.8-7.6 mg/L at time 0, 7.1-7.6 mg/L after 3 M and 8.5-8.9 mg/L after 6 M.

Oxygen measurement

Oxygen spots (Pst3, PreSens, Regensburg, Germany) were placed in several vials (control and oxygen vials) to avoid invasive measurements and used to monitor the oxygen uptake rate (Coetzee *et al.*, 2016). The oxygen consumption was monitored in C and Ox samples for the first 70-75 hours after oxygen addition. Vials were stored in the dark to avoid possible damage to the spots.

Colour and phenolic measurements

Spectrophotometric analysis

The colour and phenolic composition were analysed at time 0 M in each of the three extracts. In addition, these parameters were measured in C and Ox samples at 3 M, 6 M and 9 M of storage. At each time point, three vials of each treatment were opened and analysed. The colour density (CD), total red pigments (TRP) and total phenols (TP) of the samples were measured by spectrophotometric analysis (Somers & Evans, 1974; Boulton, 2001). TRP and TP were obtained from the absorbance units (AU) at 280 nm and 520 nm from wine-like samples diluted in 1M HCl. Tannin concentrations of the samples were then detemined by the methyl cellulose precipitation (MCP) method (Sarneckis *et al.*, 2006) and the results are expressed (in catechin equivalents) in mg/L.

Reversed phase high performance liquid chromatography (RP-HPLC)-DAD analyses

The analysis of individual and polymeric phenolic compounds were also performed at 0 M and for all treatments after 3 M, 6 M and 9 M of storage using RP-HPLC according to Garrido-Bañuelos *et al.* (2019). WL samples were centrifuged for 5 min at 8000 rpm and 20 µL of the supernatant was injected. Calibrations were done for the following phenolic standards with additional compounds quantified as equivalents indicated in brackets: gallic acid, (+)-catechin ((-)-epicatechin, B1, polymeric phenols), caffeic acid (GRP, caftaric acid), p-coumaric acid, quercetin-3-glucoside (quercetin-3-glucuronide, quercetin-3-galactoside), quercetin, myricetin, kaempferol from Sigma-Aldrich Chemie (Steinheim, Germany), and malvidin-3-glucoside (delphinidin-, cyanidin-, malvidin-, peonidin-, petunidin- 3-glucosides, -3-acetyl-glucosides, -3-p-coumaryl-glucosides, polymeric pigments) from Extrasynthese (Lyon, France). The identification of the compounds was done based on retention times of standards and the UV-Vis spectra (acquired by injection of standards or from the literature). To simplify the large set of data, certain individual compounds were grouped, namely the sum of total hydroxycinnamic acids, total flavonols, the total glucosylated-anthocyanins, total acetylated-anthocyanins and total coumaroylated-anthocyanins.

Statistical analysis

All analyses were carried out using Statistica 13.2 (TIBCO Statistica software, Palo Alto, CA, USA). Significant differences were judged on a 5% significance level ($p \leq 0.05$) with Fisher LSD Post Hoc tests. Principal Component Analysis (PCA) was performed with SIMCA 14.1 software (Sartorium Stedim Biotech - Malmö, Sweden).

RESULTS AND DISCUSSION

Colour and phenolic extraction in the different extracts

Based on previous trials, all extractions were performed for nine days in order to allow for a better extraction from the grape seeds. At 0 M (after nine days of extraction) the colour density (CD) and the total red pigments (TRP) were similar between the three extracts. As expected, higher TP and tannin concentration were found in the SKSD and especially SK4SD samples prepared with more seeds, compared to SK (prepared with only skin tannins). Considering the individual phenolic compounds, especially gallic acid and catechin concentrations were significantly different between the different extracts at 0 M (Table 1).

Influence of a different phenolic extract on oxygen consumption

The oxygen consumption (mg/L) was firstly monitored for the C and Ox samples at 0 M, and only for Ox samples in

TABLE 1
Colour and phenolic composition of the three extracts at 0 M.

	SK	SKSD	SK4SD
Spectrophotometric			
420 nm (AU)	3.04	3.45	3.30
520 nm (AU)	6.75	7.48	6.76
620 nm (AU)	1.18	1.39	1.34
CD (AU)	10.96	12.31	11.40
TRP (AU)	24.02	26.23	24.01
TP (AU)	32.37	39.86	48.99
Tannins (mg/L)	450.99	587.86	1092.77
HPLC (mg/L)			
Gallic acid	2.67	31.92	86.13
Catechin	8.60	59.76	154.14
B1	16.35	47.50	88.28
Polymeric phenols	245.66	403.40	392.75
Total hydroxycinnamic acids	4.82	7.07	7.78
Total flavonols	67.24	85.09	75.15
Total glucosylated anthocyanins	230.42	284.08	245.09
Total acetylated anthocyanins	116.79	144.60	121.15
Total coumaroylated anthocyanins	65.38	90.96	90.94
Polymeric pigments	14.24	25.50	32.02
Total anthocyanins	426.83	545.14	489.20

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the following oxygenations after 3 M and 6 M of storage. The oxygen consumption of the following oxidations was only monitored during the first three days (70-75 hours). As illustrated in Figure 1, the different extracts, which probably differ in concentration and chemical nature, clearly played a role as the oxygen consumption rates differed between the storage times and treatments. These differences in the oxygen depletion rates could possibly be explained by changes occurring in the phenolic profile and concentration of the extracts over time. Firstly, as shown in Figure 1 A1, there was a quick depletion of the low amount of oxygen present in C samples (probably due to a minimal oxygen intake during sample preparation). From Figure 1 A2, which illustrates the oxygen consumption in Ox vials at 0 M, minimal differences were found between the extracts. At 0 M, the higher phenolic levels, together with possible differences in the nature of the phenols, did not seem to influence oxygen consumption, differing from the findings of Pascual *et al.*, (2017). In both cases, the dissolved oxygen in the different extracts was depleted after a few hours. On the other hand, the oxygen consumption rate varied over time, as the phenolic profiles of the extracts evolved. Oxygen consumption measured after 3 M was generally slower compared to 0 M (Figure 1 A3). Interestingly, after 3 M the oxygen consumption was slower in the SK4SD samples. We consider that the initial excess of seed phenols may have had an influence on the formation rate of new polymeric forms involving oxidative reactions during the early stages of storage. This may have led to an extract composition with lower levels of compounds susceptible to oxidation after 3 M. Furthermore, oxygen consumption observed in Figure 1 A4 was even slower for all three extracts after 6 M, probably also as a consequence of a lower substrate availability to react with oxygen in the WL media.

Colour and phenolic evolution of the final extracts

Differences were observed in terms of the colour parameter and phenolic levels as determined spectrophotometrically at 0 M, as well as a function of storage time. Storage time played a particularly important role, as significant differences were also found in colour and the phenolic concentration between the different extracts over time. Whilst the extract was the most important factor in determining TP levels and, obviously, the tannin concentration, oxygen had a large influence on the colour parameter such as the TRP and CD, especially the absorbance at 420 nm for the latter. Furthermore, time was also a determining factor, especially for the TRP.

The evolution of the phenolic parameters determined spectrophotometrically as a function of time (Table 1 supplementary) for the three extracts and C and Ox samples are shown in Figure 2. The cumulative effect of all the studied parameters drives a clear separation between the samples. In Figure 2 A1, it can be seen that the different extracts were separated along the PC1 axis (54.4%). As previously mentioned, the TP and tannin concentration were mainly influenced by the respective extract composition. The scores plot and the corresponding loadings plot

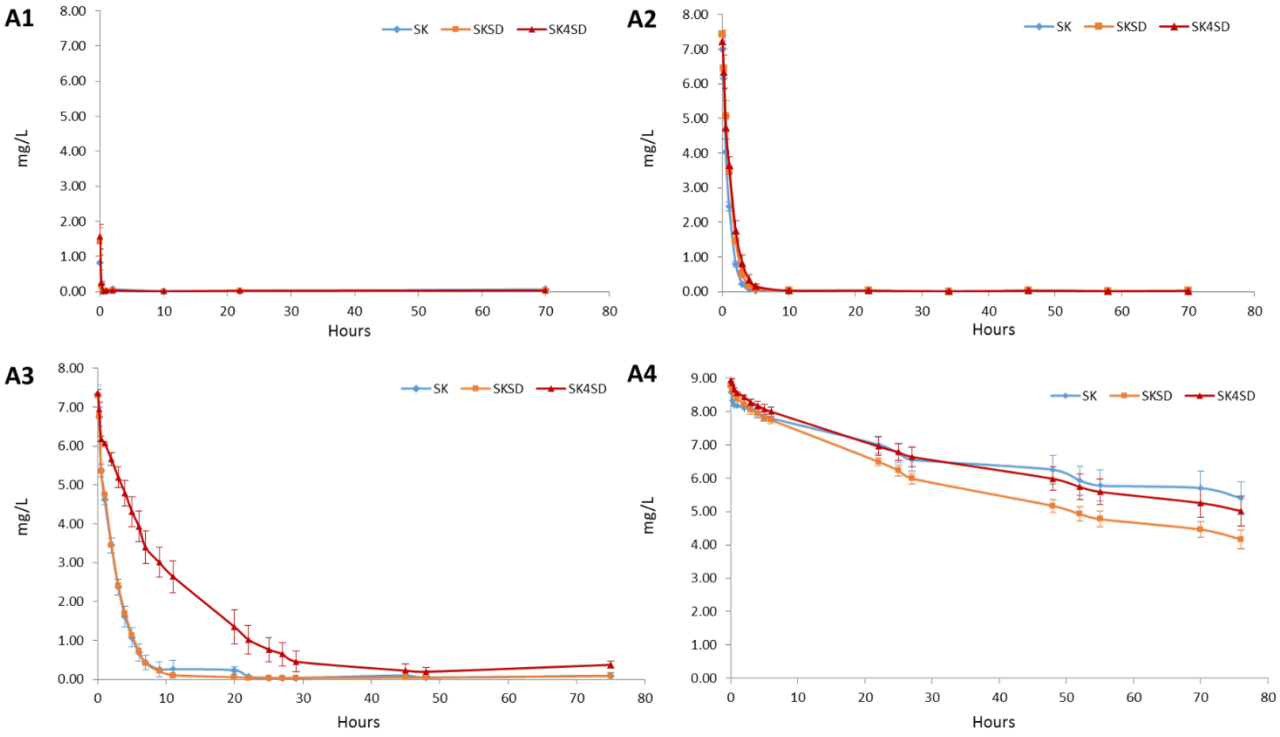

FIGURE 1

Oxygen consumption for the various treatments (SK, SKSD, and SK4SD). A1) oxygen consumption in C samples at 0 M. A2) oxygen consumption of Ox samples at 0 M. A3) oxygen consumption of Ox samples at 3 M. A4) oxygen consumption of Ox samples at 6 M.

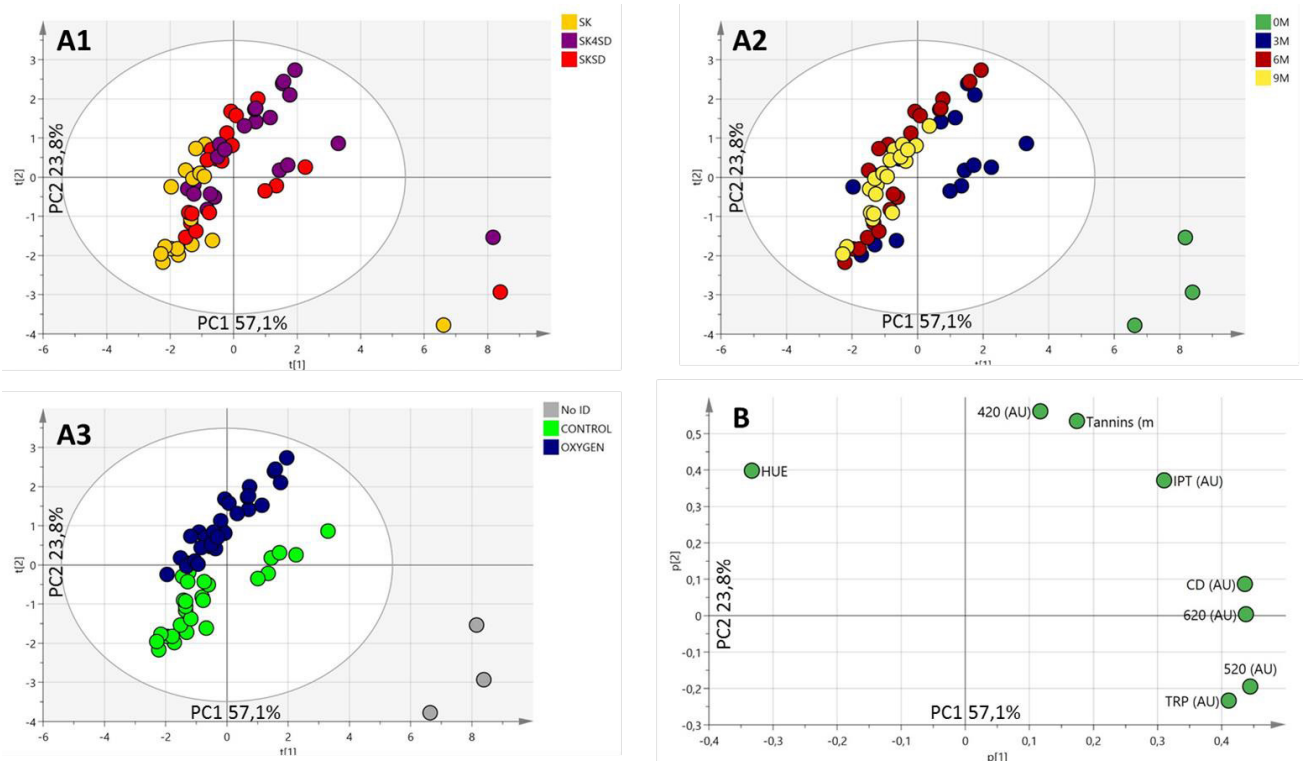

FIGURE 2

PCA score plots of the sample distribution based on the spectrophotometric data. A1) PCA score plot coloured according to the three different extracts. A2) PCA score plot coloured according to the sampling stage. A3) PCA score plot coloured according to the C/Ox treatments. B. Loadings plot of the samples distribution based on the spectrophotometric data.

(Figure 2- A1 and B) showed a general higher phenolic content in SK4SD treatments, especially compared to SK treatments. Over the course of time, these differences between the extracts became smaller, especially after 9 M, probably as a consequence of phenolic degradation, but also as a result of over-polymerisation reactions and subsequent precipitation of insoluble phenolic compounds. In Figure 2 A2, the samples are coloured according to the sampling stages (0 M, 3 M, 6 M and 9 M). After 9 M, the extracts were more closely distributed along the PC1 axis (54.4%). When the samples were coloured according to the C/Ox treatment, the samples were distributed along the PC2 axis (21.3%), with Ox samples being characterised by a generally higher phenolic and especially tannin concentrations (Figures 2 and 3). Contrary to the findings of Geldenhuys *et al.* (2012), oxygen was also found to play an important role in tannin concentrations (Figure 2 A3). However, Geldenhuys *et al.* (2012) applied progressive micro-oxygenation, whereas in this study a large amount of oxygen was added at a time.

A general loss of colour and reduction in phenolic levels was found over time, especially pronounced from 0 M (Table 1) to 3 M, except for the total tannin concentration as determined by the MCP method (Figure 3). As an example, TRP levels decreased in all the samples during the first 3M, especially in most of the C treatments (Table 1 supplementary). Oxygen seemed to have enhanced the polymerisation between certain compounds, thereby possibly limiting the degradation of certain red pigments. The TRP content was significantly higher in the SKSD and SK4SD Ox treatments at 3 M (Table 1 supplementary). From then onwards, the differences between C and Ox treatments and between the extracts became less over time.

Conversely, the tannin content showed different patterns from 0 to 3 M within the different treatments. As illustrated in Figure 3 (values at 0 M are specified on the Y axis), clear differences were found between C and Ox samples. While the MCP tannin levels were relatively constant from 0 M to 3 M in C samples (except for a slight increase in SKSD), an increase in the tannin concentration was observed in Ox (SKSD and SK4SD) samples during the same period. However, after 3 M, the tannin levels were only significantly higher in SK4SD-Ox samples compared to the corresponding C samples (Figure 3). During the following three months, the C treatments showed a progressive decrease in tannin concentration, except for the SK treatment (constant from 3M to 9M), while not changing significantly up to 9M. (Figure 3). On the other hand, the Ox treatments' tannin levels increased (SK and SKSD) or remained stable (SK4SD) up to 6M of storage, which might also explain the different oxygen consumption rates observed for the second oxidation step after 3M of storage. From then, all the Ox extracts experienced a general decrease in tannin concentration towards the last sampling stage (9M). This decrease can possibly be explained by the formation of larger and/or more unstable polymers which are no longer soluble in the hydroalcoholic solution. Thus, the oxygen had an impact on the tannin polymerisation reactions, and likely the reactivity of the polymerisation reaction products towards

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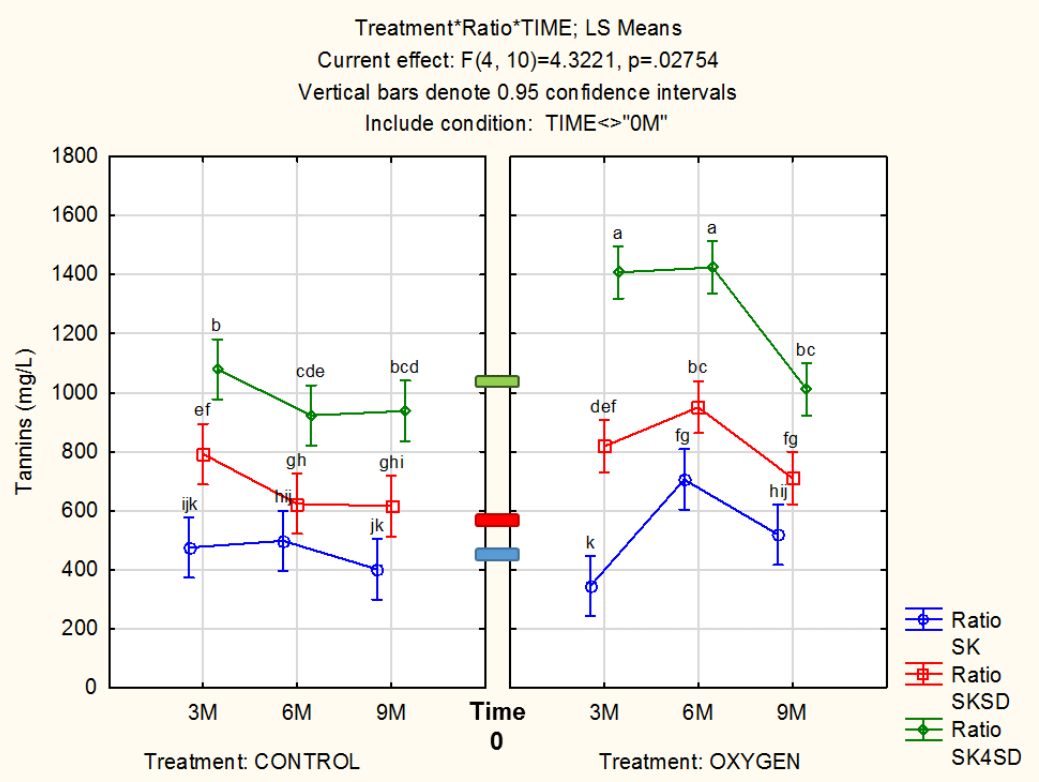

FIGURE 3

Evolution of the MCP tannin levels (mg/L) of all extracts (SK, SKSD and SK4SD) over time. Values at time 0 (0 M) are marked on the Y axis. The different letters indicate significant differences (ANOVA, $p < 0.05$) between the A/T ratios, oxygen treatments and storage times.

methylcellulose (Figure 3). The significant role of oxygen in tannin polymerisation has been widely documented in literature (Singleton, 1987; Castellari *et al.*, 2000; Atanasova *et al.*, 2002; Waterhouse & Laurie, 2006; Gambuti *et al.*, 2013; Quaglieri *et al.*, 2017).

Oxygen also influenced the evolution of the amount of TRP in the extracts. In the presence of oxygen, higher phenolic levels might compete for reaction with oxygen, favouring specific polymerisation reactions. Thus, the higher pigment content can be explained by the depletion of oxygen as a consequence of the reaction of other phenolic compounds with oxygen, instead of the anthocyanins/pigments.

HPLC data for individual phenolics

Results obtained for the RP-LC analysis of selected individual phenolic concentrations are summarised in Tables 1 and 2. The different extracts, the presence/absence of oxygen and storage time played a role in affecting the phenolic composition of the treatments. Large differences in gallic acid concentrations were found between the three extracts. Higher amounts of seeds led to an obvious increase in gallic acid content (Table 1) at 0 M. From time 0 M to 3 M, a consistent decrease in the gallic acid concentration was observed for all samples, possibly linked to the formation of new polymeric forms (especially in SK4SD), precipitation or degradation reactions. The hypothetical interaction between gallic acid quinones and flavonol units has recently been reported (Mouls & Fulcrand, 2015). The concentration of polymeric phenols was also significantly higher in SKSD and SK4SD compared to SK samples (Table 2). These differences between the extracts remained over time. Over the storage time investigated (especially from 6 M), the polymeric phenol content was generally higher in Ox treatments. Therefore, the presence and reactivity of seed derived compounds and oxygen may influence polymerisation reactions.

Higher total flavonol contents were found in the Ox samples; however, the total hydroxycinnamic acid concentrations were higher in the C samples. Unexpectedly, the total hydroxycinnamic acid content seemed to slightly increase over time (Table 2), although in some cases not significantly. Literature reports a general decrease of hydroxycinnamic acid concentrations during storage (García-Falcón *et al.*, 2007). However, an increase of certain hydroxycinnamic acids has also been observed (García-Falcón *et al.*, 2007; Arapitsas *et al.*, 2014), possibly as a result of copigment degradation expected to occur over time (Bimpilas *et al.*, 2016).

Likewise, a large decrease was observed in the anthocyanin concentrations of all treatments from 0 M (Table 1) to 3 M (Table 2). The larger decrease in anthocyanin levels observed in the C treatments was not associated with the formation of higher polymeric pigments (Table 2). Nevertheless, the HPLC results confirmed the idea of certain oxidative reactions between phenols being favoured in the presence of oxygen. The oxidation of ethanol and tartaric acid could possibly have led to the formation of ethyl bridged structures between tannins moieties, thereby leading to lower reactivity of free anthocyanins. This may

TABLE 2

Levels of the individual and classes of phenolic compounds (mg/L) for all treatments over time. Different letters indicate significant differences (ANOVA, $p < 0.05$) between the A/T ratios, oxygen treatments and storage times.

		CONTROL						OXYGEN					
		SK		SKSD		SK4SD		SK		SKSD		SK4SD	
Gallic acid	**3M**	1.14 ± 0.04	j	8.73 ± 0.02	gh	28.78 ± 0.11	c	1.28 ± 0.01	ij	9.04 ± 0.09	fg	30.24 ± 0.13	b
	6M	1.51 ± 0.04	ij	8.55 ± 0.15	h	27.69 ± 0.54	d	1.17 ± 0.01	ij	9.21 ± 0.25	f	30.40 ± 0.04	b
	9M	1.53 ± 0.05	i	8.78 ± 0.03	gh	28.57 ± 0.32	c	1.27 ± 0.01	ij	9.58 ± 0.22	e	30.78 ± ± 0.41	a
Catechin	**3M**	8.28 ± 3.03	de	8.57 ± 1.30	de	20.47 ± 1.07	b	6.73 ± 0.19	efg	15.38 ± 3.43	c	35.70 ± 2.80	a
	6M	5.88 ± 2.41	efg	6.60 ± 1.61	efg	12.49 ± 0.50	cd	3.94 ± 0.85	fg	3.12 ± 0.18	g	13.37 ± 6.03	c
	9M	20.73 ± 3.01	b	6.91 ± 0.35	efg	8.93 ± 1.68	de	6.46 ± 1.50	efg	11.78 ± 1.66	cd	8.95 ± 2.52	de
B1	**3M**	14.02 ± 4.15	ij	19.97 ± 1.32	gh	66.58 ± 0.48	a	11.55 ± 2.00	jk	33.07 ± 4.73	e	69.49 ± 4.90	a
	6M	7.38 ± 0.63	k	22.68 ± 0.88	fg	44.62 ± 2.28	d	11.30 ± 1.99	jk	25.91 ± 0.39	f	46.59 ± 4.65	d
	9M	8.61 ± 0.33	k	25.88 ± 4.25	f	52.26 ± 3.70	c	16.38 ± 2.26	hi	35.17 ± 1.89	e	61.40 ± 2.14	b
Polymeric phenols	**3M**	513.75 ± 1.55	k	707.86 ± 5.50	ef	1002.65 ± 20.15	a	525.20 ± 0.54	k	731.42 ± 20.87	de	1010.29 ± 22.25	a
	6M	442.05 ± 32.68	l	586.18 ± 48.70	i	689.75 ± 7.32	fg	529.33 ± 23.93	jk	656.95 ± 27.41	gh	832.36 ± 31.70	c
	9M	490.99 ± 16.63	k	645.19 ± 3.77	h	758.85 ± 28.89	d	568.59 ± 6.63	ij	712.80 ± 15.34	ef	903.30 ± 16.05	b
∑ Hydroxycinnamic acids	**3M**	3.59 ± 0.04	hij	4.43 ± 0.06	de	3.92 ± 0.10	gh	2.97 ± 0.09	k	3.26 ± 0.42	ijk	3.61 ± 0.46	hi
	6M	4.06 ± 0.12	efg	4.52 ± 0.07	cd	4.86 ± 0.15	bc	3.19 ± 0.09	jk	3.36 ± 0.18	ij	3.58 ± 0.16	hi
	9M	4.37 ± 0.09	def	5.10 ± 0.10	b	5.54 ± 0.09	a	3.51 ± 0.14	hij	4.01 ± 0.41	fg	4.24 ± 0.07	defg
∑ Flavonols	**3M**	16.37 ± 0.61	d	18.12 ± 0.36	c	16.44 ± 0.48	d	20.15 ± 0.62	b	22.79 ± 0.76	a	20.78 ± 0.48	b
	6M	12.10 ± 0.65	fg	13.01 ± 1.01	ef	10.23 ± 0.56	hi	15.88 ± 0.44	d	16.08 ± 0.73	d	13.30 ± 0.64	ef
	9M	11.06 ± 0.52	gh	11.62 ± 0.20	g	9.58 ± 0.35	i	13.93 ± 0.77	e	14.07 ± 1.24	e	11.75 ± 0.60	g
∑ Glucosylated anthocyanins	**3M**	3.98 ± 0.37	f	4.46 ± 0.09	e	4.88 ± 0.12	d	7.53 ± 0.12	c	12.39 ± 0.42	b	18.81 ± 0.39	a
	6M	1.25 ± 0.19	ij	1.17 ± 0.04	ijk	1.07 ± 0.06	ijk	1.94 ± 0.20	h	2.46 ± 0.20	g	2.66 ± 0.10	g
	9M	0.87 ± 0.03	k	0.92 ± 0.02	jk	0.87 ± 0.02	k	1.05 ± 0.02	ijk	1.35 ± 0.15	i	1.33 ± 0.07	i
∑ Acetylated anthocyanins	**3M**	2.72 ± 0.88	ef	2.21 ± 0.11	efg	4.18 ± 1.53	d	6.24 ± 2.55	c	11.84 ± 0.54	b	16.88 ± 0.49	a
	6M	1.74 ± 0.06	fg	1.61 ± 0.06	fg	1.44 ± 0.03	fg	2.55 ± 0.13	efg	3.20 ± 0.27	de	3.39 ± 0.15	de
	9M	1.41 ± 0.11	fg	1.33 ± 0.02	g	1.28 ± 0.07	g	1.59 ± 0.04	fg	1.88 ± 0.19	fg	1.70 ± 0.10	fg

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TABLE 2 (CONTINUED)

		CONTROL						OXYGEN					
		SK		SKSD		SK4SD		SK		SKSD		SK4SD	
Σ Coumaroylated anthocyanins	**3M**	2.92 ± 0.41	d	2.95 ± 0.10	d	3.04 ± 0.08	d	4.95 ± 0.44	c	7.58 ± 0.45	b	9.46 ± 0.50	a
	6M	1.44 ± 0.07	efg	1.31 ± 0.06	fg	1.18 ± 0.03	g	1.74 ± 0.14	e	1.79 ± 0.03	e	1.63 ± 0.04	ef
	9M	1.24 ± 0.07	g	1.19 ± 0.06	g	1.14 ± 0.02	g	1.27 ± 0.06	fg	1.44 ± 0.08	efg	1.29 ± 0.05	fg
Polymeric pigments	**3M**	14.01± 0.35	ijk	16.75 ± 0.18	def	19.78 ± 0.86	b	14.83 ± 0.30	ghij	18.70 ± 0.34	bc	21.66 ± 0.41	a
	6M	12.57 ± 1.20	l	14.52 ± 1.51	hijk	13.50 ± 0.80	kl	14.56 ± 0.94	hijk	15.15 ± 1.01	ghi	15.51 ± 0.73	fgh
	9M	13.73 ± 0.51	jkl	16.08 ± 0.18	efgj	14.81 ± 0.49	ghij	15.66 ± 0.13	efgh	16.76 ± 0.52	de	17.62 ± 0.28	cd
Total anthocyanins	**3M**	23.63 ± 0.66	e	26.37 ± 0.19	d	31.88 ± 1.01	c	33.55 ± 1.30	c	50.50 ± 0.82	b	66.81 ± 0.82	a
	6M	17.01 ± 0.67	j	18.62 ± 0.88	ij	17.19 ± 0.52	j	20.79 ± 0.73	gh	22.59 ± 0.29	efg	23.19 ± 0.35	ef
	9M	17.25 ± 0.29	j	19.52 ± 0.13	hl	18.10 ± 0.32	ij	19.57 ± 0.04	hl	21.43 ± 0.10	fgh	21.94 ± 0.05	efg

explain the higher concentration of monomeric anthocyanins found after 3 M, in the treatments where higher levels of seeds were present and oxygen added. Supporting this, after 3 M of storage, SK and SKSD samples showed a greater decrease in glucosylated, acetylated and coumaroylated anthocyanins in the absence of oxygen. On the other hand, SK4SD samples initially had higher concentration of polymeric pigments, thereby influencing the polymerisation reactions. These differences between the extracts in the concentrations of polymeric pigments, for both C and Ox samples, were also found at 3 M, but disappeared after 6 M of storage. In the interpretation of these results, we cannot discard the possibility that certain polymeric pigments are not detected by the RP-HPLC method. Nevertheless, after 6 M, all treatments experienced anthocyanin degradation and differences between treatments became smaller. The decrease in anthocyanins showed different rates among the different extracts. This delay may be linked to the excess of seed phenolics, with a higher reactivity in the presence of oxygen, and the exposure to several severe oxidations. These repeated oxidations could lead to over polymerisation, forming phenolic derived compounds not stable in solution, therefore precipitating. This anthocyanin degradation over time has been widely reported in red wines, and is at least partly a consequence of the formation of pigmented polymers (Somers, 1971; Somers & Evans, 1979; Pérez-Magariño & González-SanJosé, 2004; Arapitsas *et al.*, 2014; Quaglieri *et al.*, 2017). Also, the loss of anthocyanin derived forms over time was previously reported to be lower in oxygenated wines (Atanasova *et al.*, 2002).

CONCLUSIONS

To date, a number of studies have focused on the impact of seed addition or removal on the colour, phenolic profile and sensory properties of wines (Meyer & Hernandez, 1970; Canals *et al.*, 2008; Lee *et al.*, 2008; Guaita *et al.*, 2017), but there is a lack of information on the evolution of these wine parameters with age, as well as on the role of oxygen in this process. The main goal of this study was to assess the impact of oxygen addition on the phenolic composition of WL extracts containing three different defined A/T ratios. The extract composition seemed to play a greater role than the oxygen in phenolic evolution. Our results highlight the importance of the initial A/T ratio and of the nature of these respective compound classes on the polymerisation reactions occurring during initial stages of ageing. The higher the concentration of phenols in the solution, the greater the number of molecules susceptible to polymerise, and therefore the greater the competition between these substrates. In this context, seed derived phenols showed a high reactivity to form larger polymeric structures, both in the absence or presence of oxygen. Nevertheless, as a consequence of the oxidative process, excessive seed content may enhance the polymerisation reactions between proanthocyanidins, and thereby favour remaining of free monomeric anthocyanins in solution. The increase in polymeric phenols (Table 2), together with the higher levels of TRP (Table 1 supplementary data) and of monomeric anthocyanins (total glucosylated, acetylated and coumaroylated forms) in the SK4SD-Ox samples after 3M of storage (Table 2), support this idea.

Further research needs to investigate not only the impact of different phenolic ratios on the phenolic stability, but also the polymerisation reactions in the presence of different grape polysaccharides and protein proportions.

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