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Effect of Wine pH and Bottle Closure on Tannins

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ABSTRACT: The impact of wine pH and closure type on color, tannin concentration, and composition was investigated. A single vintage of Cabernet Sauvignon wine was divided into three batches, the pH was adjusted to 3.2, 3.5 or 3.8, and the wines were bottled under screw caps with either SaranTin (ST) or Saranex (Sx) liners. After 24 months, the tannin concentration, tannin percent yield (relating to the proportion of acid-labile interflavan bonds), and the mean degree of polymerization (mDp) had decreased significantly, all of which can contribute to the softening of wine astringency with aging. The higher pH wines contained less percent (−)-epicatechin 3-*O*-gallate subunits, whereas the Sx pH 3.2 wines were significantly lower in percent yield and mDp than the other wines. Overall, the tannin structure and wine color of the lower pH wines (pH 3.2) bottled under Sx screw caps changed more rapidly with aging than those of the higher pH wines (pH 3.8) bottled under ST screw caps.

KEYWORDS: *screw-cap liners, tannin composition, wine aging, wine pH, wine tannin*

INTRODUCTION

As red wines age they change in physicochemical properties, particularly those associated with color and astringency. Anecdotal evidence suggests that red wines often decrease in astringency with aging, and this has been corroborated by red wine aging trials[1] and trials involving sensory analysis of vertical series.[2] Hypotheses for the change in red wine astringency with wine aging have been based on analysis of vertical series or on model wine studies and have been ascribed variously to the reduction in tannin concentration due to precipitation,[3] the reduction in tannin size through depolymerization,[2,4] and a reduction in protein binding of aged wine tannins due to a change in tannin structure over time.[1,5] Research into the effect of aging on tannins has been limited to analysis of total phenolic concentrations[1] and analyses of small polyphenol monomers or oligomers.[6,7] To our knowledge, there have been no trials specifically monitoring the actual changes in tannin concentration *and* composition in red wines over time, and therefore the overall effects of aging on wine tannins remain unknown. The color of young red wines is generally a deep purple associated with high concentrations of monomeric anthocyanins, particularly malvidin-3-glucoside,[8] as well as some derived anthocyanins.[9] Red wine aging trials have indicated that with aging, wine color changes to a red-orange due to the decrease in anthocyanin concentrations and the increase in pigmented polymer concentrations from condensation reactions between anthocyanins and proanthocyanidins[10,11] and, to some extent, the formation of pyranoanthocyanins.[8,12−14] The impact of oxygen and pH on anthocyanin concentrations has been explored previously,[1,6,15] although not in the context of the impacts of pigmented polymer formation on tannin composition.

The rates of changes in wine physicochemical properties are dependent upon many factors including the level of oxygen exposure and wine pH.[6] Oxygen can oxidize ethanol to acetaldehyde, which reacts readily with flavan-3-ols to increase polymerization[6] and alter the structures of the polymers relative to direct condensation reactions.[16] Studies on wine microoxygenation (MOX) have indicated that the rate of decline in anthocyanin concentration is more rapid when the wine is exposed to more oxygen during winemaking.[13,15] MOX has also been shown to stabilize wine color by promoting the formation of pyranoanthocyanins[17] and pigmented polymers.[18] Oxygen ingress via closures, sometimes referred to as "nano-oxygenation", can affect wine mouthfeel, with greater rates of oxygen ingress reducing astringency intensity after 42 months of aging.[1] Screw caps currently dominate the Australian wine market and can provide different rates of oxygen ingress. Saran Tin (ST) closures are highly impervious to oxygen (up to 0.00043 mg/L/day), whereas the multiple layers of the Saranex (Sx) closures allow slight oxygen ingress (up to 0.0273 mg/L/day).[19,20] Variations in the level of oxygen entering the wine through the closure have been shown to influence the flavor and aroma of red wines and, in some cases, low levels of oxygen, preserving positive fruity aromas[21] and enhancing color stability in rosé wines,[22] although insufficient oxygen ingress can potentially promote reductive aromas.[20] The impact of different screw-cap closures on wine color as well as tannin concentrations and compositions in red wine with aging has not been investigated.

The more acidic media of lower pH wines increase the reaction kinetics for many of these reactions, and therefore the concentrations of anthocyanins and small polyphenols decrease more rapidly than in wines of higher pH.[6,23] More acidic wines have also been shown to exacerbate the impact of oxygen exposure on astringency,[1] further suggesting some more rapid reactions in wines with a lower pH. The impact of pH on wine tannin structure has not been explored with wine aging. This project investigated the impacts of wine aging, wine pH, and different screw-cap closures on wine color, tannin concentration, and composition in a single-vintage Cabernet Sauvignon over 24 months of bottle aging.

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MATERIALS AND METHODS

Chemicals. All solvents used were of high-performance liquid chromatography (HPLC) grade, all chemicals were of analytical reagent grade, and water was obtained from a Milli-Q purification system. Acetic acid (100%), acetonitrile, ethanol, formic acid (98−100%), HCl (32%), and H_2SO_4 (95−98%) were all purchased from Merck Australia (Kilsyth, VIC, Australia). Acetaldehyde, ammonium sulfate, ascorbic acid, lithium chloride (LiCl), methyl cellulose polymer, *N*,*N*-dimethylformamide (DMF), potassium metabisulfite, phloroglucinol, sodium acetate, sodium chloride (NaCl), sodium hydroxide (NaOH), and tartaric acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Wines. Cabernet Sauvignon (Clare Valley, South Australia) wines were prepared according to standard winemaking practices for both primary and secondary fermentations, with a final alcohol concentration of 14% v/v ethanol. After cold stabilization, the wine was divided into three batches (26 L each), and the pH was adjusted to either 3.2, 3.5, or 3.8. H_2SO_4 was used to reduce the pH to 3.2 (18 M, 36.7 mL, giving a final concentration of 0.025 M H_2SO_4 in wine) and to pH 3.5 (1.8 M, 9.75 mL). NaOH was used to give pH 3.8 (1.25 M, 241.4 mL, giving a final concentration of 0.012 M NaOH in wine). The differences in the final concentrations of the acid and base additions were <10-fold and therefore considered insufficient to significantly alter the final ionic strength of the wines.[24] Potassium metabisulfite solution (5%, 13.7 mL) was added to each batch prior to bottling to give an equal addition of 15 ppm of SO_2. The free and total SO_2 concentrations (FSO_2 and TSO_2, respectively) of each batch were measured by titration:[25] pH 3.2, FSO_2 = 40 mg/L, TSO_2 = 83 mg/L; pH 3.5, FSO_2 = 38 mg/L, TSO_2 = 82 mg/L; pH 3.8, FSO_2 = 42 mg/L, TSO_2 = 85 mg/L. Wines were filtered using Ewkip Z6 polishing grade pad and sterile membrane filters and bottled in 750 mL colorless bottles and stored at approximately 15 °C away from light. Differentiation in the level of nano-oxygenation was achieved with two different screw-cap closures: Saran Tin (ST), allowing minimal oxygen ingress (up to 0.00043 mg/L/day), and Saranex (Sx), allowing slightly greater oxygen ingress (up to 0.0273 mg/L/day).[20] Wine and bottles were sparged with N_2 prior to bottling, and levels of dissolved oxygen (DO) and headspace oxygen (HSO) were monitored in triplicate samples (at the start, middle, and end of each batch) for wines at 3 × pH and 2 × closure type using oxyluminescence (PST 3 and PST 6 sensors) via a PreSens meter (Nomasens oxygen analyzer, Nomacorc, SA).[1] DO levels prior to bottling were 0.33 ± 0.01 mg/L, and these concentrations increased to 0.40 ± 0.04 mg/L after bottling for all wines. Headspace volumes were consistently 6.4 mL, and initial HSO was 0.87 ± 0.32 mg/L. Total package oxygen (TPO) levels for all samples were 1.49 ± 0.40 mg/L.

For the wine and tannin analyses, a total of nine bottles of wine were analyzed immediately post bottling, with triplicate wines at 3 × pH (the impact of the different screw caps was considered negligible at this stage), and 18 bottles of wine were analyzed at the 6 and 24 month time points (i.e., triplicate wines at 3 × pH and 2 × closure type). Results pertaining to the general trends of wine aging used the mean and standard deviation of nine wines at bottling and all 18 wines at the other time points. Results relating to the impacts of wine pH or closure type used the mean and standard deviation of the triplicate results for wines at each variable. When no difference was observed for results from wines of different closure type but the same pH, the mean and standard deviation of all six wines of the same pH was used to highlight trends pertaining to wine pH alone.

Wine Composition. Wines at each pH and of each closure type were analyzed for tannin concentration and composition, wine color, anthocyanin concentration, and acetaldehyde concentration at 0, 6, and 24 months post bottling (from triplicate samples). Tannin concentration was measured using the methyl cellulose precipitable (MCP) tannin assay.[26,27] Briefly, polymer solution (H_2O/0.04% methyl cellulose) or H_2O for a control (300 μL) was reacted with the wine sample (25 μL) in a 96-well plate for 3 min (shaken using a platform shaker for 1 min and settled for 2 min). Saturated ammonium sulfate solution (200 μL) was then added, and each reaction was diluted to 1 mL with H_2O, shaken for 1 min, settled for 10 min, and centrifuged at 2000 rpm for 5 min. The tannin concentration was calculated on the basis of the 280 nm absorbance of the reaction mixture supernatant compared with the control per sample as previously described.[26,27]

Wine color was analyzed using the modified Somers color measurements as previously reported.[26,27] These analyses gave the wine color density (WCD, the combined absorbance of the wine at 420 nm and at 520 nm, referred to as A_{420} and A_{520}, respectively), the hue (A_{420}/A_{520}), and the SO_2 nonbleachable pigments ($A_{520:sulfite}$, A_{520} after reaction with a buffer solution containing 0.375% w/v sodium metabisulfite, 0.5% w/v tartaric acid in 12% v/v EtOH). The total anthocyanin concentrations of the wine samples were determined by comparing the A_{520} after reaction with 1 M HCl solution and the $A_{520:sulfite}$ with a malvidin-3-glucoside (M3G) standard curve (to give concentration in mg/L M3G equivalents), and total phenolics were measured as the absorbance at 280 nm after reaction with 1 M HCl (A_{280}). M3G concentrations were determined using high-performance liquid chromatography (HPLC) as previously described.[26−28]

For the acetaldehyde concentration analysis, wine samples (10 mL) were spiked with internal standard (100 μL) containing d_4-acetaldehyde and d_7-acetoin in a sealed vial prior to SPME GC-MS analysis using an Agilent 6890N gas chromatograph equipped with a Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5973 mass selective detector. An SPME fiber was exposed to each sample at 35 °C for 10 min and then injected into a split/splitless inlet fitted with an SPME inlet liner (0.75 mm i.d.), and the sample was allowed to desorb for 10 min (during which the inlet was held at 220 °C in splitless mode). Separation was achieved with a Restek Stabilwax-DA column (30 m × 0.180 mm, 0.18 μm film thickness) using helium (ultrahigh purity) as the carrier graph with a linear velocity of 43 cm/s and a flow rate of 1.4 mL/min in constant flow mode. The oven temperature was held at 40 °C for 4 min, increased to 90 °C at 5 °C/min, then heated at 40 °C/min to 240 °C, and held for 5 min. The mass spectrometer quadrupole temperature was set at 150 °C, the source was set at 230 °C, and the transfer line was held at 250 °C. Positive ion electron impact spectra at 70 eV were recorded in selective ion monitoring (SIM) mode (relative EM volts).

Tannin Isolation and Fractionation. Tannin was isolated from each wine at each sample point using Toyopearl media as previously described.[29] Briefly, Toyopearl HW-40F size exclusion medium (Optigen Scientific Pty Ltd., Port Adelaide, SA, Australia) in a glass column (50 × 450 mm) was equilibrated with H_2O/0.1% v/v formic acid wine prior to loading wine (600 mL). The column was then washed with H_2O/0.1% v/v formic acid (2 L) followed by 1:1 MeOH/H_2O with 0.1% v/v formic acid (approximately 6 L). Tannin was eluted with 2:1 acetone/H_2O with 0.1% v/v formic acid (1 L). The solvent was removed by rotary evaporator (30 °C) followed by freeze-drying. Tannin samples were stored under nitrogen at −80 °C.

To further measure changes in tannin structure, tannin was also isolated and fractionated with solid phase extraction using OASIS HLB SPE cartridges (Waters) as previously described.[26,30] Briefly, cartridges were activated with MeOH and equilibrated with H_2O prior to loading 1 mL of wine. After the cartridges were dried with N_2 and then washed with acetonitrile containing 5% v/v 0.01 M HCl (40 mL), the first tannin fraction (F2) was eluted with MeOH containing 0.1% v/v formic acid (5 mL) and the second tannin fraction (F3) with formic acid (0.3 mL) followed by MeOH containing 5% v/v H_2O (2.7 mL). Tannin fractions were dried under nitrogen at 30 °C and dissolved in either model wine (14% v/v EtOH,1 mL) for quantification by MCP analysis, as described above, or MeOH (100 μL) for characterizing the tannin structure, as described below.

Tannin Characterization. Isolated tannin and tannin fractions were characterized using gel permeation chromatography (GPC) for average tannin molecular mass[31] and using depolymerization reactions with phloroglucinol for subunit composition.[32] For GPC analysis, tannin samples (10 g/L MeOH) were diluted 1:4 with DMF and analyzed using a series of two columns (PLgel, 300 × 7.5 mm, 5 μm, 500 Å then 10^3 Å, Polymer Laboratories, USA), with an isocratic mobile phase of DMF solution (DMF/0.15 M LiCl/10% acetic acid).

Table 1. Tannin Concentration (Measured Using the MCP Tannin Assay) and Composition over 24 Months of Wine Aging[a]

	at bottling[b]	6 months[c]	24 months[c]
tannin concentration (g/L EC equiv)	1.86 ± 0.02a	1.69 ± 0.14a	1.29 ± 0.07b
% yield[d]	55.3 ± 3.3a	43.9 ± 4.8b*[g]	37.6 ± 4.4c*
% (−)-epigallocatechin subunits[d]	27.5 ± 0.8	27.5 ± 0.5	26.8 ± 0.6
% (−)-epicatechin-3-*O*-gallate subunits[d]	7.4 ± 0.1a	6.8 ± 0.3b*	6.1 ± 0.6c*
mean degree of polymerization (mDp)[d]	10.0 ± 0.1a	10.4 ± 0.5a	9.1 ± 0.7b
MM (g/mol)[e]	2854 ± 62a	2729 ± 144ab	2598 ± 134b*[g]
% colored (520:280)[e,f]	6.1 ± 0.3a	6.7 ± 0.4b	7.6 ± 0.4c

[a]Results in the same row that are significantly different ($p < 0.05$) are indicated with different letters. [b]Results are given as the mean of nine wine samples (triplicate wines at 3 × pH) ± one standard deviation. [c]Results are given as the mean of the 18 wine samples (triplicate wines at 3 × pH and 2 × closure) ± one standard deviation. [d]Calculated using phloroglucinolysis. [e]Average molecular mass as determined using gel permeation chromatography (GPC) at 50% tannin elution. [f]GPC peak area at 520 nm as a percentage of peak area at 280 nm. [g]An asterisk denotes results that were influenced by pH and/or closure type after 24 months.

Retention times were compared to a standard curve of fractionated preveraison grape skin tannins as previously described.[26,33] The average molecular weight was deemed to be the retention time at the elution of 50% of the tannin peak area, relative to the standard curve. For the depolymerization reactions, tannin samples (25 μL, 10 g/L in MeOH) were reacted 1:1 with phloroglucinol solution (phloroglucinol (100 g/L) in MeOH with 20 g/L ascorbic acid and 0.2 N HCl) at 50 °C for 20 min, prior to the addition of sodium acetate solution (70 mM, 150 μL). Reaction products were analyzed using HPLC as previously described[34] to identify and quantify subunits. These results gave the mean degree of polymerization (mDp, proportion of extension to terminal subunits), the percent of (−)-epigallocatechin subunits in the polymer, the percent of (−)-epicatechin 3-*O*-gallate subunits (% ECG), and the percent yield of the reaction, which was calculated by subtracting the total concentration of individual subunits from the concentration of tannin used in the reaction.[32]

Statistical Analysis. All significance tests were conducted using GraphPad Prism statistics software. Student's *t* test was used for comparing differences in wine composition with aging by analyzing the means and standard deviations of all wines at each time point (regardless of pH or closure type). These results were incorporated into Tables 1−3. ANOVAs and Tukey analyses were used for comparing the triplicate results from wines of different pH value and closure type. Results were incorporated into Tables 4 and 5.

RESULTS AND DISCUSSION

General Aging-Related Trends Associated with Wine Tannin and Color. The color and tannins of the single-vintage Cabernet Sauvignon (CAS) wines bottled at a range of wine pH and under two types of screw-cap closures were monitored for 24 months. Changes were observed as a consequence of wine aging, and in some cases these changes were influenced by wine pH and/or closure type. In this section, results are highlighted that indicate general trends in aging, particularly for tannin concentration and composition, which have not previously been studied in detail. Wine and tannin parameters that varied significantly with pH and/or closure type are discussed in greater detail in later sections.

To compare the general age-related trends in wine tannins, the results from each of the 18 bottles of wine at each sample point were averaged, and the means and standard deviations of all samples are reported in Table 1. Wine tannin concentrations changed substantially over the 24 months of wine aging. Tannin concentrations decreased significantly as measured using the MCP assay (Table 1), and this correlated well with the gravimetric recovery ($R^2 = 0.913$). The decline in concentration in this experiment suggested that the tannin in these wines may have precipitated or degraded into small oligomers as previously suggested.[3,4] Tannin concentration has been directly linked to astringency intensity[35,36] and, therefore, the reported decrease in wine astringency with aging [2] may be due, at least in part, to less tannin in the wine.

The structure of the wine tannins also changed substantially with wine aging, most notably in the percent yield (proportion of acid-labile bonds), molecular masses (MM) as measured at 50% elution by GPC, color incorporation, and subunit composition (Table 1). Most notably, the percent yield of tannin decreased significantly over the 24 months. The percent yield is calculated from the concentrations of cleaved catechin, epicatechin epigallocatechin, and epicatechin gallate subunits after depolymerization reactions and compared to the original concentration of tannin in the reaction. Reactions that occur as wines age will reduce the percent yield of wine tannins including oxidation, intramolecular bond formation,[37] and A-type linkages or ether linkages involving the B-ring,[38] as well as the incorporation of anthocyanins via either direct A-T or more complex interactions.[39−41] The formation of pigmented tannins was observed in this instance as a significant increase in the colored proportion of wine tannin, as measured by the GPC peak area at 520 nm as a percentage of peak area at 280 nm, over the 24 months post bottling (Table 1). This was consistent with previous reports of a greater "degree of redness" in more aged red wine tannin compared with younger wine tannin.[29] The reduction in percent yield and the increase in tannin color incorporation were pH- and closure-dependent as described in the later section.

The MM of the wine tannins as measured by GPC decreased slightly yet significantly after 24 months of aging, although there were no appreciable differences in size distributions (Table 1). The mDp as determined using depolymerization reactions also decreased slightly with aging. These results were pH-dependent as discussed later. Measuring the MM of wine tannins using either method has limitations. A decrease in calculated mDp alone can be indicative of oxidation and structural rearrangements that result in a reduction in the proportion of the polymer chain that can be cleaved (percent yield), rather than an actual decrease in overall MM,[37,42] as has been observed in previous analyses of vertical series.[26] This would lead to a disproportionate number of terminal subunits compared with extension subunits (those that react with phloroglucinol upon cleavage during the depolymerization reactions), reducing the determined mDp. Conformational changes in the tannin, such as polymer branching, may induce a different GPC response when measured at the same concentration. In this instance, the different CAS wine tannins showed no differences in GPC peak area, and the MMs as measured using both phloroglucinolysis and GPC indicated

Table 2. Characteristics of Tannin Fractions, F2 and F3, over 24 Months of Wine Aging As Measured Using the MCP Tannin Assay (% Total Tannin) and Phloroglucinolysis[a]

	F2		F3	
	bottling[b]	24 months[c]	bottling[b]	24 months[c]
% total tannin	9.8 ± 1.2a	23.4 ± 2.6b	90.2 ± 1.2c	76.6 ± 2.6d
mDp	5.3 ± 0.3a	3.4 ± 0.2b	10.8 ± 0.3c	7.9 ± 0.6d*[d]
% yield	55.3 ± 7.8a	28.6 ± 6.0b	55.4 ± 3.1a	36.4 ± 4.6c*
% (−)-epigallocatechin subunits	14.8 ± 2.6a	17.8 ± 2.5a	27.0 ± 0.3b	25.0 ± 1.1c
% (−)-epicatechin-3-*O*-gallate subunits	7.0 ± 0.7a	2.7 ± 0.3b	7.8 ± 0.3a	5.7 ± 0.6cd

[a]Results in the same row that are significantly different ($p < 0.05$) are indicated with different letters. [b]Results are given as the mean of nine wine samples (triplicate wines at 3 × pH) ± one standard deviation. [c]Results are given as the mean of the 18 wine samples (triplicate wines at 3 × pH and 2 × closure) ± one standard deviation. [d]An asterisk denotes results that were influenced by pH and/or closure type after 24 months.

Table 3. Red Wine Color (Determined Using the Modified Somers Color Assay), Acetaldehyde, and SO_2 Concentrations over 24 Months of Wine Aging[a]

	at bottling[b]	6 months[c]	24 months[c]
WCD (AU)	10.9 ± 0.8a	10.3 ± 0.4a	8.2 ± 0.2b
hue (AU)	0.60 ± 0.02a	0.63 ± 0.03a	0.71 ± 0.01b
total phenolics (AU)	42.0 ± 0.4a	43.5 ± 1.6a	36.7 ± 0.8b
NB pigments (AU)[d]	2.73 ± 0.04a	2.86 ± 0.05b	2.71 ± 0.06a
% NB pigments (% TP)[e]	6.50 ± 0.06a	6.57 ± 0.18a	7.39 ± 0.23b*[g]
total anthocyanins (mg/L M3G equiv)[f]	385.5 ± 4.7a	368.2 ± 26.4a	219.3 ± 23.7b*
acetaldehyde (mg/L)	4.73 ± 0.42a	1.44 ± 0.69b	nd[h]
free SO_2 (mg/L)	42.0 ± 4.3a	28.1 ± 2.1b	18.3 ± 2.9cc
total SO_2 (mg/L)	84.3 ± 2.6a	64.0 ± 3.4b	53.8 ± 3.3c

[a]Results in the same row that are significantly different ($p < 0.05$) are indicated with different letters. [b]Results are given as the mean of nine wine samples (triplicate wines at 3 × pH) ± one standard deviation. [c]Results are given as the mean of the 18 wine samples (triplicate wines at 3 × pH and 2 × closure) ± one standard deviation. [d]SO_2 nonbleachable pigments. [e]Calculated as a percent of the total phenolics (%TP). [f]Total anthocyanin concentrations calculated as malvidin 3-glucoside equivalents. [g]An asterisk denotes results that were influenced by pH and/or closure type after 24 months. [h]Not determined for these samples.

that the overall tannin MM decreased with wine aging over 24 months. This may be due to the cleavage of interflavan bonds in the mildly acidic wine medium[43] or potentially from some precipitation. Smaller tannins have both been associated with softer astringency,[29,44] and pigmented tannins have been rated as less astringent than noncolored tannins.[45] Therefore, the decrease in tannin MM and increase in color incorporation into tannin with wine aging may also contribute to the reduction in perceived astringency. The proportion of (−)-epicatechin-3-*O*-gallate moieties, subunits that are found more prominently in grape seeds than skins,[33,46] also decreased significantly over 24 months of aging. These reductions were pH- and closure-dependent as described in the later section. The proportions of the grape skin tannin-like subunits, (−)-epigallocatechin moieties, were unaffected. Tannin with (−)-epicatechin-3-*O*-gallate subunits are reportedly coarser compared with those containing more (−)-epigallocatechin subunits,[47] and grape skin tannin concentrations have been positively associated with wine quality in young red wines.[48] Thus, the reduction in the proportion of grape seed tannin-like subunits relative to the grape skin-like tannin subunits may also contribute to the softer astringency of aged wine.

To delve deeper into changes in tannin compositions, wine tannin from each sample was separated into two fractions, F2 and F3, using solid phase extraction (SPE),[26,30] and the concentrations and compositions of each fraction were analyzed (Table 2). F3 tannins were consistently more abundant and significantly larger than the F2 tannins. At bottling, the proportion of F2 tannin accounted for only 9.8 ± 1.2% w/w of the wine tannin, and this proportion increased significantly to 23.4 ± 2.6% w/w after 24 months of aging, which was similar to the ratios observed in CAS wine of ≥2 years old.[26] Tannin fractions separated using liquid−liquid fractionation with water and *n*-butanol have produced fractions with characteristics similar to those of F2 and F3.[26,29] Sensory analysis of these liquid−liquid fractions indicated that the more hydrophilic F3-like tannins were more astringent compared to F2-like tannin fractions that were more bitter. Therefore, the increase in the proportion of F2 tannin with wine aging, and consequent decrease in the proportion of F3 tannin, may contribute to a decrease in overall astringency. The mDp and percent yield for both fractions decreased significantly after 24 months (Table 2) as was observed for the total wine tannin (Table 1). The reduction in percent (−)-epicatechin-3-*O*-gallate subunits was more pronounced in the F2 tannins, whereas the percent (−)-epigallocatechin subunits remained relatively constant in both fractions. The impact of closure type and pH on F2 and F3 tannins is discussed in the later section.

Wine color was measured using the modified Somers color assay[27] to give wine color density (WCD), hue, total phenolics, the amount of SO_2 nonbleachable pigments, and total anthocyanins (Table 3). WCD decreased significantly over the 24 months of aging, and hue increased significantly, which was consistent with previous studies on red wine aging.[1,26] Total phenolics concentrations decreased in proportion with the decline in tannin as measured using the MCP tannin assay, further confirming the decrease in tannin concentration. The amount of total phenolics directly influences the proportion of SO_2 nonbleachable pigments (% NB pigments), as wine tannin includes pigmented polymers. By correcting for the decrease in

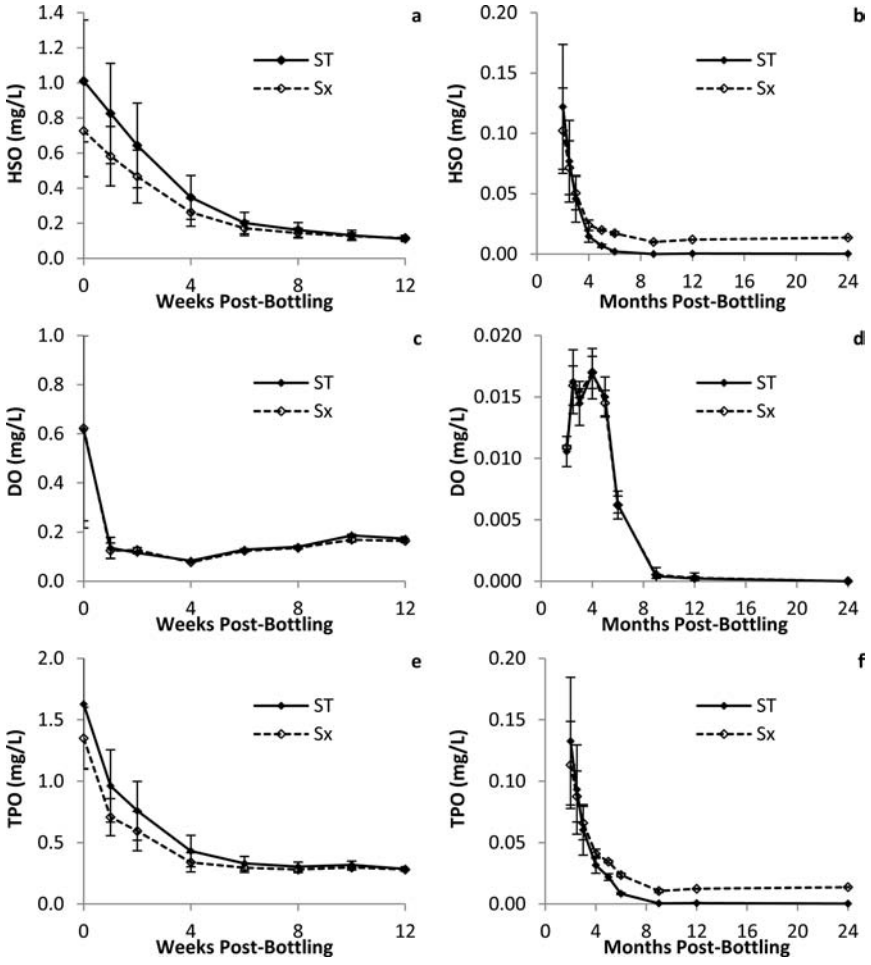

Figure 1. In-bottle oxygen concentrations across the aging trial. Initial readings (higher O_2 concentrations) were taken using the PST3 sensor for (a) headspace oxygen (HSO), (c) dissolved oxygen (DO), and (e) total package oxygen (TPO). Later readings (lower O_2 concentrations) were measured using the PST6 sensor for (b) HSO, (d) DO, and (f) TPO. Results are given as the mean of nine wine samples (triplicate wines at 3 × pH) ± one standard deviation.

total phenolics, the % NB pigments increased over the 24 months (Table 3), aligning well with the proportion of color in wine tannin as measured using GPC (Table 1). The concentrations of total anthocyanins were reduced to around half of the original concentrations within 24 months of wine aging with the formation of more stable pigments,[49,50] and this decrease was pH-dependent, as described in the later section. The rate of pigmented tannin formation is enhanced by the incorporation of fermentation and oxidation products, such as acetaldehyde, into the polymer.[51,52] Acetaldehyde concentrations decreased significantly with wine aging, and the rate of decline was pH- and closure-dependent at 6 months, as discussed in detail in the later section. Sulfur dioxide (SO_2) concentrations were initially adjusted to minimize the differences in free and total SO_2 concentrations induced by creating wines of different pH values. The level of both free and total SO_2 decreased significantly over the 24 months of aging with no significant variation due to pH or closure type. These values were similar to those obtained for Shiraz wines with low O_2 exposure after 360 days,[53] highlighting the low rate of oxygen ingress of these samples.

Impact of Closure Type on Wine Oxygen Concentrations. To minimize the amount of HSO in the wines, both the headspace and screw-cap closure were sparged with nitrogen before the bottles were sealed, and so the HSO that was measured after bottling was mainly due to oxygen ingress through the closure. Both HSO and DO were monitored over 24 months of aging (Figure 1). HSO declined continually from bottling over the 2 year trial. Initial variations in HSO between Sx and ST were not statistically significant, and within 2 months, all wines contained similar HSO levels of 0.15 ± 0.04 mg/L (Figure 1a). After 5 months, significant differences in HSO levels for the two screw-cap closures were revealed using the more sensitive PST6 sensor, with 0.020 ± 0.001 and 0.007 ± 0.001 mg/L for the Sx and ST wines, respectively (Figure 1b). HSO levels remained fairly constant for both closures from 9 months onward, with 0.012 ± 0.002 mg/L for the Sx wines and no detectable HSO for the ST wines.

Wine DO levels did not vary with closure or pH but did show some fluctuations across the 24 months (Figure 1c,d). The DO initially decreased in the first 4 weeks post bottling (Figure 1c) and then began to gradually increase from 0.011 ± 0.001 mg/L to peak at 0.017 ± 0.002 mg/L at 4 months of aging (Figure 1d). This may have been due to the rate of oxygen absorption into the wine being greater than the rate of consumption over this time.[53] The DO of all wines continued to decline after 4 months to 0.0003 ± 0.0002 mg/L at 12 months and contained no detectable DO by 24 months. The total package oxygen (TPO) for all wines showed similar oxygen concentrations for both closure types in the first 12

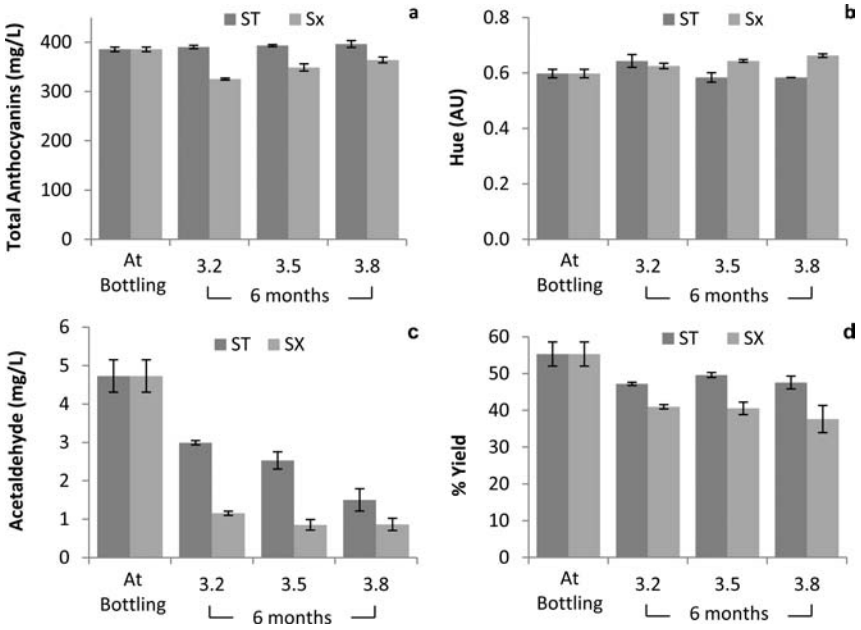

Figure 2. Wine and tannin characteristics influenced by pH and closure type after 6 months bottle aging: (a) total anthocyanins (mg/L malvidin 3-glucoside equivalents); (b) wine hue; (c) acetaldehyde concentrations (mg/L) for the wines bottled under SaranTin (ST) or Saranex (Sx) after 6 months of aging at pH 3.2, 3.8, and 3.8 and at bottling for comparison; (d) tannin percent yield (related to the proportion of acid-labile interflavan bonds). Results shown as means ± one standard deviation of nine wines at bottling (triplicate wines at 3 × pH) and triplicate wines for each variable at 24 months.

weeks after bottling (Figure 1e), and from 5 months onward (Figure 1f), Sx wines were exposed to significantly higher oxygen concentrations than the ST wines (0.03 ± 0.00 and 0.02 ± 0.001 mg/L, respectively).

Impact of Closure Type and pH on Tannin Composition and Wine Color. The single-vintage CAS wine was divided into three batches just prior to bottling, and the pH was adjusted to 3.2, 3.5, or 3.8 using either NaOH or H_2SO_4. Each batch was bottled under two different screw-cap closures: Sx, to allow slight oxygen ingress, and ST, to minimize oxygen exposure. Analysis of the wine immediately post bottling indicated that the pH adjustment did not cause a rapid change in tannin or color, because all samples produced similar characteristics.

In the first 6 months post bottling, both wine pH and closure type began to affect wine tannin structures and color attributes (Figure 2). The percent yields (proportions of acid-labile interflavan bonds) of the Sx wine tannins were significantly lower than those of the ST wine tannins (Figure 2d), and this corresponded to lower acetaldehyde and lower anthocyanin concentrations in these wines. Anthocyanins are not observed as cleaved subunits using the phloroglucinol assay[32,34] and therefore are not accounted for in the percent yield calculations. Thus, the incorporation of anthocyanins into the tannin structure, forming pigmented tannin, will lower the percent yield. Acetaldehyde-modified oligomers can also be incorporated into the wine tannin, inducing structural rearrangement reactions that also reduce the tannin percent yield.[16,54]

The Sx wines contained fewer anthocyanins than the ST wines (Figure 2a), further indicating that polymerization reactions had incorporated some anthocyanins into the pigmented tannin structure. The decrease in anthocyanin concentrations is also associated with the formation of pyranoanthocyanins as well as the degradation of anthocyanins, as has been previously noted for more stable pigments, such as indicating that oxygen exposure can facilitate the degradation of anthocyanins and may enhance formation of more stable pigments, including pigmented tannins and, as previously reported for micro-oxygenation (MOX) trials.[6] Wine pH also influenced the concentration of anthocyanins in the Sx wines, with lower anthocyanin concentration in the Sx pH 3.2 wines compared to the Sx pH 3.8 wines. This effect was not observed in the ST wines, highlighting that a combination of small amounts of oxygen and low pH promotes pigmented tannin formation.[49,55] Wine hue began to increase in some wines after 6 months of aging (Figure 2b). This change was pH-dependent, although the effect was different for the different closure wines. In the Sx wines, hues were slightly greater for pH 3.8 wines than for pH 3.2 wines, whereas the reverse was the case for the ST wines (Figure 2b), highlighting the impact of low oxygen exposure on wine color. The change in hue may be indicative of oxidized polyphenol formation and the development of yellow pigments[56] increasing the 420 nm absorbance compared with 520 nm absorbance.

Acetaldehyde can be consumed in wine by reactions with flavan-3-ol monomers to form ethylidene-linked flavanol oligomers and reactions with anthocyanins to form derived pigments such as pyranoanthocyanins.[11,54,57,58] The reduction in acetaldehyde concentrations in all wines after 6 months (Figure 2c) suggested that the rate of consumption was greater than the rate of formation. Acetaldehyde is produced as a fermentation product as well as from the oxidation of ethanol.[11] Increasing the oxygen concentration of wine can therefore increase the acetaldehyde concentration, but this response has been shown to be variable[59] and can depend on the concentration of phenolics in the wine.[15] In the presence of oxygen and a catalyst, *o*-diphenol-containing flavanols will undergo one- and two-electron oxidation to form reactive molecules that facilitate polyphenol polymerization. In this experiment, slight oxygen ingress in the Sx wines resulted in

Table 4. Red Wine Color Measures (Determined Using the Modified Somers Color Assay) after 6 and 24 Months of Aging That Were Significantly Influenced by Wine pH[a]

	months	pH 3.2	pH 3.5	pH 3.8
NB pigments (AU)	6	2.88 ± 0.07	2.88 ± 0.03	2.81 ± 0.02
	24	2.76 ± 0.02	2.73 ± 0.04	2.65 ± 0.05
total phenolics (AU)	6	43.3 ± 0.4	43.9 ± 0.8	43.4 ± 1.4
	24	36.2 ± 1.2	36.6 ± 0.2	37.3 ± 0.4
% NB pigments (% TP)[b]	6	6.65 ± 0.20a	6.56 ± 0.20b	6.48 ± 0.30c
	24	7.62 ± 0.22a	7.45 ± 0.12b	7.11 ± 0.11c
total anthocyanins (mg/L M3G equiv)[c]	6	358.8 ± 3.2a	369.4 ± 7.3b	376.3 ± 5.8c
	24	192.0 ± 5.0a	218.0 ± 2.1b	247.8 ± 3.9c

[a]Results in the same row that are significantly different ($p < 0.05$) are indicated with different letters. Results are given as the mean of six wine samples (triplicate wines at 2 × closures) ± one standard deviation. [b]Calculated as a percent of the total phenolics (%TP). [c]Total anthocyanin concentrations calculated as malvidin 3-glucoside equivalents.

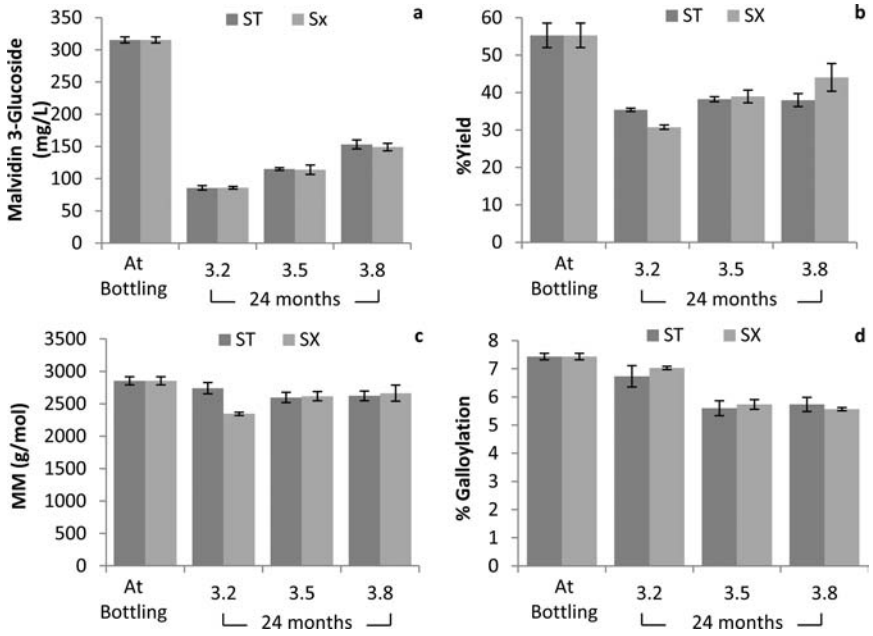

Figure 3. Wine and tannin compositional characteristics influenced by pH and closure type after 24 months of bottle aging: (a) malvidin 3-glucoside (mg/L); (b) tannin percent yield (related to the proportion of acid-labile interflavan bonds); (c) tannin molecular mass (MM, g/mol); (d) tannin percent (−)-epicatechin-3-*O*-gallate subunits (% galloylation) for the wines bottled under SaranTin (ST) or Saranex (Sx) after 24 months of aging at pH 3.2, 3.8, and 3.8 and at bottling for comparison. Results shown as means ± one standard deviation of nine wines at bottling (triplicate wines at 3 × pH) and triplicate wines for each variable at 24 months.

lower acetaldehyde concentrations than in the ST wines, presumably due to the greater rate of consumption compared with formation.[51] Wine pH influenced the amount of acetaldehyde in the ST wines with lower concentrations present in the pH 3.5 and 3.8 wines. This effect was not observed in the Sx wines, potentially due to greater reaction rates in polymer formation, mitigating the impact of wine pH on acetaldehyde.

After 24 months of bottle aging, wine pH variation produced significant differences in total anthocyanin concentrations and NB pigments (Table 4), as well as tannin MM, percent yield, and percent galloylation, with closure type predominantly influencing tannin structure at pH 3.2 (Figure 3). The difference between total anthocyanin concentrations in the pH 3.2 and 3.8 wines increased after 24 months of aging compared with 6 months as more NB pigments formed at lower pH (Table 4). M3G concentrations decreased to less than half of the original concentration at bottling in the pH 3.8 wine and to almost a fourth in the pH 3.2 wine after 24 months (Figure 3a), and other anthocyanins showed similar patterns over aging and wine pH (data not shown). The influence of wine pH on NB pigments and anthocyanins did not result in significant pH-dependent differences in overall WCD or hue, although it may have affected the tannin structure by varying the extent of anthocyanin incorporation.

Wine tannin structure was influenced by both pH and closure type, particularly for the percent yield, MM, and proportion of (−)-epicatechin-3-*O*-gallate subunits (Figure 3). The pH 3.2 wines formed tannins with significantly lower percent yields after 24 months of aging than the pH 3.5 and 3.8 wines (Figure 3b). The percent yields of the pH 3.2 Sx tannins were also significantly lower than the ST tannins at the same pH, demonstrating that low oxygen ingress at lower pH can affect tannin conformation. Wines bottled under Sx closures demonstrated initial rapid decrease in percent yield (within 6 months) along with a significant reduction in anthocyanins at pH 3.2 (Figure 2a,d), whereas ST wines demonstrated a more gradual decline in percent yield over the 24 months of aging

Table 5. Characteristics of Tannin Fractions, F2 and F3, after 24 Months of Aging That Were Significantly Influenced by Wine pH or Closure Type (SaranTin (ST) or Saranex (Sx)), As Measured Using Phloroglucinolysis[a]

tannin fraction	characteristic	closure[b]	pH 3.2	pH 3.5	pH 3.8
F2	mDp[c]	ST/Sx	3.4 ± 0.2	3.5 ± 0.2	3.4 ± 0.2
	% yield	ST/Sx	30.7 ± 4.7	27.4 ± 3.8	30.5 ± 6.5
	% (−)-ECG[d]	ST/Sx	3.0 ± 0.2a	2.6 ± 0.1b	2.4 ± 0.3b
F3	mDp	ST	7.5 ± 0.2a	8.1 ± 0.2b	8.6 ± 0.1c
		Sx	7.1 ± 0.2a	7.8 ± 0.0b	8.3 ± 0.3c
	% yield	ST	42.1 ± 3.7	38.5 ± 1.0	41.3 ± 3.5
		Sx	30.9 ± 2.2a	33.9 ± 3.5ab	38.1 ± 2.1b
	% (−)-ECG	ST/Sx	6.5 ± 0.1a	5.5 ± 0.1b	5.2 ± 0.1c

[a]Results in the same row that are significantly different ($p < 0.05$) are indicated with different letters. [b]Results from closures listed as ST/Sx are the mean of six wine samples (triplicate wines at 2 × closure) ± one standard deviation. When an individual closure type is given, the results are the mean of triplicate wines ± one standard deviation. [c]Mean degree of polymerization. [d]% (−)-epicatechin-3-*O*-gallate.

(Figures 2d and 3b). These differences in tannin reactivity due to closure and pH ultimately led to the observed differences in tannin size and percent yield after 24 months, which may increase with further aging. Tannin MM as measured by GPC was relatively consistent across the pH and closure treatments except for the tannin from the Sx pH 3.2 wines, which was significantly smaller than the other wine tannins (2345 ± 25.2 g/mol compared with around 2650 g/mol for the other wine pH and closure samples after 24 months) (Figure 3c). This indicated that the more rapid reactions involving pigmented tannin formation after 6 months may also produce smaller tannins, potentially from the cleavage of interflavan bonds in the mildly acidic wine medium with slight oxygen ingress.[43] The proportion of (−)-epicatechin-3-*O*-gallate (% ECG) decreased significantly in the pH 3.5 and 3.8 wine tannins and only slightly in the pH 3.2 wine tannin samples (Figure 3d). This was consistent across both closure types and appeared to be independent of percent yield and tannin MM. The same result was also observed in F3 tannin fractions and to a lesser extent in F2 fractions (Table 5). A decline in % ECG has also been reported with increasing grape seed maturity, and this has been related to a decrease in percent yield due to oxidation.[60] Enzymatic oxidation of pear polyphenols can potentially lead to polymerization via the galloyl group interacting directly with the catechin B-ring,[61] and a similar mechanism may be occurring in wines with aging, particularly at higher wine pH. Tannins with more (−)-epicatechin-3-*O*-gallate subunits, such as seed tannins, have been associated with coarse astringent qualities,[47] whereas skin tannins have been positively associated with wine quality.[48] The reduction in the proportion of seed-like tannin subunits with aging at the higher pH without a significant change in the proportion of skin-like tannin subunits may contribute to a softer wine mouthfeel at slightly higher pH.

The impact of wine pH and closure type on the F3 tannin fractions that were isolated from SPE demonstrated the same trends as the total tannin (Table 5), although no such trends were observed in the F2 tannins. The mDp of the F3 tannins from the pH 3.2 wines were lower than that of the other wines, especially for those bottled under Sx, and were higher in percent (−)-epicatechin-3-*O*-gallate than those fractions isolated from the higher pH wines regardless of closure type. F3 fractions were dominant in the wine tannins, accounting for around 76% w/w of the wine tannins after 24 months of aging (Table 2), which is likely to be the reason for the similar trends observed in these fractions and the total tannin. This hydrophilic portion of the wine tannin has also been associated with greater astringency.[29] Therefore, although the F2 tannins were not influenced by wine pH or closure type, winemaking and storage conditions can still alter the overall tannin composition and thus influence red wine mouthfeel.

In summary, aging red wines in bottles over 24 months significantly reduced the tannin concentration, tannin MM, tannin percent yield (proportion of acid-labile interflavan bonds), and proportion of grape seed tannin-like subunits ((−)-epicatechin-3-*O*-gallate), all of which can contribute to changes in wine astringency with aging. Closure type and wine pH influenced wine color and tannin structure, and slight differences observed after 6 months of aging were indicative of differences in the reaction kinetics of changes in tannin composition and wine color. Within 6 months post bottling, Sx pH 3.2 wines contained lower anthocyanin concentrations and more NB pigments than ST wines, and after 24 months of wine aging, the anthocyanin concentration was substantially reduced in all wines, particularly those at lower pH. Sx pH 3.2 wines contained tannins with lower mDp and percent yield and a greater proportion of (−)-epicatechin-3-*O*-gallate subunits. Overall, the tannin structure and wine color of the lower pH wines (pH 3.2) bottled under Sx screw caps changed more rapidly with aging than those of the higher pH wines (pH 3.8) bottled under ST screw caps. Further investigations will reveal the impact of these changes on sensory perception and consumer preferences. Understanding how different winemaking styles and storage conditions impact tannin composition and thus mouthfeel can enable winemakers to better manage the textures of their red wines.

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Notes

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■ ABBREVIATIONS USED

DMF, *N*,*N*-dimethylformamide; DO, dissolved oxygen; GPC, gel permeation chromatography; HSO, headspace oxygen; MCP, methyl cellulose precipitable; mDp, mean degree of polymerization; MOX, micro-oxygenation; MM, molecular mass; NB pigments, SO_2 nonbleachable pigments; SPE, solid-phase extraction; TPO, total package oxygen; Sx, Saranex liners; ST, SaranTin liners

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