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Research Article

Effect of Structural Transformations on Precipitability and Polarity of Red Wine Phenolic Polymers

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Abstract: Condensed tannins and polymeric pigments are essential red wine components since they contribute to color stability, taste, and mouthfeel. Phenolic polymers in red wine consist of flavan-3-ol monomers as well as anthocyanins and cause the perception of astringency. Due to the chemical heterogeneity of proanthocyanidin polymers, analytical tools for the determination of the polymers' structural features are limited. The incorporation of anthocyanins increases the structural complexity even more and leaves it almost impossible to assess the influence of structure on the evoked astringency. To obtain a better understanding of the structural diversity of red wine polymers, this study combines forced aging and the FLASH-fractionation of polyphenolic wine extracts to reveal the relationship between phenolic polymers and two physicochemical properties, polarity, and hydrophilicity. Red wine fractions were characterized regarding their polarity,

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octanol-water partitioning coefficient, protein precipitation assay, UHPLC-MS, and color. Tannin concentrations in wine decreased during forced aging while the concentrations were constant in the corresponding extracts, suggesting an alteration of the precipitation behavior. A simultaneous increase of precipitable polymeric pigments gives rise to the assumption that the incorporation of anthocyanins into tannin molecules alters the interactions with red wine polysaccharides and proteins, which results in lower tannin readings. Finding tannins and polymeric pigments in different FLASH-fractions indicates that precipitability of polymers is affected by the physicochemical properties, which in turn depend on the degree of polymerization as well as degree of pigmentation. The results of this study show that red wine astringency and its sub-qualities may be related to the increase in precipitable polymeric pigments during forced red wine aging and their putative enhanced interaction with wine polysaccharides and can help to better understand astringency mechanisms.

Key words: interactions, physicochemical, pigmentation, polymers, red wine, tannins

Introduction

Phenolic compounds are essential components of wine and anthocyanins and flavan-3-ols are arguably of utmost importance for red wine quality since they contribute to color and its stability as well as taste and mouth-feel properties (Cheynier et al. 2006). While monomeric flavan-3-ols contribute to bitterness, tannins and oligomeric proanthocyanidins are largely responsible for the perception of astringency (Gawel 1998, Noble 1998). The composition of the tannins, expressed by the degree of polymerization and galloylation as well as the number of trihydroxylated monomers, are the driving forces for the intensity and quality of astringency

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perception, which is explained by a loss of lubrication as a result of polyphenols precipitating saliva proteins (Noble 1998, de Freitas and Mateus 2001, Vidal et al. 2003, Harbertson et al. 2014). Anthocyanins determine the color of young red wines and are extracted during wine making. They have a key role in the modulation of color and mouthfeel properties during red wine aging.

Anthocyanins are transformed to more stable pigments which is accompanied by a loss in wine color density (Bindon et al. 2014). Together with some low molecular wine constituents and yeast metabolites, anthocyanins can form pyranoanthocyanins (Fulcrand et al. 2006) or can be incorporated into tannin-like structures. Tannins that incorporate anthocyanins during red wine aging are designated polymeric pigments (Remy et al. 2000).

Chira et al. (2012) reported an age-related decrease of tannin concentrations and mean degree of polymerization (mDP) accompanied with a decline in perceived astringency. In contrast, McRae et al. (2012) showed that tannin concentrations were not directly related to wine age and that tannin size increased during aging indicating that lower astringency ratings of aged wines do not result solely from lower tannin concentrations and mDPs. Earlier studies (Vidal et al. 2004a, Weber et al. 2013) suggested that the formation of polymeric pigments found in aged red wine attenuates astringency. Hence, the incorporation of anthocyanins may affect astringency perception even more than the concomitant increasing polymer length.

Due to similar chemical structures and the chemical heterogeneity of proanthocyanidin polymer length, sub-unit composition, and constitution, analysis of these phenolics has proved difficult. Reversed-phase HPLC-DAD-MS is commonly used to identify and quantify low molecular polyphenols, but this approach is limited regarding tannin analysis since they elute as a polydisperse hump (Ma et al. 2018). Methods that are utilized to partly characterize red wine

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polymers include tannin precipitation either by proteins in combination with bisulfite bleaching (Harbertson et al. 2002, 2003) or polysaccharides (Sarneckis et al. 2006). Acid-catalyzed cleavage of proanthocyanidins in the presence of nucleophilic agents like phloroglucinol (Kennedy and Jones 2001) is another approach to assess polymer composition. However, this method showed its limits when applied to analyze pigmented tannins (Vidal et al. 2004a) and therefore, the manifold structures of polymeric pigments have not yet been identified. Consequently, the complex composition and alteration of red wine polymers as well as their impact on astringency perception remain important issues to be studied.

To address this lack of knowledge, this study utilizes normal-phase FLASH-chromatography to fractionate red wine polyphenols according to their size and polarity. The fractions were chemically characterized including the determination of their octanol-water partitioning coefficients (K_{OW}) to measure hydrophilicity. A previous study (Merrell et al. 2018) showed that the K_{OW} is influenced by tannin composition and red wine maturity. Combining forced aging and fractionation of polyphenolic wine extracts aims at revealing the relationship between polymeric pigments as well as tannins and two physicochemical properties. Polarity and hydrophilicity were investigated to gain a better understanding of the structural diversity of red wine polymers.

Materials and Methods

Materials

Acetic acid, hexane, hydrochloric acid (HCl), potassium bisulfite, and acetonitrile were purchased from VWR International GmbH (Darmstadt, Germany). Ethanol, bovine serum albumin

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fraction V, and (+)-Catechin were purchased from Carl Roth (Karlsruhe, Germany). Silica gel 60 Å (particle size 0.063-0.2 mm, 70-230 mesh) and sodium hydroxide were purchased from Honeywell Fluka (Offenbach, Germany). Urea, maleic acid, ferric chloride, triethanolamine (TEA), and octanol were purchased from Alfa Aesar (Kandel, Germany). Sodium chloride and Amberlite XAD7 were purchased from Labochem int. (Heidelberg , Germany) and Sigma-Aldrich (Darmstadt, Germany), respectively.

Wine samples

Two different commercially available wines were chosen in this study. Six bottles each of the 2018 Cabernet Sauvignon from the Trapiche winery (Maipú, Mendoza, Argentina) and the 2016 Cabernet Sauvignon from the Salentein winery (Tunuyán, Mendoza, Argentina) were used. The wines were assessed in advance by FT-IR and in a bench tasting, which verified that both wines had no considerable differences in their general composition and sensory properties. Two different wines from two vintages were selected to investigate whether wine phenolic composition and tannin structures change differently in an older wine compared to a younger wine during forced aging. The 2018 wine was composed as follows: 13% ethanol by volume, 9 g/L glycerol, pH 3.7, titratable acidity as 5.9 g/L tartaric acid equivalents, 5 g/L residual sugars, 1935 mg/L catechin equivalents total phenolic content. The 2016 wine was composed as follows: 13.5% ethanol by volume, 10 g/L glycerol, pH 3.8, titratable acidity as 5.4 g tartaric acid equivalents/L, 5 g/L residual sugars, 2117 mg/L catechin equivalents total phenolic content. Apart from the phenolic content, that was determined according to chapter 2.5, these parameters were obtained using Fourier-transform mid-infrared spectroscopy, including the appropriate calibration method (WineScan FT120 Basic, Foss, Hilleroed, Denmark). The total phenolic contents of the wines were not

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significantly different at $p \leq 0.05$. Free and total SO_2 values were 6 mg/L and 70 mg/L for the 2016 wine and 10 mg/L and 100 mg/L for the 2018 wine determined by titration. The samples were split into three pairs. Two were kept at 35 °C for three or six weeks and were compared to the non-aged wines. All bottles were closed with screw caps and the two bottles of each sample were pooled for all experiments.

Solid phase extraction and fractionation of phenolic compounds

To obtain a polyphenol rich extract from the wines, each wine sample was diluted with water (1:2) and was loaded onto an Amberlite XAD7 column (65 mm x 450 mm; 1.5 L bed volume), which was previously washed with 250 mL of a 0.1% (w/v) sodium hydroxide solution and preconditioned with 2 L of water. After elution of the wine, the column was washed with 2 L of water (1.3 fold of the bed volume) in order to remove sugars and organic acids. The polyphenols were eluted with approximately 3 L of ethanol acidified with acetic acid (29:1 v/v) at a gravity flow rate of approximately 10 mL/min. The collected extracts were concentrated using a rotary evaporator and consecutively lyophilized. The fractionation was conducted on a self-packed silica gel 60 Å column (36 mm x 460 mm; 0.5 L bed volume) using a low-pressure chromatography pump (C-605 pump with C-615 pump manager, Büchi Labortechnik GmbH, Essen, Germany). Isocratic elution involved three solvents: 60% hexane, 40% ethanol (solvent A), ethanol with 1% formic acid (solvent B), and 50% ethanol (v/v) with 1% formic acid (solvent C). At a flow rate of 90 mL/min the column was first rinsed with solvent C for 10 minutes and then preconditioned with solvent A for another 10 minutes. Subsequently, 5 mL of extract dissolved in solvent B were loaded onto the column with a concentration of 75 g/L. Solvent A, B and C were successively applied to the column for 10 minutes each and changed manually. Elution was monitored at 280 nm and 520

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nm with a Knauer BlueShadow 50D detector and the ClarityChrom Software (Knauer, Berlin, Germany). According to the chromatogram obtained at 280 nm, the fractions were manually combined. After complete elution, solvents were evaporated, and the fractions were lyophilized. The column was washed with solvent C for 10 minutes. Prior to further analyses, the lyophilized fractions and extracts were dissolved at concentrations of 2 g/L in a wine-like solution (12% ethanol by volume, 5 g/L tartaric acid, pH 3.3 adjusted with NaOH).

Spectrophotometric analysis

Absorbance spectra were recorded in undiluted wines and sample solutions between 300 and 800 nm by a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany), using a 1 mm path-length glass cuvette (Hellma GmbH & Co. KG, Müllheim, Germany). After values were corrected to a 10 mm path length cylindrical coordinates chroma C* and hue h* were calculated with the Spectra Manager Ver.2.14G (JASCO Deutschland GmbH, Pfungstadt, Germany) according to OIV recommendations (OIV 2006).

Chemical characterization

Anthocyanins were analyzed following the protocol reported by Harbertson et al. (2009). Protein precipitation was combined with bisulfite bleaching to determine tannins and polymeric pigments (Harbertson et al. 2002, 2003) using a reformulated resuspension buffer (urea 8.3 M, 5% TEA, pH 7 adjusted with HCl) as published by Harbertson et al. (2015). To quantify total iron reactive phenolics, an aliquot of the sample is diluted with the previously mentioned resuspension buffer to a total volume of 875 µL and incubated for 10 minutes. Absorbance at 510 nm is measured before and after addition of 125 µL of ferric chloride solution. Tannins and total iron

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158 reactive phenolics were expressed as catechin equivalents (CE) according to an external calibration
159 curve.

160 *Octanol-Water partition coefficient*

161 A volume of 1 mL of the sample solution was thoroughly mixed with 1 mL of octanol and
162 vortexed for 10 seconds. For faster separation of the phases, the samples were centrifuged at 9,600*g*
163 for 10 minutes. Subsequently, an aliquot of both phases was injected into the Shimadzu Nexera
164 X2 UHPLC-DAD system (two Nexera X2 LC-30AD high-pressure gradient pumps, a Prominence
165 DGU-20A5R degasser, a Nexera SIL-30AC autosampler (15 °C, injection volume 2 µL), a CTO-
166 20AC Prominence column oven (40 °C), and a SPD-M20A Prominence diode array detector;
167 Shimadzu, Kyoto, Japan) using an ACQUITY HSS T3 column (50 mm × 2.1 mm, 1.8 µm; Waters,
168 Milford, USA). At a flow rate of 0.5 mL/min samples were eluted using the following gradient: 0
169 min, 50% B; 2 min, 100% B; 3.3 min, 100% B; 4 min, 50% B; 7 min, 50% B, with A being
170 water/formic acid (97/3; v/v) and B being acetonitrile/formic acid (97/3; v/v). The partitioning
171 coefficient was formed by the ratio of the samples' total peak area in the octanol phase and the
172 water phase, respectively, according to the chromatogram at 280 nm.

173 *UHPLC-ESI-MS/MS*

174 UHPLC-MS analysis of the fractions was performed on an Acquity UPLC I-Class system
175 (Waters, Milford, MA) consisting of a binary pump, an autosampler cooled at 10 °C, a column
176 oven set at 40 °C, and a diode array detector scanning from 190 to 800 nm. An Acquity HSS-T3
177 RP18 column (150 × 2.1 mm; 1.8 µm particle size) combined with a precolumn (Acquity UPLC
178 HSS T3 VanGuard, 100 Å, 2.1 × 5 mm, 1.8 µm), both from Waters (Milford, MA) was used for

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separation. At a flow rate of 0.5 mL/min analytes were eluted using the following gradient: 0 min, 5% B; 8 min, 10% B; 25 min, 25% B; 26 min, 100% B; 28 min, 100% B; 29 min, 5% B; 31 min, 5% B, with A being water/formic acid (97/3; v/v) and B being acetonitrile/formic acid (97/3; v/v). The injection volume was 5 µL. The UPLC was coupled to a LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Waltham, MA) equipped with an electrospray interface operating in positive ion mode for analysis of anthocyanins and anthocyanin derivatives and in negative ion mode for other polyphenols. For identification, mass spectra were recorded in the range of *m/z* 120–1500 with three consecutive mass scans (MS^2, 35% normalized collision energy; MS^3, 45% normalized collision energy). The capillary was set at 325 °C with a voltage of 40 V for ESI^+, and at 350 °C and a voltage of −44 V for ESI^-. The source voltage was maintained at 5 and 4 kV, respectively, at a current of 100 µA. The tube lens was adjusted to 70 V for ESI+ and −105 V for ESI−. For quantification, specific *m/z* values of 63 polyphenolic compounds were recorded in single ion monitoring (SIM) measurements using one scan event.

Sensory analysis

To determine the effects of alterations of tannin structures on astringency during forced aging, overall astringency of the wines was evaluated by a panel tasting. The sensory panel was composed of 14 volunteer judges that participated in three training sessions prior to the final tasting. The first session was dedicated to the differentiation between astringency, sourness and bitterness by the panelists who were familiarized with these tastes and sensations. Solutions of aluminum sulfate (2 g/L), caffeine (1.5 g/L) and tartaric acid (2 g/L) in a Pinot noir wine from 2018 used as basic wine were presented to train astringency, bitterness, and sourness perception.

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The second session was dedicated to the recognition of various aluminum sulfate concentrations (0, 0.5, 1 and 2 g/L). Panelists were advised to rank the standard solutions by ascending intensity. During the third session the panelists were introduced to the intensity scale of the final tasting which was a structured scale from 1 to 10 for "very low intensity" and "very high intensity", respectively. Two astringency standard solutions (0.5 g/L and 3 g/L) were presented and set as points 3 and 8 of the scale after panel discussion. The final tasting was held in four individual sessions and three samples were evaluated in each of them. Wine samples were presented in a balanced random order in coded glasses and were tasted in duplicate. Reference astringency solutions were provided in each session. The panelists tasted 30 mL of the wine in individual booths wearing a blindfold. They were advised to neutralize the oral cavity with water and bread and to wait 3 minutes before tasting the following sample.

Statistical analysis

Statistical analysis of the results was performed using XLSTAT (Version 2014.4.06, AddinSoft Technologies, Paris, France). For pairwise comparisons, an ANOVA with a selected significance level of $p < 0.05$ was used.

Results

Wine samples and storage

The two wines chosen for this study presented a similar initial composition and were stored at elevated temperature to accelerate reactions normally occurring during red wine aging. Two bottles of each wine were subjected to forced aging for three or six weeks. The results of the FT-IR analysis revealed only negligible changes in the wines' general composition after storage. The

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color assessed by the CIELab parameters hue and chroma (Table 1), showed that the 2018 wines exhibited greater color intensities than the 2016 samples. In contrast to the rather high ΔE values between fresh and stored samples of 4.66 and 8.93 for the 2016 and 2018 wines, respectively, the color differences were hardly perceptible. The higher ΔE value of the 2018 wines may be explained by the faster loss of anthocyanins in younger wines due to the exponential decline of anthocyanins during aging (McRae et al. 2012).

Since color intensity is correlated with anthocyanin concentration and red wine maturity, the loss of color is consistent with the fast decline of anthocyanin concentrations during storage (Figure 1A). This development can be explained by the degradation, conversion, and incorporation of anthocyanins into pyranoanthocyanins and polymeric pigments, respectively. Figures 1B and 1C indicate higher proportions of polymeric pigments in the 2016 wines compared to the 2018 samples, whereby both contain more non-precipitable polymeric pigments (PP) than precipitable PP. While the proportion of precipitable PP is increasing in both samples, the amount of non-precipitable PP is increasing only in the 2018 wine. In the 2016 wine, non-precipitable PP concentration leveled, whereas in the 2018 wine, the non-precipitable PP concentration increased. While concentrations of precipitable PP increased, tannin concentrations decreased in the wine samples (Figure 1).

Since the wines did not show considerable differences in terms of sourness and bitterness, which was also proven by the FT-IR data, only wine astringency was further assessed in the sensory analysis. The sensory evaluation of the perceived astringency revealed that the 2016 wine appears to induce higher but still moderate astringency (Table 2). A four-way ANOVA of the

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astringency rating including vintage, storage, panelist, and replicate is presented in Supplemental Table 1. The astringency of the wines slightly declined which is in line with the findings for tannin concentrations (Figure 1D). Interestingly, the astringency of the 3 weeks stored 2018 sample dropped to 3.5 but increased during another 3 weeks of storage. This coincides only partially with the tannin concentrations as tannin concentration declined constantly over time in the 2018 wine.

Isolation of a polyphenol rich extract and fractionation using silica gel

The yields of the polyphenol rich extracts obtained by solid phase extraction using Amberlite XAD7 as solid phase were 3.6±0.1 g/L for the 2018 wines and 4.1±0.1 g/L for the 2016 wines. For every wine sample, the low-pressure fractionation on silica gel was repeated 6 to 8 times to produce enough material for the following analyses. The separation with silica gel primarily works on size exclusion, but hydrogen bonding between the phenolics and the silanol groups also plays an important role. The ternary isocratic separation of the injected extracts generated three fractions and the corresponding yields, and the distribution are given in Table 3. The elution of the fractions was monitored at 280 and 520 nm.

Composition of the FLASH fractions

Table 1 presents the color metrics recorded for the fractions of all wine samples. With chroma values of 13 to 16 and a color hue of around 70, fractions 1 had a light orange to yellow coloration indicating a limited amount of red pigments. Having color hues of 28 and 35 each fraction 2 and 3 of the 2018 wine were closer to a blueish red color than fraction 2 and 3 of the 2016 wine with values of 37 and 41, respectively.

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The results of the protein precipitation assay (Figure 2A) show that the highest number of anthocyanins was found in fraction 2 of the 2018 wine. In all fractions, the amount of non-precipitable PP (Figure 2B) is higher than that of precipitable PP (Figure 2C) and tannins were only found in fractions 2 and 3. Tannins, polymeric pigments, and monomeric anthocyanins are absent in fraction 1, suggesting that fraction 1 is mainly composed of non-polar and non-phenolic substances.

Figure 3 presents the octanol water partitioning coefficients (K_{OW}) of the fractions. A K_{OW} higher than 1 implies that the fraction is lipophilic, while values below 1 express the hydrophilicity of the contained compounds. The K_{OW} of the fractions follows the elution gradient of the FLASH separation as expected, where fraction 1 showed hydrophobic properties, while fractions 2 and 3 are both hydrophilic. The highest hydrophilicity is found in fraction 3 of both vintages. Merrell et al. (2018) determined the octanol water partitioning coefficients of young and aged Cabernet Sauvignon wines and defined coefficients of around 0.19 for young wines. This is comparable to the values found in this study for the wine extracts (Figure 3A).

The results of the UHPLC-MS analyses show that fraction 1 mainly contains gallic acid, monomeric flavan-3-ols, hydroxycinnamic acids and oligomeric procyanidins, whereas malvidin-3-*O*-glucoside is the main compound in fractions 2 and 3 (Supplemental Table 2 and 3). In agreement with the color and the precipitation assay, fraction 1 is characterized by the absence of anthocyanins and their derivatives.

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Changes in the fractions during wine storage

The storage of the wines did not change the quantitative proportions of the fractions. Anthocyanins in fractions 2 and 3 declined in both vintages. The decrease in anthocyanins does not lead to a loss in color intensity (chroma), but goes along with a change in hue which indicates structural changes of pigments rather than a mere loss. Non-precipitable PP (Figure 2B) of the 2018 wine increased in fraction 2 and decreased in fraction 3. Since a less polar solvent elutes fraction 2, these developments of non-precipitable PP also indicate structural transformations of molecules, which correspond with declining polarities. In the 2016 wine, non-precipitable PP concentrations remained constant in both fractions. In fractions 2 and 3, precipitable PP (Figure 2C) increased during storage. No changes in tannin concentrations were detected except in fraction 2 of the 2016 wine, which showed a slight decrease indicating that the amount of less polar tannins of the 2016 wine decreased over time.

As a result of lower concentrations in polymeric pigments, the color of fraction 3 of the 2018 wine changed the most while the color of the other fractions (Table 1) was rather constant. It is apparent that the hydrophilicity of the fractions changed significantly during storage, however alterations are rather small with only fraction 3 of the 2018 wine undergoing considerable changes (Figure 3). Fraction 1 of the 2016 wine becomes more hydrophilic while fraction 1 of the 2018 wine shows higher hydrophobicity after storage. In fraction 2 of the 2016 wine and fraction 3 of the 2018 wine the hydrophilicity is increasing, whereas in fraction 3 of the 2016 wine and fraction 2 of the 2018 wine at the end of the 6 weeks storage no change was detected. Nevertheless, a rise

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and a decrease of water solubility in fraction 3 of the 2016 wine and fraction 2 of the 2018 wine, respectively, occurred after 3 weeks.

In contrast to the anthocyanin concentrations, the UHPLC-MS results show no changes in the concentration of anthocyanin-derived pigments like pyranoanthocyanins or anthocyanin-flavanol oligomers (Supplemental Table 2 and 3). Likewise, monomeric flavanols, benzoic acids, hydroxycinnamic acids, flavanol dimers and trimers did not decrease.

Discussion

This study was conducted to gain a deeper understanding of structural transformations of polyphenols occurring during forced red wine aging and their effects on astringency perception. Earlier studies (Boselli et al. 2004, Landon et al. 2008, Chira et al. 2011) associated red wine astringency with tannin concentrations as well as the vintage of the wines. Accordingly, the astringency of the 2018 wine was expected to be higher than that of the 2016 wine, and the wines were expected to decrease in astringency during forced aging; neither of which was actually observed (Table 2). This indicates that astringency is not only influenced by tannin concentrations but also by structural and compositional differences (Gawel 1998) like the degree of polymerization (Chira et al. 2012) and the composition of tannin sub-units, in particular their degree of galloylation and trihydroxylation on the B-ring (Vidal et al. 2003). According to Vidal et al. (2003) roughness of astringency increases with proceeding galloylation and decreases with the number of epigallocatechin subunits. To compare tannin concentration in the wines and extracts, the values obtained for the extracts were referenced to the corresponding volume of the wine considering the respective yield (Table 3). In contrast to the results obtained for the wines,

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significantly higher tannin concentrations and no significant changes of tannin concentrations were found in the XAD7 extracts of the corresponding wines. These differences may be explained by interactions of the tannins with wine polysaccharides that are eliminated by the extraction procedure. The polysaccharides can form complexes with the tannins leading to an impaired precipitability with the BSA (Mateus et al. 2004) used for tannin quantification which results in lower tannin readings. Since the differences in tannin concentrations between wines and extracts increased, these interactions appear to become more pronounced when the wine is subjected to forced aging probably due to structural changes of the tannins. Precipitable PPs can be regarded as pigmented tannins since they are part of the tannin fraction determined after precipitation with BSA. The results show increasing precipitable PP ratios combined with decreasing or constant tannin levels indicating a progressive incorporation of anthocyanins into tannin molecules. Sommer et al. (2016) investigated the haze formation in red wines when treated with carboxymethyl cellulose (CMC). They found that CMC forms haze with wine proteins rather than with tannins and proposed a protein-bridged reaction between anthocyanins and CMC that leads to their precipitation. Accordingly, the incorporation of anthocyanins into tannin molecules changes the interaction between tannin sub-units and polysaccharides as well as proteins camouflaging them from analysis. Polysaccharides may also interact directly with BSA (de Freitas et al. 2003), which is used for tannin precipitation and might be another reason for the underestimation of tannins in wine samples. Astringency perception is also affected by wine polysaccharides that interact with red wine tannins and salivary proteins (Vidal et al. 2004b , Watrelot et al. 2017). Panelists were only requested to rate the overall astringency intensity that was compared to the drying mouthfeel evoked by aluminum sulfate. Future research should look

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345 at the perception of different astringency sub-qualities to investigate whether the decrease of
346 astringency rather represents a change in sub-qualities towards a less harsh mouthfeel. These
347 results show that the tannin concentration may not be the only factor that should be considered for
348 an evaluation of astringency and the sensory quality of the wine in general. Weber et al. (2013)
349 showed that gel permeation chromatography fractions containing the highest number of polymeric
350 pigments and rather small tannin concentrations elicited the lowest astringency as well as green
351 and dry tannins intensity. A continuously increasing precipitable PP/tannin ratio of the wines may
352 have favored the perception of a softer astringency.

353 The mechanism of astringency perception is based on tannin-protein interactions leading to
354 insoluble precipitates, increasing friction and a loss of lubrication in the oral cavity (Baxter et al.
355 1997). Charlton et al. (2002) proposed a model for protein precipitation that is initially driven by
356 hydrophobic interactions between the proline residues of proline-rich proteins and the aromatic
357 flavonoid rings. These soluble aggregates are further stabilized through hydrogen bonding leading
358 to cross linked tannin-protein complexes and their precipitation, suggesting that hydrophilicity is
359 an important factor determining the astringency of distinct compounds.

360 The ratio of the concentration of lipophilic to hydrophilic compounds in the fractions is
361 reflected by the octanol water partitioning coefficient (K_{OW}). The generally higher anthocyanin
362 concentrations in fraction 2 of all samples raised the expectation to observe higher hydrophilicities
363 of this fraction compared with fraction 3. Since this was not the case, other compounds, like
364 polymeric pigments and tannins, apparently contribute more to the overall hydrophilicity of the
365 fractions. Hagerman et al. (1998) investigated the effect of growing tannin polymer lengths on the
366 precipitability and the K_{OW}. They stated that tannins with higher degrees of polymerization

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367 exhibited lower octanol water partitioning coefficients compared to their corresponding flavan-3-
368 ol subunits. Hence, a higher degree of polymerization results in higher hydrophilic properties and
369 precipitability. The hydrophobic character of fractions 1 is the result of the presence of monomeric
370 flavan-3-ols, oligomeric procyanidins as well as benzoic and hydroxycinnamic acids.

371 The leveling concentrations of non-precipitable PP in fractions 2 and 3 of the 2016 wine lead
372 to the assumption that the wines reached a maximum of non-precipitable PP which was already
373 reported by Merrell et al. (2018) and may have two explanations. First, the formation and
374 degradation processes of non-precipitable PP reached an equilibrium or, second, the formation of
375 polymeric pigments in the older red wine that was subjected to forced aging favors the
376 development of high molecular pigments that are not included into the non-precipitable PP
377 measurement. Harbertson et al. (2014) showed that precipitation with BSA increases with polymer
378 size of the tannins indicating that polymeric pigments that are resistant against SO_2 bleaching and
379 that are not precipitated with BSA include oligomeric anthocyanin adducts as well as
380 pyranoanthocyanins. The UHPLC-MS results show no considerable changes in the concentration
381 of pyranoanthocyanins and anthocyanin-flavanol dimers (Supplemental Table 2 and 3). Hence, the
382 protein precipitation assay indicates that anthocyanins are incorporated into existing polymeric
383 structures to form polymeric pigments rather than forming new oligomeric pigments that grow in
384 size. This is supported by earlier studies (Haslam 1980, Salas et al. 2003, Salas et al. 2004) that
385 demonstrated that direct adducts of tannins and anthocyanins are formed after the preceding acid-
386 catalyzed cleavage of procyanidins. The products formed during this reaction may still be regarded
387 as polymeric structures although they might be of lower molecular weight due to the breakdown
388 process.

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389 The decline of tannins in fraction 2 of the 2016 wine together with a rise of precipitable PP
390 results in the increase of hydrophilicity. This indicates that tannins initially found in fraction 2 of
391 the 2016 wine are rather small and, thus, non-polar and hydrophobic, whereas the proceeding
392 incorporation of anthocyanins during forced aging leads to more water soluble polymeric pigments
393 (Singleton and Trousdale 1992, Merrell et al. 2018). Since the tannin concentration of fraction 3
394 of the 2016 wine remains constant, the corresponding partitioning coefficients follow the
395 developments of precipitable PP, showing that fraction 3 of the 2016 wine contains large and polar
396 tannins that were progressively pigmented during storage. In the 2018 wines, tannin concentrations
397 in fractions 2 and 3 show no changes over time and accordingly, hydrophilicity seems to be
398 affected by the compositional changes of precipitable PP and non-precipitable PP. As the
399 determination of polymeric pigments is based on their absorption at 520 nm, the protein-
400 precipitation assay does not distinguish between polymers of different intramolecular
401 compositions (Weber et al. 2013). Hence, no conclusion can be drawn about the exact size of the
402 molecules and the proportion of anthocyanins incorporated. Weber et al. (2013) examined the
403 chemical composition of red wine polymers obtained by gel permeation chromatography that is
404 based on the separation of molecules due to their size and polarity. Combining several analytical
405 techniques, they stated that early eluting fractions were composed of large and less pigmented
406 polymers. Further retention on the column eluted polymers with decreasing molecular size and
407 increasing anthocyanin incorporation followed by less pigmented proanthocyanidin-like
408 oligomers. Together with the results of the present study, the changes in hydrophilicity as well as
409 the distribution of polymeric pigments between the fractions visualize the compositional
410 transformations of red wine polymers. The hydrophilicity of fraction 2 of the 2018 wine decreased

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411 during the first 3 weeks while the amount of precipitable PP increased. Because fraction 2 contains
412 less polar and smaller polymers compared to fraction 3, this suggests an increase in the amount of
413 smaller precipitable PP rather than an increase in the proportion of incorporated anthocyanins, i.e.
414 the degree of pigmentation.

415 In contrast, the increase in hydrophilicity after 6 weeks resulted from the increase in non-
416 precipitable PP or rather the augmented pigmentation of non-precipitable PP. The progressive
417 increase in hydrophilicity of fraction 3 of the 2018 wine is caused by the ongoing new formation
418 of larger precipitable PP or the continuous pigmentation of already existing large precipitable PP,
419 and the simultaneous decrease of smaller non-precipitable PP that are less pigmented.

420 The different sub-qualities of astringency perception are explained by the varying
421 manifestation of the physico-chemical interactions between tannins and proteins, which are
422 specific and dependent on the molecular weight, the 3D structure and the water-solubility of
423 tannins, that is, according to Haslam (1996), one of the main factors for tannin complexation
424 (Simon et al. 2003). Being of a certain size, polyphenols can act as multidentate ligands binding
425 more than one site of the protein (de Freitas and Mateus 2001) leading to the formation of protein-
426 tannin networks and eventually precipitation (Cala et al. 2010). The formation of such networks
427 and resulting astringent sensations were shown to be influenced by stereochemistry and
428 conformation of procyanidins, because intramolecular stacking hinders the development of
429 protein-tannin aggregates (Cala et al. 2010, Quijada-Morín et al. 2012). An earlier study (McRae
430 et al. 2010) showed that the interactions between red wine tannins and a prolin-rich peptide
431 changed with wine age towards less pronounced hydrophobic interactions. The authors attribute
432 this to the change of tannin structures, like the incorporation of anthocyanins.

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In 2013, McRae et al. showed that tannins obtained by liquid liquid extraction with butanol were smaller in size, more hydrophobic and comprise more red pigments than the aqueous fractions, which was inversely correlated with the perceived astringency. The findings of McRae et al. (2013), the results published by Weber et al. (2013), and the results of the present study argue for the concept of pigmented tannins being less astringent than non-pigmented tannins. Accordingly, a higher degree of pigmentation is not necessarily resulting in lower hydrophobicity since other structural features also contribute to the overall hydrophobicity of the tannins. The higher hydrophobicity of the butanol tannins may be due to a greater oxidation and an increased amount of intramolecular bonds possibly leading to a reduced number of binding sites, hence, a reduced astringency (McRae et al. 2013). The interim decline of astringency of the 2018 wine stored for 3 weeks might be the consequence of the considerably higher non-precipitable PP in fraction 2 and the increase in hydrophopbicity of this fraction at this point of forced aging, while the further alterations of the tannins lead to an increase in astringency after 6 weeks of storage.

Finding tannins, PP in both, fraction 2 and fraction 3, indicates that not only the size of these polymers is important for their protein precipitability. It is affected by the physicochemical properties, which in turn depend on the size of tannin molecules and the ratio of incorporated anthocyanins, among others. However, it has still to be investigated how the elongation of polymers by anthocyanins as well as flavanols influences the protein precipitability.

Conclusion

The present results reveal that a wide structural variety of pigments can be found within the classification of polymeric pigments into two categories. This variety is based on the differences

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454 of sub-units as well as chain length and ratio of incorporated anthocyanins and leads to polymers
455 of different physicochemical properties that can be visualized by the octanol-water partitioning
456 coefficient and the FLASH fractionation. The change of polarity of polymeric pigments in turn
457 alters their ability to interact with wine polysaccharides and saliva proteins. Since the presumed
458 proceeding incorporation of anthocyanins into tannin molecules, which can be assumed by the
459 presented increase in precipitable PP, appears to reduce the measurability of precipitable tannins
460 during forced aging, a special role may be assigned to the interactions of precipitable PP with
461 polysaccharides and proteins. The formation of precipitable PPs during forced red wine aging and
462 their putative enhanced interactions with wine polysaccharides obviously play a key role in the
463 perception of red wine astringency. In particular, the perception of different sub-qualities of
464 astringency seems to be related to the proportion of precipitable PP and polysaccharides, which
465 needs to be addressed in the course of continuing research.

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Table 1 CIELab parameters of Cabernet Sauvignon wines and silica gel fractions at the various stages of storage at 35°C.

Sample		Wine		Fraction 1		Fraction 2		Fraction 3	
	weeks	h°	C*	h°	C*	h°	C*	h°	C*
2016	0	14.97	29.12	69.82	15.21	36.23	53.21	40.39	45.44
	3	15.88	23.94	72.84	15.96	37.36	53.08	40.67	47.99
	6	16.13	24.62	72.84	13.80	37.81	52.22	42.75	46.86
2018	0	20.17	38.88	70.18	14.52	27.36	50.47	32.56	47.08
	3	17.6	30.1	71.16	13.09	28.44	51.15	35.19	41.75
	6	18.07	30.54	71.49	13.60	29.54	51.33	37.25	42.29

Table 2 Astringency ratings (left) of Cabernet Sauvignon wines at the various stages of storage at 35°C (means presented with standard deviation; n = 14;). Means within columns and between tannin concentrations having the same letters are not significantly different at $p \leq 0.05$. Tannin concentrations of the wines and the corresponding extracts (right); means presented with standard deviation; n = 3; Concentrations with different capital letters are significantly different between the wines and the extracts ($p \leq 0.05$).

Sample	Weeks	Wine		Extract
		Astringency	Tannins (mg/L CE)	Tannins (mg/L CE)
2016	0	6.52 ± 1.50 a	692.05 ± 3.24 B	732.72 ± 6.67 A
	3	6.27 ± 1.86 ab	669.48 ± 20.68 BC	729.49 ± 11.56 A
	6	5.46 ± 2.48 ab	654.98 ± 12.39 C	727.63 ± 7.38 A
2018	0	5.50 ± 2.13 ab	607.66 ± 4.15 D	587.15 ± 2.12 D
	3	3.56 ± 1.09 c	535.39 ± 18.45 E	585.92 ± 5.29 D
	6	4.53 ± 1.47 bc	508.49 ± 8.64 E	605.81 ± 2.61 D

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Table 3 Yields and proportions (in parentheses) of silica gel chromatography fractions of Cabernet Sauvignon XAD7 extracts after storage at 35°C (means presented with standard deviation; n = 6-8).

Sample	**2016**			**2018**		
	Yield (mg/g) (Proportion (%))					
Weeks	0	3	6	0	3	6
F1	130.2 ± 29.4 (21.0 ± 4.7)	162.2 ± 0.8 (25.1 ± 0.2)	162.1 ± 3.7 (24.9 ± 0.6)	154.0 ± 45.1 (24.8 ± 7.2)	158.7 ± 39.7 (23.4 ± 5.9)	145.9 ± 44.5 (21.7 ± 6.6)
F2	396.8 ± 6.8 (64.0 ± 1.2)	368.4 ± 31.6 (57.0 ± 4.5)	378.7 ± 12.4 (58.1 ± 1.9)	421.2 ± 29.1 (67.7 ± 4.7)	450.4 ± 2.3 (66.4 ± 0.3)	451.1 ± 71.2 (67.1 ± 10.6)
F3	93.4 ± 10.8 (15.1 ± 3.8)	114.6 ± 39.9 (17.7 ± 5.8)	112.6 ± 8.4 (17.3 ± 1.3)	48.3 ± 17.8 (7.8 ± 2.9)	70.1 ± 19.4 (10.3 ± 2.9)	75.7 ± 20.7 (11.3 ± 3.1)

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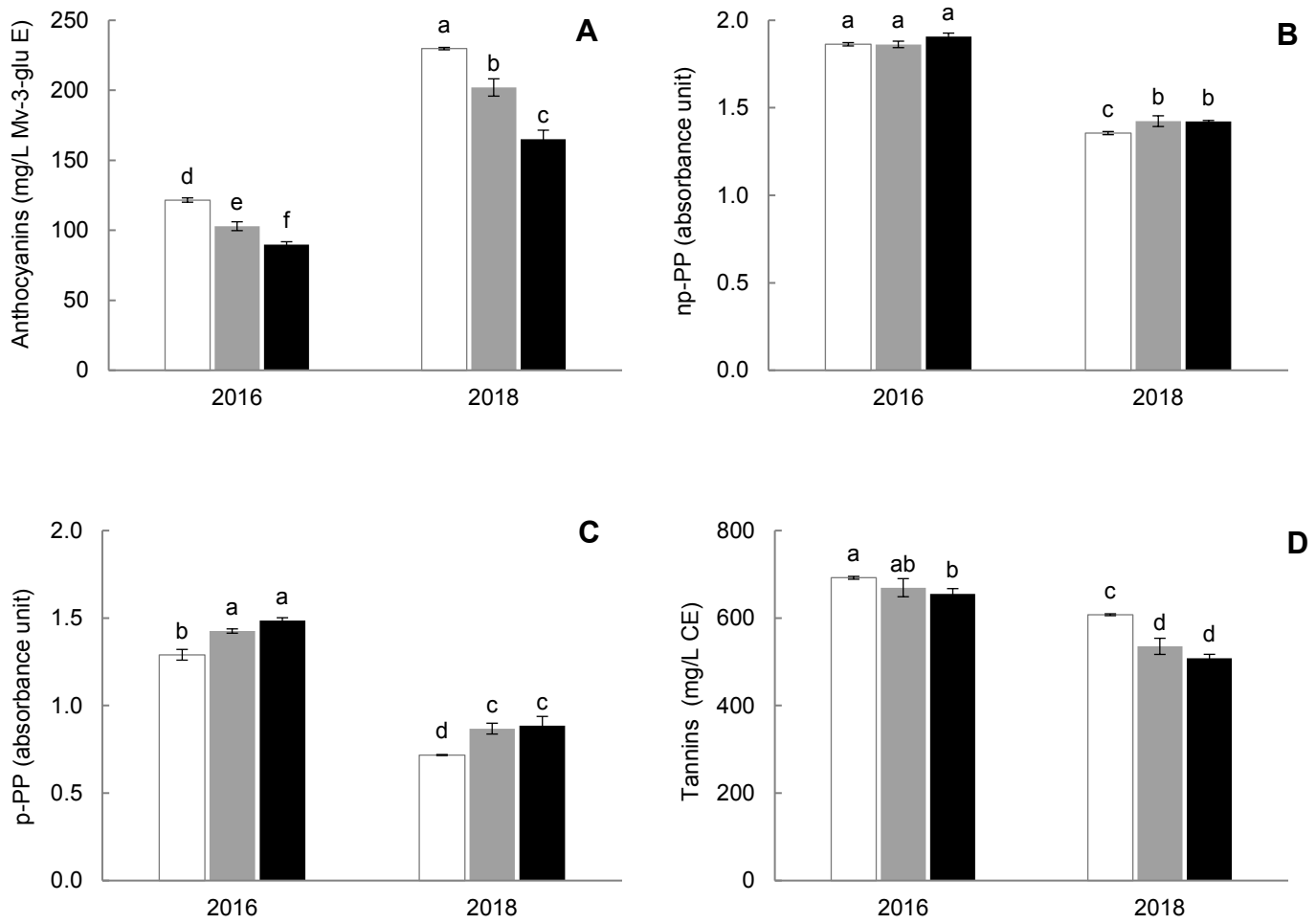

Figure 1 Phenolic composition including total anthocyanins (**A**), non-precipitable polymeric pigments (np-PP; **B**), precipitable polymeric pigments (p-PP; **C**), and total tannins (**D**) of Cabernet Sauvignon wines at the various stages of storage at 35°C: no storage (□), 3 weeks (▒), and 6 weeks (■). Results obtained by photometric assays (Harbertson et al. 2002, 2003, 2009, 2015). Means presented with standard deviation; n = 3. Means having the same letters are not significantly different at $p \leq 0.05$.

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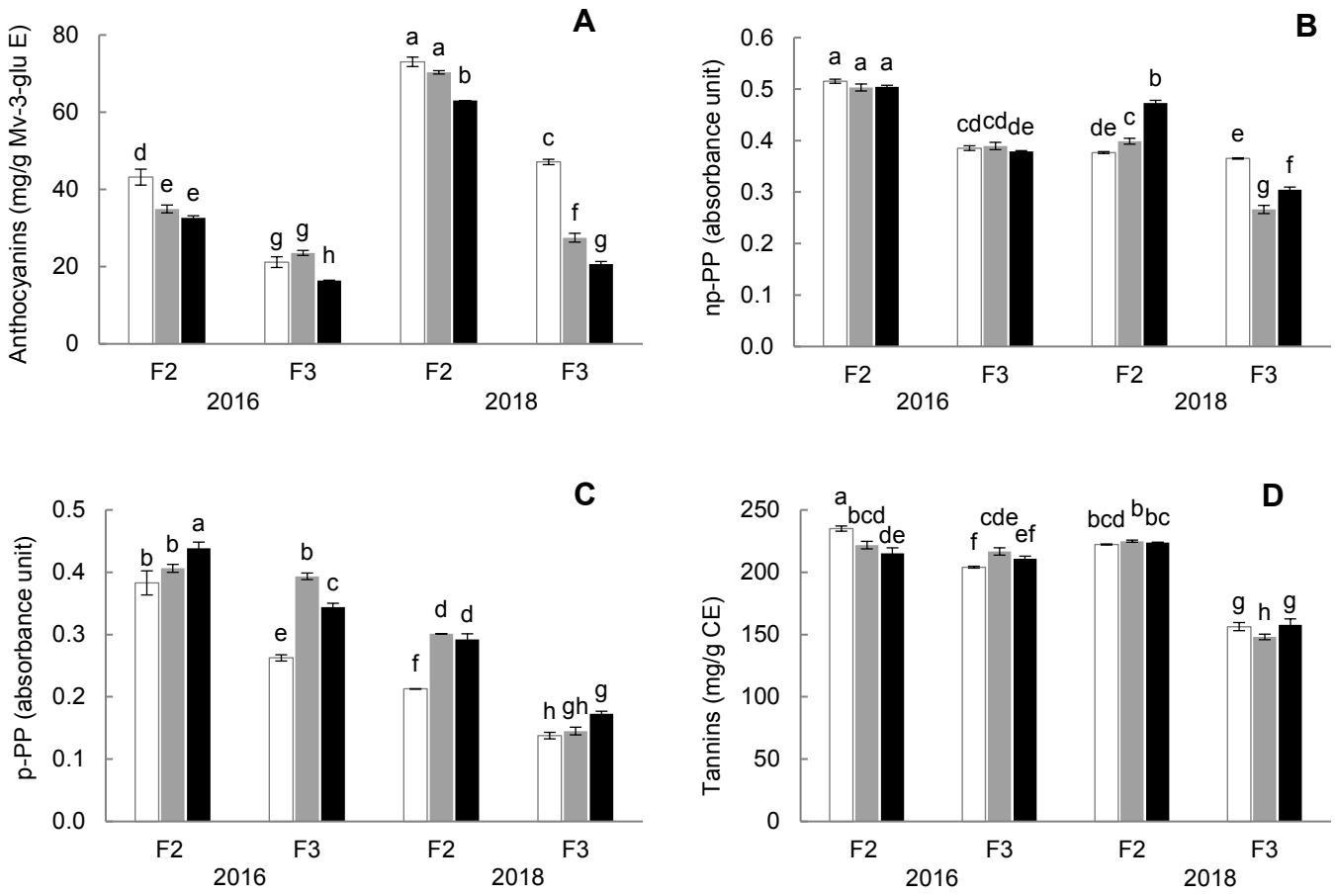

Figure 2 Phenolic composition including total anthocyanins (**A**), non-precipitable polymeric pigments (np-PP; **B**), precipitable polymeric pigments (p-PP; **C**), and total tannins (**D**) of silica gel chromatography fraction 2 (F2) and fraction 3 (F3) of Cabernet Sauvignon XAD7 extracts at the various stages of storage at 35°C: no storage (□), 3 weeks (▒), and 6 weeks (■). Restults obtained by photometric assays (Harbertson et al. 2002, 2003, 2009, 2015). Means presented with standard deviation; n = 3. Means having the same letters are not significantly different at $p \leq 0.05$.

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Figure 3

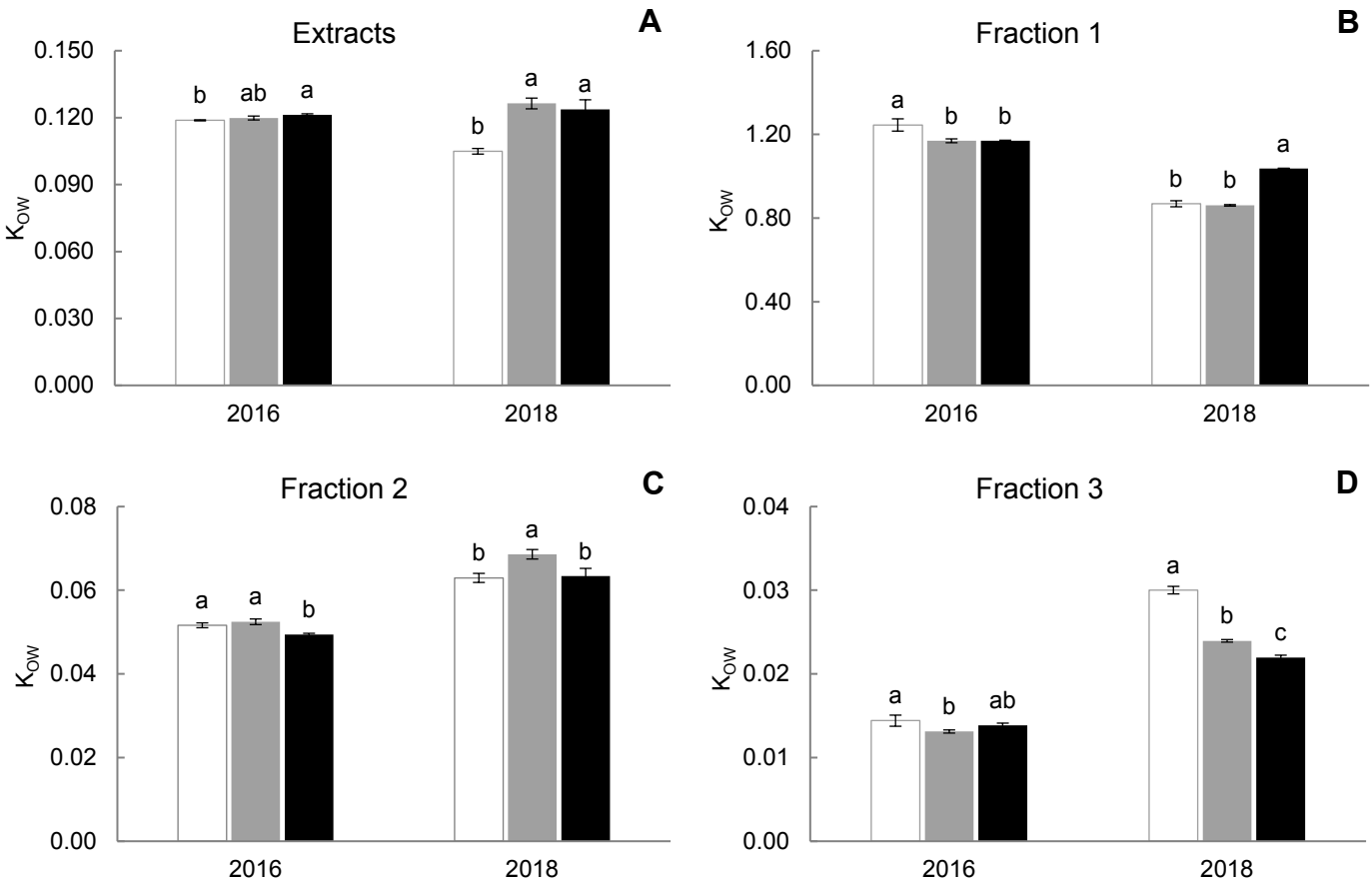

Figure 3 Octanol water partitioning coefficients (K_{OW}) of XAD7 extracts (**A**), and silica gel chromatography fraction 1 (**B**), fraction 2 (**C**), and fraction 3 (**D**) of Cabernet Sauvignon wines at the various stages of storage at 35°C: no storage (□), 3 weeks (▒), and 6 weeks (■). Means presented with standard deviation; n = 3. Means within columns having the same letters are not significantly different at $p \leq 0.05$.

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Supplemental Table 1 Fourway-ANOVA of the astringency rating including vintage, storage, panelist, and replicate of the tasting showing that the vintage of the wines and the panelist have a significant impact on the astringency rating at $p \leq 0.05$.

Source	Degrees of freedom	Sum of squares	Mean of squares	F-value	p-value
Vintage	1	48.747	48.747	12.861	0.000
Storage	2	20.859	10.430	2.752	0.068
Panelist	13	100.143	7.703	2.032	0.023
Replicate	1	0.547	0.547	0.144	0.705

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Supplemental Table 2 Heat map of the low molecular phenolic composition of 2016 Cabernet Sauvignon wine fractions at the various stages of storage at 35°C determined with UHPLC-MS/MS. Means presented with mean standard deviation (mSD) for substance classes, n = 3.

Substance (mg/g)	**Fraction 1**			**Fraction 2**			**Fraction 3**		
weeks	0	3	6	0	3	6	0	3	6
Anthocyanins (±0.06 mSD)									
Delphinidin-3-glucoside	n.d.	n.d.	n.d.	0.39	0.36	0.27	0.62	0.44	0.41
Cyanidin-3-glucoside	n.d.	n.d.	n.d.	0.07	0.06	0.05	0.06	0.03	0.03
Petunidin-3-glucoside	n.d.	n.d.	n.d.	0.92	0.78	0.62	0.73	0.56	0.50
peonidin-3-glucoside	n.d.	n.d.	n.d.	0.89	0.68	0.61	0.22	0.22	0.15
Malvidin-3-glucoside	n.d.	n.d.	n.d.	13.06	9.46	9.00	4.16	4.05	2.79
Delphinidin-3-(6-acetyl)glucoside	n.d.	n.d.	n.d.	0.09	0.09	0.06	0.18	0.12	0.11
Petunidin-3-O-(6-O-acetyl)glucoside	n.d.	n.d.	n.d.	0.28	0.23	0.18	0.23	0.16	0.14
Malvidin Formiat	n.d.	n.d.	n.d.	0.28	0.26	0.26	0.12	0.08	0.06
Peonidin 3-O-acetylglucoside	n.d.	n.d.	n.d.	0.40	0.28	0.24	0.07	0.07	0.04
Delphinidin-3-(p-coumaroyl)glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Malvidin-3-O-(6-O-acetyl)glucoside	n.d.	n.d.	n.d.	5.43	3.95	3.36	1.23	1.15	0.80
Petunidin-3-(p-coumaroyl)glucoside cis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	n.d.	n.d.
Petunidin-3-(p-coumaroyl)glucoside trans	n.d.	n.d.	n.d.	0.06	0.05	0.04	n.d.	n.d.	n.d.
Malvidin-3-O-(6-O-p-coumaroyl)glucoside cis	n.d.	n.d.	n.d.	0.08	0.05	0.05	n.d.	n.d.	n.d.
Peonidin-3-(6"-p-coumaroylglucoside)	n.d.	n.d.	n.d.	0.14	0.10	0.09	n.d.	n.d.	n.d.
Malvidin-3-O-(6-O-p-coumaroyl)glucoside trans	n.d.	n.d.	n.d.	1.16	0.83	0.76	0.22	0.22	0.17
Pyranoanthocyanins (±0.01 mSD)									
Petunidin-3-glucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.05	0.05	0.05	0.04	0.04	0.04
Peonidin-3-glucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.05	0.05	0.05	n.d.	n.d.	n.d.
Malvidin-3-O-glucosid pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.23	0.20	0.20	0.10	0.11	0.11

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Malvidin-3-O-acetyglucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.16	0.15	0.16	n.d.	n.d.	n.d.
Malvidin-3-glucoside-vinyl-catechin	n.d.	n.d.	n.d.	0.06	0.05	0.06	n.d.	n.d.	n.d.
Mv-3-glc-4-vinylcatechol (Pinotin)	n.d.	n.d.	n.d.	0.50	0.49	0.59	0.23	0.28	0.29
Malvidin-3-glucoside-vinyl-epicatechin	n.d.	n.d.	n.d.	0.09	0.08	0.09	n.d.	n.d.	n.d.
Malvidin-3-glucoside-4-vinylphenol (Pinotin)	n.d.	n.d.	n.d.	0.53	0.84	0.55	0.13	0.32	0.23
Anthocyanin flavanol adducts (±0.01 mSD)									
Malvidin-3-glucoside-gallocatechin	n.d.	n.d.	n.d.	0.23	0.21	0.20	0.06	0.05	0.04
Peonidin-3-glucoside-(epi)catechin	n.d.	n.d.	n.d.	0.08	0.08	0.07	n.d.	n.d.	n.d.
Malvidin-glucoside-(epi)catechin	n.d.	n.d.	n.d.	0.75	0.73	0.68	0.17	0.19	0.15
Malvedin-acetylglucoside-(epi)catechin	n.d.	n.d.	n.d.	0.13	0.12	0.11	n.d.	n.d.	n.d.
Malvidin-coumaroylglucoside-(epi)catechin	n.d.	n.d.	n.d.	0.06	0.06	0.05	n.d.	n.d.	n.d.
Flavanols (±0.87 mSD)									
Catechingallat	1.89	2.02	2.61	0.98	0.70	0.48	0.52	0.45	0.30
(-)-Gallocatechin	7.57	6.08	9.67	0.71	0.92	0.64	0.18	0.15	0.15
Epicatechingallat	1.29	1.13	1.63	0.70	0.43	0.34	n.d.	n.d.	n.d.
(-)-Epigallocatechin	2.57	1.89	2.75	0.16	0.18	0.12	0.05	0.04	0.04
Catechin	48.55	38.99	48.28	3.31	4.11	2.47	1.02	0.96	0.85
Epicatechin	33.78	23.75	28.66	1.80	2.28	1.54	0.72	0.65	0.62
Proanthocyanidins (±0.30 mSD)									
Flavanol trimer	0.60	0.50	0.58	0.42	0.18	0.15	0.37	0.32	0.29
Flavanol dimer	12.59	11.04	14.65	3.60	2.59	1.94	0.99	0.96	0.78
Flavanol dimer	4.04	3.19	4.47	0.54	0.38	0.23	0.05	0.07	0.06
Flavanol trimer	1.31	1.31	1.86	0.71	0.44	0.35	0.09	0.08	0.07
Flavanol trimer	0.99	1.10	1.61	0.43	0.30	0.22	0.03	0.03	0.03
Flavanol dimer	2.74	1.93	2.33	0.23	0.17	0.11	n.d.	n.d.	n.d.
Flavanol trimer	0.85	0.90	1.25	0.42	0.25	0.20	n.d.	n.d.	n.d.

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Flavanol dimer	13.85	10.12	13.80	2.23	1.59	1.17	0.58	0.56	0.46
Flavanol dimer gallat	0.07	0.18	0.23	0.16	0.15	0.13	n.d.	n.d.	n.d.
Flavanol dimer gallat	0.02	0.06	0.09	0.09	0.09	0.07	n.d.	n.d.	n.d.
Flavanol trimer	1.69	1.54	2.08	0.52	0.32	0.22	0.07	0.06	0.05
Flavonols (±0.23 mSD)									
Dihydromyricetin-3-rhamnoside	0.10	0.14	0.19	0.17	0.15	0.12	n.d.	n.d.	n.d.
Myricetin-3-glucuronide	0.13	0.18	0.18	2.00	1.98	2.00	n.d.	n.d.	n.d.
Quercetin-3-O-glucuronide	0.91	1.23	1.19	4.99	3.95	4.31	0.60	0.50	0.57
Laricitrin-3-galactoside	n.d.	n.d.	n.d.	0.22	0.14	0.13	n.d.	n.d.	n.d.
Syringetin-3-glucoside	0.03	0.05	0.05	3.01	2.16	2.04	0.46	0.41	0.44
Benzoic acids (±1.49 mSD)									
Gallic acid	62.69	53.79	70.25	4.05	4.13	3.77	0.55	0.61	0.85
Vanillic acid	1.81	1.31	1.62	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hydroxycinnamic acids (±0.35 mSD)									
Cis-Caftaric acid	0.97	1.38	1.16	0.29	0.20	0.15	n.d.	n.d.	n.d.
Cis-Caffeic acid	6.16	5.17	5.77	2.42	2.03	1.89	n.d.	n.d.	n.d.
Trans-Caftaric acid	6.92	5.73	6.74	2.34	1.85	1.81	0.12	0.12	0.14
Hydroxy-caffeic acid dimer isomer	1.73	1.56	1.39	0.68	0.59	0.61	n.d.	n.d.	n.d.
Ferulic acid	0.85	0.55	0.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cis-Coutaric acid	1.94	1.52	1.74	0.43	0.33	0.30	n.d.	n.d.	n.d.
p-Coumaric acid	13.64	11.81	13.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
trans-Coutaric acid	7.00	5.41	8.06	1.09	0.88	0.80	0.06	0.06	0.06
Trans-Caffeic acid	18.10	13.51	17.13	0.26	0.26	0.20	n.d.	n.d.	n.d.
cis-Ethylcaffeic acid	2.72	2.25	2.28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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Supplemental Table 3 Heat map of the low molecular phenolic composition of 2018 Cabernet Sauvignon wine fractions at the various stages of storage at 35°C determined with UHPLC-MS/MS. Means presented with mean standard deviation (mSD) for substance classes, n = 3.

Substance (mg/g)	**Fraction 1**			**Fraction 2**			**Fraction 3**		
weeks	0	3	6	0	3	6	0	3	6
Anthocyanins (±0.06 mSD)									
Delphinidin-3-glucoside	n.d.	n.d.	n.d.	0.62	0.54	0.44	2.73	1.09	0.94
Cyanidin-3-glucoside	n.d.	n.d.	n.d.	0.13	0.11	0.09	0.32	0.12	0.08
Petunidin-3-glucoside	n.d.	n.d.	n.d.	2.20	1.81	1.49	5.12	1.92	1.41
peonidin-3-glucoside	n.d.	0.05	n.d.	3.66	2.82	2.29	1.14	0.66	0.57
Malvidin-3-glucoside	0.06	0.37	0.03	30.00	26.71	22.49	12.78	8.34	7.99
Delphinidin-3-(6-acetyl)glucoside	n.d.	n.d.	n.d.	0.10	0.08	0.07	0.55	0.21	0.19
Petunidin-3-O-(6-O-acetyl)glucoside	n.d.	n.d.	n.d.	0.45	0.36	0.30	1.06	0.42	0.30
Malvidin Formiat	n.d.	n.d.	n.d.	0.82	1.00	0.65	0.23	0.15	0.13
Peonidin 3-O-acetylglucoside	0.03	0.04	n.d.	1.23	0.95	0.75	0.32	0.20	0.15
Delphinidin-3-(p-coumaroyl)glucoside	n.d.	n.d.	n.d.	0.13	0.11	0.09	0.42	0.19	0.12
Malvidin-3-O-(6-O-acetyl)glucoside	0.20	0.31	0.17	13.69	11.25	9.36	3.73	2.83	2.18
Petunidin-3-(p-coumaroyl)glucoside cis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.67	0.29	0.17
Petunidin-3-(p-coumaroyl)glucoside trans	n.d.	n.d.	n.d.	0.44	0.33	0.27	n.d.	n.d.	n.d.
Malvidin-3-O-(6-O-p-coumaroyl)glucoside cis	n.d.	n.d.	n.d.	0.59	0.39	0.29	0.13	0.10	0.06
Peonidin-3-(6"-p-coumaroylglucoside)	n.d.	n.d.	n.d.	0.89	0.77	0.58	0.20	0.15	0.10
Malvidin-3-O-(6-O-p-coumaroyl)glucoside trans	n.d.	n.d.	n.d.	6.99	5.74	4.56	1.50	1.21	0.88
Pyranoanthocyanins (±0.01 mSD)									
Petunidin-3-glucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.04	0.04	0.03	0.13	0.04	0.03
Peonidin-3-glucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.04	0.05	0.04	n.d.	n.d.	n.d.
Malvidin-3-O-glucosid pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.22	0.19	0.19	0.09	0.09	0.08

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Malvidin-3-O-acetyglucoside pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.15	0.15	0.14	n.d.	n.d.	n.d.
Malvidin-3-glucoside-vinyl-catechin	n.d.	n.d.	n.d.	0.05	0.05	0.05	n.d.	n.d.	n.d.
Mv-3-glc-4-vinylcatechol (Pinotin)	n.d.	n.d.	n.d.	0.09	0.15	0.18	0.19	0.11	0.09
Malvidin-3-glucoside-vinyl-epicatechin	n.d.	n.d.	n.d.	0.08	0.08	0.07	n.d.	n.d.	n.d.
Malvidin-3-glucoside-4-vinylphenol (Pinotin)	n.d.	n.d.	n.d.	0.69	1.15	0.75	0.47	0.15	0.20
Anthocyanin flavanol adducts (±0.01 mSD)									
Malvidin-3-glucoside-gallocatechin	n.d.	n.d.	n.d.	0.28	0.32	0.29	0.11	0.07	0.06
Peonidin-3-glucoside-(epi)catechin	n.d.	n.d.	n.d.	0.14	0.16	0.14	0.07	0.04	0.04
Malvidin-glucoside-(epi)catechin	n.d.	n.d.	n.d.	0.86	1.07	0.91	0.36	0.24	0.25
Malvedin-acetylglucoside-(epi)catechin	n.d.	n.d.	n.d.	0.19	0.21	0.18	0.05	0.04	0.04
Malvidin-coumaroylglucoside-(epi)catechin	n.d.	n.d.	n.d.	0.16	0.20	0.17	0.05	0.04	0.04
Flavanols (±0.87 mSD)									
Catechingallat	3.26	3.27	3.26	1.55	1.44	1.05	0.47	0.97	0.71
(-)-Gallocatechin	9.01	6.67	8.16	0.94	1.01	0.70	0.23	0.14	0.13
Epicatechingallat	2.38	2.03	1.87	1.07	0.99	0.67	n.d.	n.d.	n.d.
(-)-Epigallocatechin	2.79	2.55	2.69	0.18	0.21	0.15	0.06	0.04	0.04
Catechin	56.66	51.39	55.98	3.87	4.33	3.59	1.32	0.90	0.86
Epicatechin	47.43	36.74	39.62	2.62	2.45	1.90	1.12	0.67	0.64
Proanthocyanidins (±0.30 mSD)									
Flavanol trimer	1.32	1.41	1.04	0.87	0.83	0.44	0.35	0.37	0.38
Flavanol dimer	20.22	18.91	18.54	6.49	6.96	4.43	1.98	1.01	1.04
Flavanol dimer	6.97	5.98	5.32	1.18	1.13	0.67	0.13	0.06	0.06
Flavanol trimer	3.36	2.87	2.81	1.50	1.31	0.87	0.22	0.09	0.09
Flavanol trimer	2.47	2.27	2.06	0.87	0.81	0.65	0.08	0.05	0.04
Flavanol dimer	5.45	3.90	3.98	0.62	0.54	0.29	0.08	0.06	0.07
Flavanol trimer	1.74	1.56	1.51	0.67	0.64	0.52	n.d.	n.d.	n.d.

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Flavanol dimer	19.64	17.54	17.33	4.44	3.86	2.65	1.18	0.64	0.60
Flavanol dimer gallat	0.25	0.44	0.39	0.34	0.37	0.30	0.06	0.03	0.03
Flavanol dimer gallat	0.10	0.19	0.16	0.18	0.20	0.18	n.d.	n.d.	n.d.
Flavanol trimer	3.91	3.35	3.22	1.28	1.04	0.69	0.20	0.08	0.08
Flavonols (±0.23 mSD)									
Dihydromyricetin-3-rhamnoside	0.05	0.07	0.07	0.09	0.08	0.06	n.d.	n.d.	n.d.
Myricetin-3-glucuronide	0.30	0.43	0.26	2.70	2.18	2.03	n.d.	n.d.	n.d.
Quercetin-3-O-glucuronide	11.28	11.22	8.32	25.36	22.56	19.87	6.31	4.86	3.98
Laricitrin-3-galactoside	0.03	0.14	0.03	0.90	0.79	0.62	0.20	0.12	0.10
Syringetin-3-glucoside	0.08	0.57	0.10	3.64	3.49	2.80	0.85	0.68	0.55
Benzoic acids (±1.49 mSD)									
Gallic acid	67.91	65.59	66.14	4.68	3.96	3.12	1.85	0.63	0.74
Vanillic acid	3.05	3.19	3.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hydroxycinnamic acids (±0.35 mSD)									
Cis-Caftaric acid	2.70	1.16	0.62	0.54	0.41	0.18	0.04	0.08	0.04
Cis-Caffeic acid	8.85	9.32	10.49	2.55	2.73	2.15	n.d.	n.d.	n.d.
Trans-Caftaric acid	10.72	10.68	11.94	2.90	2.90	2.34	0.47	0.22	0.22
Hydroxy-caffeic acid dimer isomer	1.55	1.90	1.65	0.46	0.59	0.43	n.d.	n.d.	n.d.
Ferulic acid	0.95	0.95	0.99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cis-Coutaric acid	3.33	2.72	2.68	0.65	0.64	0.38	0.08	0.04	0.04
p-Coumaric acid	14.11	14.46	14.75	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
trans-Coutaric acid	9.27	7.73	8.93	1.10	1.05	0.75	0.15	0.07	0.07
Trans-Caffeic acid	15.89	15.10	15.00	0.16	0.09	0.08	n.d.	n.d.	n.d.
cis-Ethylcaffeic acid	2.33	2.44	2.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.