Composition and astringency of Cabernet Sauvignon red wines: Implications of polyphenol-polysaccharide interactions

Dissertation

zur Erlangung des Doktorgrades (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

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aus

Fürstenfeldbruck

Bonn

Mai 2024

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

Gutachter/Betreuer: Prof. Dr. Andreas Schieber

Gutachter: Prof. Dr. Matthias Wüst

Tag der Promotion: 15.07.2024

Erscheinungsjahr: 2024

“We especially need imagination in science. It is not all mathematics, nor all logic, but is somewhat beauty and poetry.”

Maria Mitchell

Abstract

Red wine quality is based on organoleptic properties like color, color stability, and mouthfeel, among others. The specifications for these attributes vary depending on cultivar, wine style, and personal taste. Ideally, red wines should come with a well-balanced taste and mouthfeel without any unpleasant spikes of acidity, bitterness, or astringency.

The role of red wine polyphenols in these characteristics is well known, with anthocyanins providing color for young red wines, polymeric pigments stabilizing the color in the long term, and condensed tannins eliciting the mouthfeel, more precisely the astringency. However, due to the complex mechanisms behind the astringency perception and difficulties to characterize polymeric flavonoids, the exact factors influencing astringency are not yet fully understood. Furthermore, information on the impact of grape-derived pectic polysaccharides on red wine astringency is limited.

Therefore, the aims of this thesis were to develop a new approach to further characterize condensed tannins and polymeric pigments, their structural changes during red wine aging, and to examine the implications for changes in the astringency perception. Furthermore, the influence of pectic polysaccharides on the condensed tannin and polymeric pigment composition was investigated to gain knowledge about their role in the precipitability of these polymers and ultimately the astringency.

The determination of the polarity and hydrophobicity of condensed tannins and polymeric pigments visualized the complex structural transformations these polymers undergo during red wine aging. Medium sized and hydrophilic, pigmented tannins seemed to be a factor in the attenuation of astringency, which may be attributed to their higher density of positively charged flavylium cations and enhanced interactions with dissociated pectin fragments. The polymerization of these molecules resulted in their increased polarity and hydrophilicity, possibly strengthening the interactions with salivary proteins, which resulted in higher astringency ratings.

In general, the formation of polymeric pigments correlated with an age-related decrease in astringency, highlighting their role in the attenuation of astringency. However, depending on the composition of wine pectic polysaccharides, polymeric pigments did not comprise of conventional tannins that incorporated anthocyanins but rather of pigmented polysaccharide-polyphenol aggregates. Therefore, this thesis revealed that pectic polysaccharides could significantly alter the polyphenolic profile of red wines. Furthermore, they were unambiguously connected with astringency-related mouthfeel attributes, emphasizing their importance for red wine quality.

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Preliminary remarks

List of abbreviations

AG	Arabinogalactan
AGP	Arabinogalactan-protein
APS	Acidic polysaccharides
BSA	Bovine serum albumin
CW	Cell wall
DA	Degree of acetylation
DAD	Diode array detector
DM	Degree of methylation
ESI	Electrospray ionization
Fuc	L-Fucose
GalAc	α-D-Galacturonic acid
HG	Homogalacturonan
K_{ow}	Octanol-water partitioning coefficient
LPP	Large polymeric pigments
MCP	Methyl cellulose precipitation
mDP	Mean degree of polymerization
MLF	Malolactic fermentation
MS	Mass spectrometry
MW	Molecular weight
np-PP	Non-precipitable polymeric pigments
NPS	Neutral polysaccharides
PG	Polygalacturonase
PME	Pectin methylesterase
p-PP	Precipitable polymeric pigments
PRAGs	Polysaccharides rich in arabinose and galactose
PRP	Proline-rich proteins
Rha	L-Rhamnose
RG-I/II	Rhamnogalacturonan I/II
SEC	Size exclusion chromatography
SPE	Solid phase extraction
SPP	Small polymeric pigments
TA	Titratable acidity
TSP	Total soluble polysaccharides
UHPLC	Ultra high-performance liquid chromatography

List of publications

Weilack, I., Schmitz, C., Harbertson, J. F., & Weber, F. (2021). Effect of structural transformations on precipitability and polarity of red wine phenolic polymers. *American Journal of Enology and Viticulture*, 72(3), 230–239.
DOI: 10.5344/ajev.2021.20064

Hensen, J.-P., Hoening, F., Weilack, I., Damm, S., & Weber, F. (2022). Influence of grape cell wall polysaccharides on the extraction of polyphenols during fermentation in microvinifications. *Journal of Agricultural and Food Chemistry*, 70(29), 9117–9131.
DOI: 10.1021/acs.jafc.2c02697

Feifel, S., Hensen, J.-P., Weilack, I., Weber, F., Wegmann-Herr, P., & Durner, D. (2023). Impact of climate change on grape cluster structure, grape constituents, and processability. *BIO Web of Conferences*, 56, 01016.
DOI: 10.1051/bioconf/20235601016

Weilack, I., Mehren, L., Schieber, A., & Weber, F. (2023). Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines. *Current Research in Food Science*, 6, 100506.
DOI: 10.1016/j.crfs.2023.100506

Feifel, S., Weilack, I., Markusevics, E., Zimmermann, D., Wegmann-Herr, P., Weber, F., Richling, E., & Durner, D. (2024). Influence of potential alcohol in grapes on phenolic and sensory characteristics of red wine. *Journal of Agricultural and Food Chemistry*, 72(22), 12725–12737.
DOI: 10.1021/acs.jafc.4c01035

Weilack, I., Mehren, L., & Weber, F. (2024). Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines. *Food Hydrocolloids*, 157, 110402.
DOI: 10.1016/j.foodhyd.2024.110402

Conferences

Weilack, I., Schmitz, C. & Weber, F. (2019). Novel approach for stable food colorants from red wine or winery by-products. 13th INSAH World Congress on Polyphenols Applications, Valletta, Malta, September 30 – October 1, 2019, *Abstracts Book*, p. 47. [Short oral presentation]

Weilack, I., Schmitz, C., Harbertson, J. F. & Weber, F. (2021). Effect of structural transformations on precipitability and polarity of red wine phenolic polymers. 72nd ASEV National Conference, online conference, June 21 – 24, 2021, *Technical Abstracts*, p. 10. [Oral presentation]

Weilack, I., Feifel, S., Wegmann-Herr, P., Durner, D. & Weber, F. (2022). Role of the tannin to anthocyanin ratio in the formation of polymeric pigments and its influence on red wine mouthfeel. 73rd ASEV National Conference, San Diego, California, USA, June 19 – 22, 2022, Technical Abstracts, p. 71. [Oral presentation]

Chapter 1

General introduction

1 Red wine

First indications of grape juice date back to around 6000 – 5000 BC and were found in potteries from the Caucasus region. Grape juice is easily fermented, making it very likely that these findings indicate the early consumption of wine from fortuitous vinification. Systematic viticulture and intentional wine production requires a certain degree of sedentism, which was probably not achieved until one or two thousand years later. Clear evidence of wine production dating back to around 3000 BC is provided by wine presses and press residues discovered in Egypt and Crete. Supported by its socio-religious function, the expertise in viticulture and winemaking was brought to Europe and eventually to the rest of the world (Robinson & Harding, 2006; Jackson, 2020).

Nowadays, 74.5 million tons of grapes are harvested globally, 34.1 million tons of which are used to produce 258 million hectoliters of wine (OIV, 2023). The Eurasian grape species *Vitis vinifera* L., which belongs to the *Vitaceae* family, is of highest economic importance. This family comprises of 14 genera with around 900 species, including the two *Vitis* sub-genera *Euvitis* and *Muscadinia.* While the Eurasian group of the sub-genus *Euvitis* only consists of one species, which is *Vitis vinifera*, the American and Asian groups include 30 to 50 species. Thereby, especially the American species have an important role in rootstock breeding, because most *Vitis vinifera* varieties are grafted onto North American rootstocks to increase the resistance against pests, diseases and varying soil conditions (Wen, 2007; Rahemi et al., 2022).

Grape cultivars can be classified according to their skin color – white and red. Grape skin color is the first determinant of the vinification process and therefore the style of the wine. The red cultivar Cabernet Sauvignon ranks as the most frequently cultivated wine variety (Anderson & Nelgen, 2020). Surprisingly, Cabernet Sauvignon is a hybrid of a red and a white cultivar, namely Cabernet Franc and Sauvignon Blanc (Bowers & Meredith, 1997), originating from Gironde in France before the mid eighteenth century. Cabernet Sauvignon grapes ripen slowly,

which is why they need temperate to hot climate to fully mature. The berries of this cultivar are small, acidic, and seedy. Their thick skins have a strong blueish, almost black pigmentation, which produce deeply colored, acidic, and tannic wines, making them very popular among consumers (Robinson et al., 2012; Jackson, 2020).

The annual vineyard and vine growth cycle starts with the planting of vineyards and bud break in early spring. Thereby, it takes three years for the newly planted vine to begin producing fruits. Flowering and fruit set, during which flowers transition to grape berries, take place in early summer followed by veraison. Veraison denotes the beginning of the berry ripening process when acidity declines, skin color changes from green to red, and sugar increases. Depending on grape variety and climatic conditions, it takes up to 70 days until the harvest of grape berries, which starts in late summer in hot climates and in autumn in cool climates. Once all the leaves have fallen, the vine goes into dormancy, allowing for pruning up to the next growing season (Robinson & Harding, 2006).

It does not take much more than the presence of wild yeasts to turn grape juice into wine. Grape berries will start self-fermentation when left unattended for some time, which results in the rupture of grape skins releasing grape juice. The skin's yeast flora will colonize the grape juice and turn sugar, which is easily accessible for yeasts, into ethanol. However, unless sugars were not fully fermented and oxygen supply was not reduced, spoilage yeasts and bacteria will promote the production of vinegar (Jackson, 2020).

Therefore, wine production methods were developed, continuously improved, and adapted to ensure wine stability and generate different wine styles. In general, the winemaking process starts with grape picking and destemming to minimize the extraction of polyphenols from the stems, which are associated with increased bitterness and greenness of the wines. This step is often accompanied by the crushing of grape berries to break down cell walls, releasing grape juice, and facilitate the fermentation process. Red wine making differs from that of white and rosé wines by undergoing a maceration step instead of immediate pressing to produce grape juice. This step involves leaving the must, which consists of grape juice and solids, to macerate. SO_2 is added to the must to stabilize the microbial flora and prevent spoilage. During maceration, red wine constituents like polyphenols and polysaccharides are extracted to the must, and by adding fermentation yeasts, the conversion of sugar to ethanol begins. Polyphenols are essential for red wine quality characteristics because they elicit color, color stability, taste, and astringency. However, their extraction rates vary from almost immediate extraction of anthocyanins from grape skins to several days for skin tannins to weeks for seed tannins (Waterhouse et al., 2016; Unterkofler et al., 2020). The latter correlates with the ethanol

concentration of the musts, which facilitates seed tannin extraction (Casassa & Harbertson, 2016).

Once the wine has reached the desired ethanol concentration, the must is drained and pressed to separate wine and pomace. Depending on the wine style, the wine can be left for further fermentation until dryness. Subsequently, a secondary fermentation, the malolactic fermentation (MLF), may be desired to convert malic acid, which is perceived as harshly sour, to lactic acid by adding lactic acid bacteria (Waterhouse et al., 2016; Unterkofler et al., 2020).

Between fermentation and bottling, red wines can undergo a maturation phase, in which they are stored in either steel tanks or oak barrels to promote reactions of polyphenols. Depending on the availability of oxygen, these reactions include oxidation and (in-)direct condensation of anthocyanins, flavan-3-ols, and tannins. Oak barrels ensure the slow diffusion of ambient oxygen into the wine, leading to oxidation reactions, whereas micro-oxygenation may be applied with tank storage. This leads to stabilized wines in general, but more specifically to the stabilization of red wine color, and the attenuation of astringency. When stored in oak barrels, hydrolyzable tannins, their monomeric constituents, and flavor compounds are extracted from the wood, adding vanilla, coconut, and smoky nuances, among others, to the aroma of the wine. In wines stored in steel tanks, this can be achieved by adding oak wood chips. (Waterhouse et al., 2016; Unterkofler et al., 2020; Ribéreau-Gayon et al., 2021).

After maturation, the wines are racked, clarified, stabilized, and bottled. Once bottled, reactions of polyphenols continue as red wines age. Unless the bottles are closed with natural or synthetic cork, which allows a considerable oxygen ingress during aging, the residual oxygen limits the occurrence of oxidation reactions (Ribéreau-Gayon et al., 2021).

Consequently, three factors significantly influence the polyphenolic composition of red wines: First, the composition of grape berries, second, the extraction protocol applied in winemaking, and third, the polyphenolic reactions during aging.

2 Red wine composition

2.1 General composition

Red wines consist primarily of two compounds: water and ethanol. Together, both account for approximately 97 % (w/w) of the wine. The remaining 3 % (w/w) comprise of a nearly uncountable number of chemical compounds, the precise composition of which differs with variety, wine style, harvest conditions, and vintage (Robinson & Harding, 2006; Waterhouse et al., 2016).

In terms of abundance, glycerol follows water and ethanol and is usually found in concentrations of 5 – 10 mg/L and higher in high sugar fermentations. Glycerol is a fermentation by-product produced by yeasts and was thought to play a role in the fullness of red wines. However, considering it's concentration in wine, the effect on wine mouthfeel appears to be minor (Waterhouse et al., 2016; Ribéreau-Gayon et al., 2021).

Other major red wine components which significantly affect wine quality are organic acids, monomeric sugars, and phenols (sorted by abundance). All three classes of compounds have an impact on red wine taste and mouthfeel as they elicit sourness, sweetness, bitterness, and astringency, respectively. Tartaric acid and malic acid account for the majority of organic acids and are readily extracted from grape pulp cells during must pressing, whereby malic acid is often turned into lactic acid through MLF (Cheynier & Sarni-Manchado, 2010; Waterhouse et al., 2016).

The sourness of wine correlates with the titratable acidity (TA) expressed as tartaric acid equivalents. Besides contributing to wine sourness, organic acids determine the wine pH (usually between 3 and 4), which in turn influences wine color as well as microbial and chemical stability (Waterhouse et al., 2016).

The main monomeric sugars found in grapes and wines are glucose and fructose. Both are the primary substrate for yeasts, which convert them into ethanol during the alcoholic fermentation. The perception of sweetness of red wines depends on the residual sugar content, which usually ranges from 1 to 4 g/L in dry red wines, and the acidity, which can offset the sweetness (Robinson & Harding, 2006; Waterhouse et al., 2016). Polymeric sugars are referred to as polysaccharides. Their composition and influence on red wine mouthfeel are described later in this dissertation (**Chapters 2.3 and 4**).

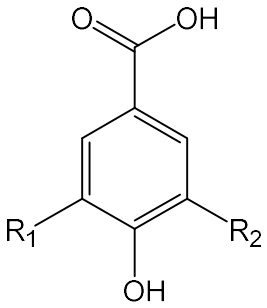
One of the most discussed topics in red wine research is arguably the polyphenolic composition and its contribution to quality characteristics like color and astringency. Therefore, the following chapter (**Chapter 2.2**) is dedicated to red wine polyphenols.

2.2 Polyphenols

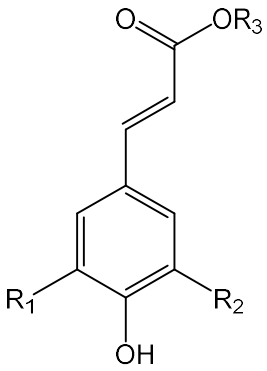
The basic building block of polyphenols is phenol (C_6-OH skeleton), hence, the polyphenolic structure comprises of an aromatic ring with one or more hydroxyl groups (Fulcrand et al., 2006; Robinson & Harding, 2006). They are classified into flavonoids and non-flavonoids, whereby the latter ones include simple phenols like hydroxybenzoic acids (C_6-C_1 skeleton) and hydroxycinnamic acids (C_6-C_3 skeleton), and stilbenes (C_6-C_2-C_6 skeleton) (**Figure 1-1**). Major

representatives of these groups are gallic acid, caffeic acid, and resveratrol, respectively (Cheynier, 2005; Iriti & Faoro, 2009).

In contrast to aged wines, young wines do not contain simple hydroxycinnamic acids because they are extracted from grape berries, in which the acids are esterified with tartaric acid. Due to the acidic pH (3 – 4) of red wine, these esters are hydrolyzed during aging. Similarly, in young wines gallic acid is mainly esterified with glucose, forming hydrolysable tannins, specifically gallotannins, which are extracted from oak wood during maturation. This group of tannins is easily hydrolyzed, releasing gallic acid to the wine (Waterhouse, 2002).

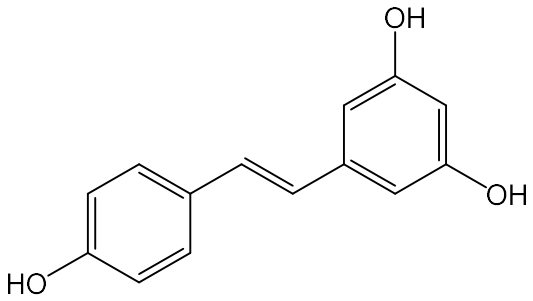


Hydroxybenzoic acids	R_1	R_2
Gallic acid	-OH	-OH
Vanillic acid	-H	$-OCH_3$
Syringic acid	$-OCH_3$	$-OCH_3$



Hydroxycinnamic acids	R_1	R_2	R_3 = Tartaric acid
Caffeic acid	-H	-OH	Caftaric acid
Coumaric acid	-H	-H	Coutaric acid
Ferulic acid	-H	$-OCH_3$	Fertaric acid

Stilbenes



Trans-Resveratrol

Figure 1-1 Structures of most abundant non-flavonoid polyphenols including hydroxybenzoic acids, hydroxycinnamic acids, and stilbenes (Iriti & Faoro, 2009; Waterhouse et al., 2016).

Flavonoids represent the majority of red wine polyphenols and combine structures that provide red wine with color, color stability, and mouthfeel. Due to their abundance and multifunctionality, flavonoids (C_6-C_3-C_6 skeleton) are of prime importance for red wine quality.

They comprise of two polyhydroxylated aromatic rings (A and B ring), which are connected by a pyran ring (C ring) (Fulcrand et al., 2006).

Wine flavonoids share a *meta*-hydroxyl group on the A ring at positions 5 and 7 and a hydroxyl group at position 3 of the C ring. Depending on the oxidation state of the heterocyclic C ring, flavonoids are classified into different groups, of which anthocyanins and flavanols are arguably the most valuable (Cheynier, 2005; Waterhouse et al., 2016).

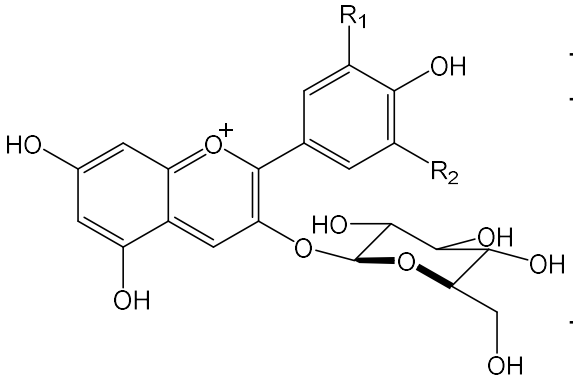
The C ring of flavanols is fully saturated and they are often referred to as flavan-3-ols to indicate the position of the hydroxyl group at the C ring. In contrast, anthocyanins possess an aromatic, positively charged C ring, which causes anthocyanins to have a red color (Waterhouse et al., 2016). Besides anthocyanins and flavanols, flavonols are found in the grape berry skin, where they serve as UV screen. They carry a keto group at position 4 of the C ring and elicit a yellow color, which is mostly covered up by the red color of anthocyanins in red wines (Castillo-Muñoz et al., 2009).

The high electron density and hydroxyl substitution patterns of flavonoids promote oxidation and electrophilic substitution reactions, while the positive charge of anthocyanins gives way for nucleophilic addition on the C ring (Fulcrand et al., 2006). Therefore, red wine flavonoids undergo several reactions during aging, creating oligomers and polymers, which increases the high number of identified flavonoids.

2.2.1 Anthocyanins

A wide variety of fruits and flowers throughout the plant kingdom are adorned by anthocyanins. They provide red, blue, and purple colors, which is why they act as attractants for pollinating insects. Due to their fully unsaturated structure, they can serve as filters against harmful irradiation. With the exception of *teinturier* varieties, which possess colored flesh, anthocyanins are usually found in the skin of red grape berries only (Burns et al., 2002).

The term anthocyanin refers to an anthocyanidin (aglycone) linked to a sugar moiety, which makes the anthocyanin more stable and water-soluble. In European red wine cultivars, five aglycone structures with differing substitution patterns at the B ring and their 3-*O*-monoglucosides have been identified (**Figure 1-2**). In other *Vitis* species, the presence of anthocyanidin-3,5-diglucosides was reported (Cheynier, 2006). In addition to the glucosylation, the C6 atom of the glucose moiety can be esterified with acetic, coumaric, or caffeic acids. With proportions of 50 – 90 %, malvidin-3-glucoside is the most abundant anthocyanin in red wine (Waterhouse et al., 2016; Ribéreau-Gayon et al., 2021).



Anthocyanidin-3-*O*-glucoside	R_1	R_2
Cyanidin	-OH	-H
Delphinidin	-OH	-OH
Peonidin	$-OCH_3$	-H
Petunidin	$-OCH_3$	-OH
Malvidin	$-OCH_3$	$-OCH_3$

Figure 1-2 Structure of anthocyanidin-3-*O*-glucosides and the substitution patterns of the five major red wine anthocyanins (Kennedy et al., 2006).

The state in which anthocyanins are positively charged is responsible for their red color and is referred to as flavylium cation. This form of anthocyanins is highly unstable and pH dependent. In acidic solutions with a pH < 2, the predominant form of anthocyanins is the flavylium cation. However, red wine pH is considerably higher (pH 3 – 4), leading to the protonation and hydration of anthocyanins (Brouillard & Delaporte, 1977; Brouillard & Dubois, 1977). As a result, a quinoidal base and a colorless hemiketal are formed, respectively, of which the latter is the primary state of anthocyanins in red wines (Cheynier, 2006; Fulcrand et al., 2006). Additionally, at wine pH and with an excess of bisulfite, anthocyanins are susceptible for its nucleophilic addition at positions 2 and 4 of the C ring, which results in a colorless sulfonate adduct (Jurd, 1964; Berké et al., 1998).

Altogether, this shows the importance of color-stabilizing interactions and reactions of anthocyanins, whereby the latter are related to red wine aging. Interactions between anthocyanins and other planar red wine components, like flavanols, hydroxybenzoic and hydroxycinnamic acids, is referred to as copigmentation. Copigmentation results from the π-π interactions of the delocalized electrons of the phenolic benzene rings, leading to the stacking of the pigment and the copigment. This complexation reduces the discoloration of anthocyanins by preventing the formation of the colorless hemiketal. Moreover, copigmentation causes an increase in absorbance (hyperchromic shift) and a shift of the absorbance maximum towards higher wavelengths (bathochromic shift) (Boulton, 2001; Fulcrand et al., 2006).

One of the aging related reactions involves wine compounds with a polarizable double bond, such as hydroxycinnamic acids and pyruvic acid deriving from the yeast metabolism, which form a second pyran ring between C4 and the C5 hydroxyl group. Due to the presence of the

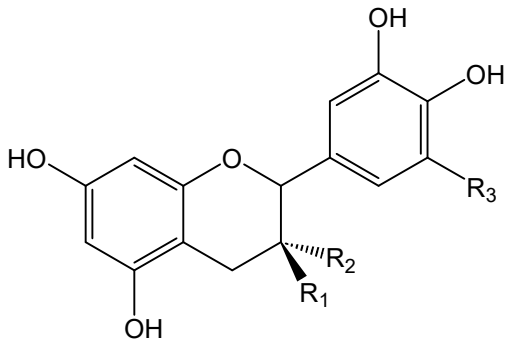
second pyran ring, these pigments are referred to as pyranoanthocyanins, which are stable against pH changes and bleaching (Fulcrand et al., 1998; Cheynier, 2006).

Further stabilizing reactions include the (in-)direct condensation of anthocyanins with flavanol monomers and polymers to form polymeric pigments, which is discussed in depth later in this dissertation (**Chapter 2.2.4**).

2.2.2 Flavanols

Monomeric and polymeric flavanols are extracted from grape berry skins and seeds during maceration, whereby the latter contain particularly high amounts of monomeric flavanols. In red wine, monomers elicit bitterness, which decreases with the increasing degree of polymerization (Noble, 1998; Kennedy et al., 2006).

The structural features of flavanol monomers (**Figure 1-3**) alone are highly diverse. The positions 2 and 3 of the C ring are chiral carbon atoms, which give rise to two isomers for each position. Usually, flavanols have a *2R* stereochemistry and the prefix “epi-“ refers to the *cis* form of the hydroxyl substitution at C3 relative to the B ring. The flavanol B ring can either bear two or three hydroxyl groups, whereby the latter is referred to as (epi-)gallocatechin because of its similarity to gallic acid. An additional galloylation of the hydroxyl group at position 3 leads to the formation of the corresponding gallates (Waterhouse et al., 2016).



Flavanols	R_1	R_2	R_3
(+)-Catechin	-OH	-H	-H
(-)-Epicatechin	-H	-OH	-H
(+)-Gallocatechin	-OH	-H	-OH
(-)-Epigallocatechin	-H	-OH	-OH

Figure 1-3 Structure of monomeric flavanols (Waterhouse et al. 2016).

The number of monomeric flavanols found in grape material is rather low, with 2 % and 11 % of total flavanols being present in skins and seeds, respectively. Instead, flavanols are mainly present as oligo- and polymers (Prieur et al., 1994; Souquet et al., 1996). Polymeric flavanols are known by various names such as condensed tannins and proanthocyanidins as a

reference to the release of anthocyanidins in hot and acidic conditions. In the following, polymeric flavanols will be referred to as condensed tannins.

2.2.3 Condensed tannins

While monomeric flavanols appear more bitter than astringent, astringency is increasing with the chain length of condensed tannins, overshadowing the decreasing bitterness of flavanol units (Noble, 1998; Kennedy et al., 2006). In contrast to hydrolyzable tannins, condensed tannins result from the condensation of two or more flavanol units and are cleavable in hot and acidic conditions. As mentioned before, this results in the release of anthocyanidins, more precisely in the release of cyanidin or delphinidin depending on the hydroxylation pattern of the B ring. Condensed tannins with units that carry two hydroxyl groups at the B ring ((+)-catechin, (-)-epicatechin, and (-)-epicatechingallate) are referred to as procyanidins, whereas condensed tannins with trihydroxylated units ((+)-gallocatechin and (-)-epigallocatechin) are called prodelphinidins (Thompson et al., 1972).

The composition of grape skin and seed tannins differs in the mean degree of polymerization (mDP), trihydroxylation at the B ring, and degree of galloylation. Skin tannins are highly polymerized (mDP ~30), with 90 % of tannins having an mDP > 10, around 30 % of skin tannins are prodelphidins, and the degree of galloylation is rather low (<6 %) (Souquet et al., 1996, 2000). In contrast, seed tannins are less polymerized (mDP <10), do not contain trihydroxylated units, and are therefore only procyanidins; their degree of galloylation is at around 30 % (Prieur et al., 1994; Souquet et al., 2000).

Due to the hydroxylation pattern of flavonoids, positions 6 and 8 at the A ring are partially negatively polarized, creating two nucleophilic sites. This results in the formation of interflavan linkages that connect C4 and C6 or C8 of the flavonoids, respectively. Dimeric flavanols with one C4-C6 or C4-C8 interflavan linkage are referred to as B-type proanthocyanidins. Among there, 8 B-type procyanidins (B_1-B_2) were reported most frequently (Cheynier, 2006; Ribéreau-Gayon et al., 2021). In the B-type series, the number of possible isomers increases exponentially with the number of monomeric units. A-type dimers have an additional ether bond creating a second C2-O-C5 or C2-O-C7 linkage. (Cheynier, 2005).

Skin and seed tannins are extracted during maceration, which highlights the structural heterogeneity of condensed tannins in young red wines. However, like anthocyanins, condensed tannins are highly reactive under the conditions prevalent in red wines, which leads to an even wider range of polymeric structures in aged wines. In addition to the two nucleophilic sites at the A ring, cleavage of condensed tannins results in the release of a flavanol

carbocation, which enables the flavanol to act as an electrophile; hence, condensed tannins can take part in electrophilic substitutions, as nucleophile and electrophile, which is further polymerized (Haslam, 1980; Vidal et al., 2002). However, this was only observed in model solutions with lower pH ranges (pH 2 and 3.2) and not at pH 3.8, indicating that these reactions are limited to acidic red wines (Vidal et al., 2002; Salas et al., 2003). Besides these reactions, leading to direct interflavan linkages, flavanols can be connected through methyl methine bonds after the condensation with acetaldehyde (Fulcrand et al., 2006; Cheynier & Sarni-Manchado, 2010).

In red wines, condensed tannins and anthocyanins are present side-by-side, making reactions between both compound classes inevitable. As a result, new polymers, which are referred to as polymeric pigments, are formed.

2.2.4 Polymeric pigments

During the aging of red wines, anthocyanin concentrations decline while the color density remains largely similar. Only the change of color hue reveals the structural transformations of wine pigments (Somers, 1968). Moreover, the responses to pH changes and bisulfite addition of these wine pigments differ, as the color of the pigments is less affected. While young wines contain 0 – 5 % of these non-bleachable pigments, concentrations increase constantly during aging (Somers & Evans, 1977; Bindon, McCarthy, et al., 2014). Due to their polymeric character, which is a result of the reaction between anthocyanins and polymeric flavanols, the pigments are called polymeric pigments and can be considered as pigmented tannins (Somers, 1971).

Anthocyanins can react with monomeric and polymeric flavanols, which leads to mainly two types of adducts: anthocyanin-flavanol (A^+-F) and flavanol-anthocyanin (F-A^+) adducts, depending on whether the anthocyanin acted as electrophile or nucleophile forming the upper or lower unit, respectively. Both forms of pigments are stable against pH and bisulfite bleaching (Salas et al., 2003; Fulcrand et al., 2006).

The latter form of adduct requires the acid-catalyzed cleavage of condensed tannins, which releases a positively charged carbocation, followed by the nucleophilic attack of an anthocyanin (C4-C8 adduct). In this case, the anthocyanin is present in the hemiketal form, making a subsequent dehydration necessary to obtain the colored flavylium cation. As described earlier, the cleavage of condensed tannins is limited to low pH media. Therefore, the formation of these adducts is less likely at red wine pH but could still be obeserved in wine samples at small concentrations (Salas et al., 2003, 2004).

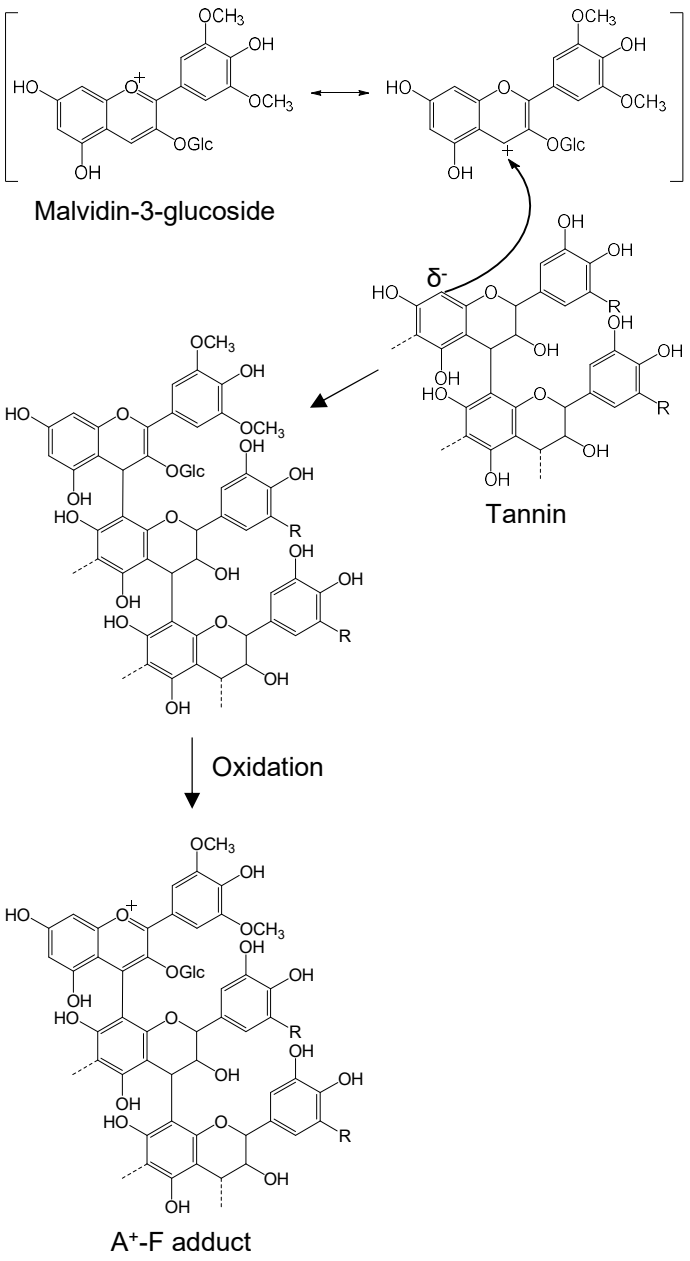


Figure 1-4 Formation of an A^+-F adduct as exemplified by the reaction between malvidin-3-glucoside and a simplified tannin structure (Fulcrand et al. 2006).

At a higher pH value (pH 3.8), anthocyanin-flavanol (A^+-F) adducts were identified, which are formed through the nucleophilic attack of the flavylium cation (C4) by the flavanol (C6/C8). The subsequent oxidation of the colorless flavene intermediate results in the pigmented C4-C6/C8 adduct (**Figure 1-4**). Besides these B-type dimers, colorless A^+-F adducts can occur through the intramolecular reaction of the flavene intermediate forming an additional ether bond. This results in a bicyclic A-type product (Remy-Tanneau et al., 2003; Salas et al., 2003).

Because the flavylium cation and hemiketal form of the anthocyanins are both present at wine pH, they can both act as nucleophile and electrophile and undergo this reaction with each other. Pigments that result from this are anthocyanin-anthocyanin (A^+-AOH) adducts and are of oligomeric nature (Salas et al., 2003; Bindon, Kassara, et al., 2014).

During fermentation and wine storage, yeast metabolism and oxidation produce acetaldehyde from ethanol, respectively, which can alter red wine pigment composition by accelerating the polymerization of anthocyanins and flavanols (Cheynier, 2006; Waterhouse et al., 2016). In the acidic red wine milieu, acetaldehyde undergoes protonation, which renders it positively charged, leading to a nucleophilic attack on behalf of a flavanol molecule. After dehydration, another flavanol molecule acts as nucleophile and again attacks the newly formed carbocation. Considering that the resulting methyl methine linked flavanol dimers can be linked through positions C6-C6, C8-C8, and C6-C8, whereby the C6-C8 dimer bears an asymmetric carbon, this gives rise to four isomers. The diversity of the products increases with the inclusion of both (+)-catechin and (-)-epicatechin and the ongoing oligomerization and polymerization (Fulcrand et al., 1996; Es-Safi et al., 1999).

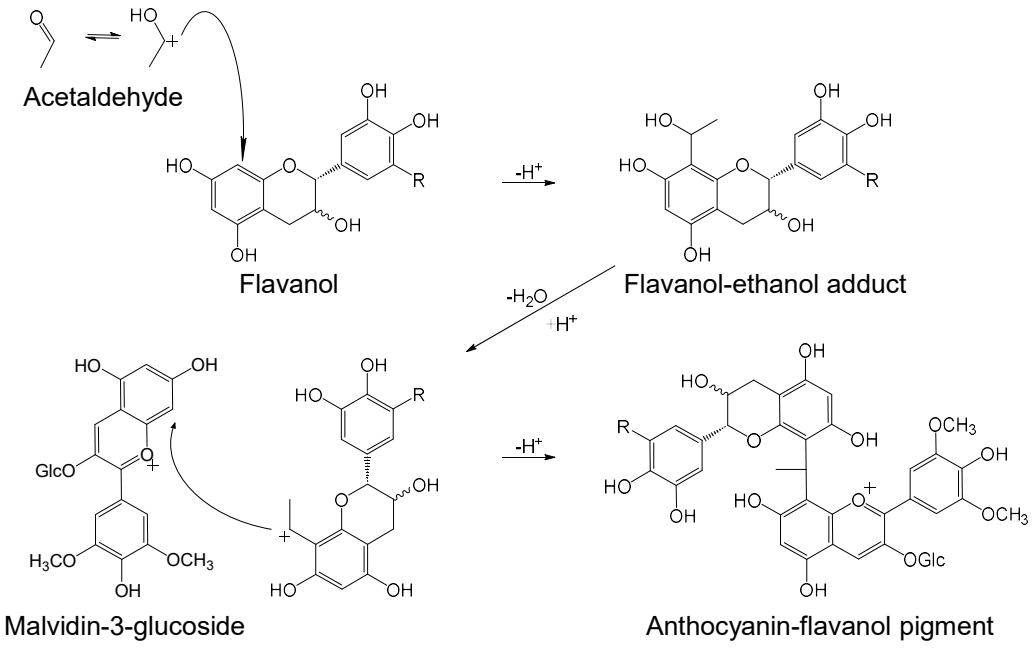


Figure 1-5 Formation of a methyl methine linked anthocyanin-flavanol pigment as exemplified by the reaction between malvidin-3-glucoside and a monomeric flavanol (Timberlake & Bridle, 1976).

When incorporating anthocyanins into those structures, which leads to the formation of methyl methine linked polymeric pigments, the diversity of the resulting oligomers and polymers is even more so enhanced. Like flavanols, positions 6 and 8 of the A ring are partially negatively charged. Therefore, anthocyanins can act as nucleophile and attack both carbocations, the

protonated acetaldehyde and the flavanol/anthocyanin-ethanol adduct (**Figure 1-5**). This results in the formation of methyl methine linked anthocyanin-anthocyanin and anthocyanin-flavanol pigments, which then undergo oligomerization with an unknown proportion of anthocyanin subunits. In comparison to anthocyanin monomers, the color of these pigments shifts towards a deep purple hue contributing to red wine color stabilization (Timberlake & Bridle, 1976; Atanasova et al., 2002).

2.3 Polysaccharides

Red wine polysaccharides derive from two sources: Yeasts release mannoproteins during the fermentation and pectic polysaccharides are extracted from the grape berries, whereby the latter is more abundant in red wines (Vidal, Williams, et al., 2003). Grape-derived polysaccharides are found in the primary cell wall (CW) and the middle lamella of berry skin and pulp. They find their way into the wine during maceration. The primary CW serves many purposes like providing structure (strength and shape) and protection against environmental factors, as well as controlling cell expansion. Around 90 % of the primary CW comprise of polysaccharides, most of which is pectin (40 %) followed by cellulose (35 %) and hemicellulose (15 %). Structural proteins account for the remaining 10 % of the CW (Hanlin et al., 2010; Goualo et al., 2012).

Pectin is embedded in a network of cellulose and hemicellulose functioning as gel forming polysaccharide, which regulates water and ion transportation, and the flexibility of the CW (Voragen et al., 2009). Pectic polysaccharides are highly complex molecules, of which some structural features are not yet fully understood. Available information indicates that there are specific domains covalently linked with each other, which are referred to as smooth and hairy region, insinuating the linear and branched nature of these pectin domains, respectively. The most prominent pectin structure (~60 %) and representative of the smooth region is a chain of 1,4-linked α-D-galacturonic acid (GalAc) units, which form the homogalacturonan (HG). The GalAc units are partially esterified with methanol and acetic acid at the C6 carboxyl group and the C2 or C3 hydroxyl groups, respectively. The degree of methylation (DM) determines the net charge of pectin molecules, because the carboxy group of unesterified GalAc units dissociates at wine pH and leaves a negative charge. In highly esterified pectins, more than 50 % of GalAc units are esterified and a chain of HG is estimated to comprise of 100 – 200 GalAc units (Robledo et al., 2019; Ropartz & Ralet, 2020).

Other structures of the smooth region are substitutions of HG and include rhamnogalacturonan II (RG-II), xylogalacturonan, and apiogalacturonan. The latter two carry a xylose or apiose

substitution at the hydroxyl group of C3 and/or C2, respectively. They are less common, as their abundance is restricted to a few plant species (Mohnen, 2008). RG-II is arguably the most widespread pectic structure in the plant kingdom and makes up for around 10 % of pectin. This substituted HG comprises of a minimum of seven partially methyl esterified GalAc units bearing four specific side chains, which are composed of 20 sugar moieties. Of these sugar moieties, six are rarely found in nature and characteristic for the RG-II structure, like 2-*O*-methyl-fucose and 2-*O*-methyl-xylose, making them suitable for analytical purposes (Pérez et al., 2003; Mohnen, 2008; Yapo, 2011). In the presence of boron, RG-II occurs as monomer (~5 kDa) and dimer (~10 kDa), as it can form borate diester cross-links (Doco & Brillouet, 1993; Pellerin et al., 1996).

The hairy region of the pectin comprises of rhamnogalacturonan I (RG-I), which presents a backbone of alternating GalAc and α-L-rhamnose (Rha) units (1,2-linked). Like HG, RG-I shows acetylation at the C2 and C3 hydroxyl groups of the GalAc units, but no methylation. Instead, linear and branched side chains of arabinose (α-1,5-arabinans), galactose (β-1,4-galactans), or both (arabinogalactans) are attached at the C4 hydroxyl group of the rhamnosyl residue. Arabinogalactans (AG) are characterized by their β-D-galactose backbones, whereby type I AGs and type II AGs show 1,4- and 1,3-linkages, respectively. Type I AGs are substituted with arabinose units and/or arabinan chains at the C3 hydroxyl group with several variations. Type II AGs are highly branched because the galactose backbone is substituted with other galactose units (1,6-linked) and/or short β-1,6-galactose chains. The galactose backbone and side chains may be substituted with arabinose (α-1,3-/ α-1,4-/ α-1,6-linked) (Voragen et al., 2009; Yapo, 2011; Ropartz & Ralet, 2020). In addition to the neutral sugar substitution, 3 – 8 % of type II AGs are associated with proteins, giving them the name arabinogalactan proteins (AGP). AGPs show an *O*-glycosylation of the AG II sugar moieties with one or more hydroxyl groups of the hydroxyproline residues, which is one of the main amino acids of the AGPs. With a proportion of more than 90 %, AG II is still the main component of AGPs (Seifert & Roberts, 2007; Voragen et al., 2009). Due to their compositional characteristics, arabinans, AGs, and AGPs are classified as polysaccharides rich in arabinose and galactose (PRAG). Furthermore, wine polysaccharides are often categorized according to their acidity into neutral (NPS; AGs and AGPs) and acidic polysaccharides (APS; homo- and rhamnogalacturonans) (Ayestarán et al., 2004; Guadalupe et al., 2007; Martínez-Lapuente et al., 2020).

The specific composition of plant CWs depends on the plant species and other factors like fruit maturity, which changes the structural characteristics of polysaccharides due to endogenous enzyme activities (Minic & Jouanin, 2006; Ortega-Regules et al., 2008). In grape berry CWs, the HG accounts for around 80 % of pectic polysaccharides, followed by RG-I and RG-II with around 15 % and 5 %, respectively (Vidal et al., 2001). Comparing these numbers with the

amounts of pectic polysaccharides found in red wines, their composition differs significantly. While in grape berries the main pectin fragment is HG, the majority of red wine pectic polysaccharides comprises of AGPs, which account for around 40 %. Concerning grape-derived polysaccharides, AGPs are followed by RG-II (~20 %) and small amounts of RG-I and HG fragments (Pellerin et al., 1995, 1996; Gao et al., 2015).

These differences in composition result from the solubility of pectin fragments related to the structural features of the polysaccharides. During grape ripening, endogenous enzymes degrade the CW polysaccharides, which is accompanied by an increasing solubility of pectin fragments. Thereby, the AGPs as well as the de-esterified and depolymerized pectin components are readily extracted with water, whereas the polymerized and higher esterified fragments are more soluble with increasing ethanol concentration (Nunan et al., 1998; Gao et al., 2015).

Due to the solubilization of pectin fragments, they are extracted in the must and wine, where they can interact with polyphenols. These interactions determine the extractability and the composition of red wine polyphenols, which will be discussed in the following chapter.

3 Interactions between red wine polyphenols and macromolecules

3.1 Interactions between polyphenols and polysaccharides

The driving forces for the interactions between polysaccharides and polyphenols are of hydrophobic and electrostatic nature, whereby the latter includes hydrogen and ionic bonding. These interactions result in the formation of non-covalent binding and complexation of polyphenols (Weber, 2022). The type and strength of interaction depends on the structural features of both, polyphenols, and polysaccharides. Regarding the polyphenols, the substitution at the C ring, conformational changes, and hydroxylation of the B ring affect their complexation. At the same time, the solubility, branching complexity, degree of esterification, and porosity of polysaccharides influences the ability of binding polyphenols. Both share the influence of the molecular weight on the interactions between them (Liu et al., 2020).

Along the winemaking process, from the grape to the finished wine, there are two crucial turning points which determine the composition of must and wine polysaccharides. The first point marks the harvest date, which influences the level of berry CW degradation by endogenous enzymes. The second point is the use of exogenous enzymes during the maceration of the must (Hanlin et al., 2010). Polygalacturonase (PG), pectin methylesterase (PME) and β-galactosidase are the main enzymes involved in the pectin degradation during

grape ripening. They are responsible for the cleavage of unesterified polygalacturonans, the de-esterification of methylated galacturonic acid units, and the de-polymerization of (1→4)-β-galactan constituents of the RG-I side chains, respectively (Nunan et al., 1998; Minic and Jouanin, 2006). At commercial red wine harvest, a wide variety of DMs were reported ranging from 82.5 % (Ortega-Regules et al., 2008) to 39 % (Vicens et al., 2009).

Due to the interactions between CW polysaccharides and polyphenols, the latter are subject to constant adsorption to and desorption from CW polysaccharides (Medina-Plaza et al., 2020; Hensen et al., 2022). Pectin shows a higher adsorption effect for polyphenols than cellulose, leading to the retention of polyphenols in the must respectively pomace. Ultimately, the structural features of grape berry pectin affect the extractability of polyphenols and therefore their abundance in red wine (Gao et al., 2015; Y. Liu et al., 2019). To increase the yield of juice and polyphenol extraction, macerating enzymes, which show the before-mentioned enzyme activities as well as a pectin lyase activity, are added during red wine fermentation. The enzymatic treatment of the must was shown to disrupt the adsorption of polyphenols to the pectin fragments, keeping them in solution (Bautista-Ortín et al., 2016; Castro-López et al., 2016; Osete-Alcaraz et al., 2022). Overall, the degradation of pectic polysaccharides, either by endo- or exogenous enzymes, not only increases pectin solubility, but is also accompanied by the enhanced extraction of condensed tannins and anthocyanins. This greatly influences red wine quality characteristics like color and astringency (Ducasse et al., 2010; Hernández-Hierro et al., 2012; Bindon et al., 2016; Benucci et al., 2017).

Once polysaccharides and polyphenols are extracted to the wine, hydrophobic and electrostatic forces lead to the complexation of both. Thereby, these forces do not act individually but rather simultaneously, as can be explained using the example of malvidin-3-glucoside: At wine pH, part of the anthocyanins is positively charged, as it exists in the flavylium cation form. At the same time, dissociated GalAc units of unesterified pectin fragments are negatively charged, which allows for ionic interactions between the two (Holzwarth et al., 2012). Additionally, the hydroxyl groups of the HG and RG backbone can form hydrogen bonds with the hydroxyl groups of malvidin-3-glucoside. However, as malvidin-3-glucoside carries two methoxy substitutions at positions C3’ and C5’ of the B-ring, hydrophobic interactions with methyl esterified GalAc units also play a role in the complexation of anthocyanins (Fernandes et al., 2020).

These interactions result in an increased storage (Buchweitz et al., 2013) and pH stability of anthocyanins (Fernandes et al., 2021). Furthermore, copigmentation-like effects lead to hyperchromic and bathochromic shifts of the absorbance maximum of anthocyanins. This may be explained by a two-step mechanism, whereby the first step involves the interaction of

anthocyanins with pectin and the second step is assigned to the self-association of other anthocyanins through π-π stacking (Padayachee et al., 2012; Fernandes et al., 2016).

Flavanol monomers and condensed tannins can form aggregates through self-aggregation, which leads to the formation of colloids and haze (Poncet-Legrand et al., 2003). In the presence of pectic polysaccharides, the size evolution of these colloids was reduced, with acidic AGPs showing the highest reducing effect, whereas dimeric RG-II promoted colloid growth (Riou et al., 2002). Ultimately, the self-aggregation of tannins leads to their precipitation, which was prevented by the addition of pectin on the one hand and increased after the release of high molecular weight polysaccharides through macerating enzymes on the other hand (Osete-Alcaraz, Bautista-Ortín, et al., 2020; Osete-Alcaraz, Gómez-Plaza, et al., 2020).

In conclusion, the interactions between polysaccharides and polyphenols significantly influence the composition of the latter. Consequently, pectic polysaccharides are highly important for red wine quality, which will be discussed in more detail later in this dissertation (**Chapter 4**).

3.2 Interactions between polyphenols and proteins

Like the interactions of red wine polyphenols with polysaccharides, hydrophobic and electrostatic forces drive the interactions with proteins. In this case, however, electrostatic forces only refer to hydrogen bonding (Weber, 2022). The complexation of polyphenols and proteins is correlated with the proportion of proline residues in the amino acid sequence of proteins. Due to their heterocyclic structure, the proline residues facilitate hydrophobic interactions with the phenolic benzene ring. The noncovalent bond between the molecules is strengthened through hydrogen bonds between the polyphenolic hydroxyl groups and the protein amide group (Waterhouse et al., 2016; Vernhet, 2019).

Because of these interactions, particularly condensed tannins form insoluble precipitates with proteins. This protein precipitability is of utmost importance for red wine quality because it leads to the perception of astringency and determines the mouthfeel of red wine. Furthermore, this feature is used in the clarification and stabilization of wine, whereby condensed tannins are precipitated with proteins from various origins (Vernhet, 2019).

3.2.1 Astringency

The word astringency refers to the ability of condensed tannins to bind proteins and describes the drying in-mouth sensation (mouthfeel) after the consumption of red wine (Cheynier & Sarni-Manchado, 2010). This sensation derives from the complexation and precipitation of condensed tannins with salivary proline-rich proteins (PRP), whereby the exact mechanisms of this phenomenon are not yet fully understood. The underlying interactions take place according to the above-mentioned two-step process, where tannins and proteins first interact via hydrophobic attraction followed by the strengthening through hydrogen bonding. Subsequently, the aggregates that are formed precipitate (Charlton et al., 2002).

The most accepted mechanism of astringency perception is that this precipitation leads to a loss in lubrication of the oral cavity and the tactile sensation of increased friction. Other hypotheses suggest that the formation of protein-tannin aggregates activate mechanoreceptors or that condensed tannins can directly interact with oral epithelial cells. While all of these mechanisms seem plausible, another pending question is whether they act simultaneously or rather consecutively (González-Muñoz et al., 2022).

Because red wine astringency is determined by the interactions between polyphenols and salivary PRP, it ultimately depends on the structural features of the polyphenols. Accordingly, it was associated with the mDP of the flavanols (Chira et al., 2012), the pigmentation of tannins (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013), and the degree of galloylation and trihydroxylation of the B-ring (Vidal, Francis, et al., 2003). The latter two were reported to respectively increase and decrease the roughness of astringency (Vidal, Francis, et al., 2003). Moreover, red wine aging seems to attenuate the astringency perception, leading to a softer mouthfeel, which was assigned to decreasing tannin concentrations (Boselli et al., 2004; Landon et al., 2008; Chira, Pacella, et al., 2011). Beyond the general perception of astringency, specifications of various astringency-related sensations were established, which are referred to as astringency sub-qualities. Yet, no clear connection could be identified between these sub-qualities and red wine composition (Gawel et al., 2000; González-Muñoz et al., 2022).

3.2.2 Characterization of tannins and polymeric pigments

The complexity of condensed tannins entails many challenges in their analytical characterization. Despite their heterogeneity due to polymer length, sub-unit composition, and constitution, the structures are yet too similar, which limits the feasibility of conventional analytical tools like UHPLC-DAD-MS. Instead of chromatographically separated peaks, an

unresolved polymer hump appears in the chromatogram, hindering the identification and quantification of condensed tannins (Ma et al., 2018). Moreover, mass spectrometric detection of highly polymerized proanthocyanidins in mixed samples, like the red wine matrix, proves to be difficult due to inadequate ionization (Yanagida et al., 2003).

To compensate for these difficulties, several methods were developed to quantify condensed tannins and elucidate their structural features. Among them is the acid-catalyzed cleavage of condensed tannins in the presence of nucleophilic agents like phloroglucinol, also referred to as phloroglucinolysis, to determine the mDP of condensed tannins (Kennedy & Jones, 2001). However, this approach also has its limits when it comes to pigmented tannins because they are resistant to the acid-catalyzed cleavage (Vidal, Francis, Noble, et al., 2004).

To determine the size of condensed tannins, size exclusion chromatography (SEC) can be used to separate polymerized proanthocyanidins according to their size. This method makes use of the molecular sieve effect and can be coupled with various detectors to visualize the separation. However, polarity based adsorption of the analytes on the column material is a challenge to the development of suitable methods (Yanagida et al., 1999).

Photometric assays, which determine tannin concentrations, include the Bate-Smith Assay, the Vanillin Assay, the protein precipitation assay according to Harbertson et al. (2002, 2003), and the Methyl Cellulose Precipitation Assay (MCP Assay). Like the phloroglucinolysis, the Bate-Smith Assay uses the acid-catalyzed cleavage of condensed tannins and subsequent absorbance measurement at around 550 nm to quantify released anthocyanidins (Bate-Smith, 1975). The Vanillin Assay requires the reaction of the *meta*-substituted A-ring of monomeric flavanols with an aromatic aldehyde like vanillin. This reaction results in the formation of a red pigment, the absorption of which is subsequently measured at 500 nm. A preceding acid-catalyzed cleavage allows for the quantification of condensed tannins (Swain & Hillis, 1959). Because the absorbance maximum of anthocyanins is at around 520 nm, they can interfere with both assays, showing their limitations when it comes to complex matrices such as red wine.

Another concept of tannin quantification is the utilization of tannin precipitation by other polymers and subsequent absorbance measurement. On the one hand, protein precipitation can be employed for this purpose, whereby bovine serum albumin (BSA) is used to precipitate condensed tannins. This is followed by the resuspension of the pellet and the subsequent reaction of condensed tannins with iron chloride, which results in the formation of a chromophoric complex (Hagerman & Butler, 1978). Initially, the resuspension buffer contained sodium dodecyl sulfate, which was later replaced by urea (Harbertson et al., 2002, 2015). The photometric quantification of polymeric pigments is based on the differential measurement of

anthocyanins before and after bleaching with bisulfite (Harbertson et al., 2009). By adding a protein precipitation step, polymeric pigments can be categorized into two sub-groups: Polymeric pigments, which remain in solution after the addition of proteins, and those that do precipitate with proteins. They are called small polymeric pigments (SPP) and large polymeric pigments (LPP), respectively, assuming that the size of the polymers determines their ability to precipitate proteins (Harbertson et al., 2003). However, similar to the binding between condensed tannins and proteins, the precipitability of polymeric pigments depends not only on the mDP but also on various other factors including the pigmentation of tannins (Watrelot et al., 2017).

On the other hand, the MCP Assay uses one specific polysaccharide, methyl cellulose, to complex and precipitate condensed tannins. Due to the absorbance measurement of the sample before and after precipitation, the protocol of the MCP Assay is simpler than that of the protein precipitation assay. However, the results were approximately 3-fold higher than those obtained by protein precipitation while still showing a high correlation with these values. This was assigned to the ability of methyl cellulose to precipitate oligomers like SPP, which are not precipitable by BSA (Sarneckis et al., 2006; Mercurio & Smith, 2008).

Because the same forces govern the interactions of polyphenols with polysaccharides and proteins, respectively, all three compound classes can form ternary complexes or interfere with each other's reactions. This matter and the implications on the composition and quality of red wines will be subject of the following two chapters (**Chapter 3.3** and **Chapter 4**).

3.3 Interactions between polyphenols, polysaccharides, and proteins

Several studies investigated the impact of polysaccharides on tannin and protein interactions because of their importance for red wine astringency and quality. Different compositions of proteins and polysaccharides were selected, most of which were added to wine model solutions to react with condensed tannins. Proteins that were used for these experiments were either salivary proteins, including PRPs and α-amylase, or BSA. In contrast, the influence of a wide variety of polysaccharides like xanthan, gum arabic, and β-cyclodextrin was tested (Mateus et al., 2004; Soares et al., 2009). More importantly, pectin and pectin fractions were included in these experiments because of their natural occurrence in red wine (Mateus et al., 2004).

Regarding the effect of polysaccharides on the interactions between condensed tannins and BSA, pectin and pectin fragments, including polygalacturonic acid and arabinogalactan, were able to reduce protein precipitation. Acidic pectic polysaccharides appeared to be more

effective than neutral fragments referring to arabinogalactan. This protective effect of polysaccharides may be explained by two possible mechanisms: On the one hand, polysaccharides might form a third layer in a ternary protein-polyphenol-polysaccharide complex, which increases its solubility in hydroethanolic solutions. On the other hand, polysaccharides might compete with proteins to bind condensed tannins, leading to a lack of the latter and inhibiting protein precipitation (de Freitas et al., 2003; Mateus et al., 2004). In contrast to these results, RG-II was reported to enhance the precipitation of tannin and BSA, whereby the interactions between the three compound classes increased with higher levels of tannin pigmentation (Watrelot et al., 2017).

Results of studies on the interactions between polysaccharides, condensed tannins, and salivary proteins are contradictory. However, it appears that the effect and effectivity of polysaccharides depends on the polysaccharide structure and on the type of salivary protein. One study reported that RG-II and AGPs inhibited procyanidin B2 precipitation by PRPs (Brandão et al., 2017), whereas another study showed an enhancing effect of RG-II and neutral AGPs on the aggregation of PRPs and condensed tannins (Carvalho et al., 2006). However, in the presence of α-amylase, solely the neutral AGP fraction increased aggregation. The enhanced aggregation was assigned to the co-aggregation of polysaccharides with proteins and tannins. At the same time, negatively charged AGP fractions were reported to prevent tannin precipitation in the presence of both types of proteins (Carvalho et al., 2006). Similarly, pectin and polygalacturonic acid had a protective effect on tannin precipitation (Soares et al., 2009, 2012).

Anthocyanins are not expected to precipitate proteins, but in the concomitant presence of proteins and polysaccharides, protein bridged precipitation of anthocyanins was observed (Sommer et al., 2016). While the changes in tannin precipitability are believed to alter red wine astringency perception, the precipitation of anthocyanins may result in reduced color characteristics. Therefore, these changes can have drastic implications for red wine quality characteristics, which will be discussed in the upcoming chapter.

4 Implications for red wine composition and quality

Treating must with maceration enzymes results in an increased extraction of polyphenols and pectic polysaccharides, which was associated with an enhanced color intensity and stability during barrel aging (Guadalupe et al., 2007). Higher levels of both anthocyanins and condensed tannins may lead to an accelerated formation of polymeric pigments due to the higher availability of reaction partners. However, these pigments appear to adsorb more readily

to CW material than condensed tannins and anthocyanins, leading to a lack in stable pigments, which may decrease the potential for red wine aging (Beaver et al., 2020).

In young red wines, 30 to 50 % of color is elicited by the copigmentation of anthocyanins (Boulton, 2001), to which polysaccharides contribute after their extraction into the wine. As described above, the copigmentation-like effect induced by polysaccharides results in the stabilization of anthocyanins and an increase in their absorbance maximum. This was shown to also intensify red wine color characteristics, which were stable for six months after bottling (Osete-Alcaraz, Bautista-Ortín, et al., 2020). The colloidal stability of these complexes depends on the type of polysaccharide. While mannoproteins seemed to retain wine pigments in solution (Alcalde-Eon et al., 2014), large, neutral pectic polysaccharides led to the precipitation of anthocyanins. The latter, however, did not result in a loss of color because the pigmented aggregates showed absorbance at 520 nm (Larsen et al., 2019; Hensen et al., 2022).

The color intensifying and stabilizing effect of polysaccharides, which reduces their susceptibility to bisulfite bleaching, may alter the results of the photometric quantification of anthocyanins and polymeric pigments. Moreover, the addition of proteins, like BSA, was reported to result in the protein bridged precipitation of anthocyanins, altogether leading to shifted proportions of SPP and LPP (Sommer et al., 2016; Graves & Sommer, 2021). Due to the protective or enhancing effects on the protein precipitability of condensed tannins, polysaccharides may not only affect the determination of polymeric pigments but also the quantification of condensed tannins.

Polysaccharides were also shown to play an important role in the mouthfeel of red wines, whereby their impact was similarly high as that of polyphenols (Chong et al., 2019). However, due to the high complexity of the red wine matrix, it is difficult to assign changes in astringency unambiguously to individual components. This is why earlier studies assessed the relationship between polysaccharides and astringency either in model experiments (Vidal, Francis, Williams, et al., 2004; Wang et al., 2020) or in comparative set-ups that included many different experimental approaches and analytical methods (Guadalupe et al., 2007; Quijada-Morín et al., 2014; Chong et al., 2019; Kuhlman et al., 2022). Vidal, Francis, Williams, et al. (2004) showed that polysaccharides themselves had an influence on the astringency of model wines in the absence of tannins. Neutral AGPs and RG-II showed a reduction in all astringency related attributes and an increase in the fullness of the model wines (Vidal, Francis, Williams, et al., 2004). Kuhlman et al. (2022) reported that enzyme treatment led to a lack of pectic polysaccharides due to their degradation, which resulted in an increased overall astringency with grippy, dry, hard, grainy, and chalky sub-qualities. Together with the results of another

study (Quijada-Morín et al., 2014), which associated decreasing astringency with the polymer length of polysaccharides, this suggests that the presence of pectin fragments is important for a softer astringency perception. However, other studies (Guadalupe et al., 2007; Chong et al., 2019) showed a positive correlation between pectin fragments, both neutral and acidic, and the overall astringency, roughness, and persistence.

5 Aims of the thesis

Red wine quality characteristics include color density, color stability, and astringency, all of which are due to the polyphenolic composition of red wine. The aging potential of red wine is related to the wide range of reactions and dynamic changes of flavonoids occurring during maturation. This results in the compositional complexity of the red wine matrix (**Chapter 1**), which poses several challenges for red wine research. While the analysis of monomeric and oligomeric polyphenols is easy to achieve using UHPLC-DAD-MS, the heterogeneity of polymeric flavonoids reveals the technical limitations of established analytical tools. Ultimately, this makes it difficult to assign specific structural characteristics of condensed tannins to the perception of astringency. To extend the knowledge about the relationship between tannin structures and astringency, **chapter 2** examines the following hypothesis: Structural features of polymeric flavonoids, like size and pigmentation, determine their polarity and hydrophobicity, which are driving forces in the interactions between polyphenols and proteins. Therefore, these physico-chemical properties are valuable parameters for the characterization of condensed tannins and polymeric pigments.

Recently, the characterization of red wine polysaccharides and their role in the interactions between polyphenols and proteins has gained more interest. The presence of polysaccharides in the wine matrix appears to change the protein precipitability of condensed tannins and polymeric pigments, which in turn affects their measurability, posing another challenge for red wine research. Unlike other factors influencing the formation of polymeric pigments, like different yeast strains (Escott et al., 2018), anthocyanin-to-tannin ratio (Merrell et al., 2018), and tannin structure (Bindon, Kassara, et al., 2014), the impact of polysaccharides on this formation has not yet been investigated. However, this is of great importance as they complex anthocyanins and condensed tannins, affecting the color stability of red wines. Therefore, **chapter 3** explores the hypothesis that not only the measurability of polymeric pigments is impaired but also their composition is affected by the interactions of polysaccharides and polyphenols. Moreover, because hydrophobic and electrostatic forces drive the interactions between polysaccharides and polyphenols, the structural features of polysaccharides determine the extent to which they affect measurability and composition of polymeric flavonoids.

Because polysaccharides alter the protein pecipitability of condensed tannins and polymeric pigments, it is plausible that they are also involved in the perception of astringency. Together with the wide variety of tannin structures, this leads to an extensive sensory vocabulary describing red wine astringency (Gawel et al., 2000). Thereby, the age-related softening of astringency was associated with the proceeding pigmentation of tannins, which may be due to

the positive charge of incorporated anthocyanins rendering tannins more polar. Furthermore, little is known about the changes pectic polysaccharides are subject to during bottle-aging, both of which affect the measurability and composition of condensed tannins and polymeric pigments. This gives rise to the hypothesis that the age-related attenuation of red wine astringency is due to an increase in tannin pigmentation and the resulting changes of the interactions between condensed tannins and polysaccharides and/or proteins (**Chapter 4**).

In summary, the specific aims of this thesis (**Chapters 2-4)** are as follows:

- Characterization of tannin and polymeric pigment fractions in commercially available Cabernet Sauvignon wines by determining their polarity and hydrophobicity (**Chapter 2**)
- Determination of the structural changes of condensed tannins and polymeric pigments during simulated aging and their impact on red wine astringency perception (**Chapter 2**)
- Characterization of pectic polysaccharides in commercially available Cabernet Sauvignon wines (**Chapter 3**)
- Establishing the relationship between pectin structures and the precipitability of condensed tannins and polymeric pigments and its implications for the compositional analysis of flavonoids (**Chapter 3**)
- Characterization and determination of structural changes of pectic polysaccharides in aged red wines (**Chapter 4**)
- Determination of the impact of these changes on tannin and polymeric pigment precipitability and the implications for the compositional analysis of flavonoids and red wine astringency perception (**Chapter 4**)

Chapter 2

Effect of structural transformations on precipitability and polarity of red wine phenolic polymers

This article was published in the American Journal of Enology and Viticulture, 72(3),
Weilack, I., Schmitz, C., Harbertson, J. F., & Weber, F. (2021). Effect of structural transformations on precipitability and polarity of red wine phenolic polymers, 230–239. *
DOI: 10.5344/ajev.2021.20064

The research summarized in this chapter was reprinted in Appendix C with permission from the authors.

Author contributions: Ingrid Weilack: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Christina Schmitz**: Methodology, Investigation, Formal analysis. **James F. Harbertson**: Methodology, Writing - Review & Editing. **Fabian Weber**: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition.

Summary

Essential red wine quality characteristics are color, color stability and mouthfeel like astringency. In young wines, anthocyanins provide color to the wines, whereas during red wine aging anthocyanins degrade and color is stabilized by pyranoanthocyanins and polymeric pigments (Brouillard et al., 2003; Cheynier et al., 2006; Fulcrand et al., 2006). By combining a protein precipitation assay with bisulfite bleaching of monomeric anthocyanins, Harbertson et al. (2003) managed to fractionate polymeric pigments in small (SPP) and large polymeric pigments (LPP). LPP result from the incorporation of anthocyanins into condensed tannins during red wine aging and can therefore be referred to as pigmented tannins (Remy et al., 2000). Condensed tannins cause saliva proteins to precipitate, leading to a loss of lubrication in the oral cavity, which elicits red wine astringency (Noble, 1998; de Freitas & Mateus, 2001).

In theory, the various structural transformations which wine polyphenols undergo during aging change their physico-chemical properties and subsequently their interactions with wine polysaccharides and proteins including saliva proteins (Watrelot et al., 2017). A special role can be assigned to the formation of LPP because the incorporation of anthocyanins leads to positively charged polymers (Singleton & Trousdale, 1992). This is why earlier research hypothesized that the attenuation of astringency during red wine aging may be related to the formation of LPP (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013).

The aim of this study was to gain a better understanding of the structural diversity of red wine polymers by establishing the relationship between selected physico-chemical properties and structural features of the polymers, including condensed tannins and pigments. This was achieved by combining forced aging with several analytical techniques to determine the effect of aging-induced structural changes of wine polymers on their polarity and hydrophilicity. Sensory profiling was conducted to study the implications for red wine astringency.

Wine samples included two different commercially available wines: 2018 Cabernet Sauvignon from the Trapiche winery (Maipú, Mendoza, Argentina) and 2016 Cabernet Sauvignon from the Salentein winery (Tunuyán, Mendoza, Argentina). The wines were subjected to simulated aging at 35 °C for three or six weeks and compared to the non-aged wines. From all wines, extracts rich in polyphenols were obtained by solid phase extraction (SPE). Normal-phase FLASH-chromatography was used 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 trained sensory panel, which was composed of 14 volunteer judges, assessed overall astringency, bitterness, and sourness by sensory profiling.

As expected, wine anthocyanin concentrations dropped during the storage of the wines due to degradation, conversion, and incorporation into tannins. While SPP leveled at the concentrations of the non-aged wines, LPP proportions increased during aging. The results of the UHPLC-MS analysis showed no considerable changes in the concentrations of pyranoanthocyanins and anthocyanin-flavanol oligomers, which are considered as SPP (Harbertson et al., 2014). Together, this suggests that anthocyanins are incorporated into existing polymeric structures rather than forming new oligomers.

Tannin concentrations gradually decreased in the wines, while no changes were detected in the polyphenol-rich extracts. Since polysaccharides are removed from the wine matrix during the SPE extraction of the polyphenols, this difference may be assigned to interactions between condensed tannins, proteins and wine polysaccharides (de Freitas et al., 2003; Mateus et al., 2004; Carvalho et al., 2006). These interactions led to a reduced precipitability of condensed tannins in the wines and therefore a reduced measurability in the protein precipitation assay. The differences in tannin concentrations between wine and extracts became more pronounced with the aging of the wines, indicating that the incorporation of anthocyanins into condensed tannins enhanced the interactions with wine polysaccharides.

UHPLC-MS analysis of the fractionated polyphenols together with the K_{OW} revealed that monomeric flavan-3-ols, gallic acid, hydroxycinnamic acids, and oligomeric procyanidins are non-polar and hydrophobic. All condensed tannins were more polar and hydrophilic than their sub-units; hence, the polarity and hydrophilicity of condensed tannins was related to their degree of polymerization, with smaller tannins being less polar than larger ones. Condensed tannins, SPP, and LPP were found across two polar fractions, which are associated with different polymer sizes, indicating that size is not the only parameter determining precipitability of wine phenolic polymers. Therefore, polymeric pigments are no longer classified and named according to sizes but precipitability. Thus, they can be divided into non-precipitable (np-PP) and precipitable polymeric pigments (p-PP).

Astringency decreased in both wines during aging, whereby the 2018 wine showed a minimum of astringency after 3 weeks of aging. This pattern was only partially in line with the decreasing tannin concentrations, which have been associated with wine aging and overall astringency earlier (Landon et al., 2008; Chira, Lorrain, et al., 2011). P-PPs appeared to be less precipitable in the presence of wine polysaccharides, which indicates that polysaccharides can have a masking effect on pigmented tannins. The proceeding incorporation of anthocyanins correlated with lower astringency ratings, which may be explained by a higher polarity of p-PPs due to the positive charge introduced by anthocyanins. Therefore, pigmented tannins should be assigned a special role in the attenuation of astringency perception of aged wines.

Chapter 3

Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines

This article was published in Current Research in Food Science, 6, Weilack, I., Mehren, L., Schieber, A., & Weber, F. (2023). Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines, 100506. *
DOI: 10.1016/j.crfs.2023.100506

The research summarized in this chapter was reprinted in Appendix D with permission from the authors.

Author contributions: Ingrid Weilack: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Lea Mehren**: Methodology, Investigation, Formal analysis. **Andreas Schieber**: Resources, Supervision, Writing - Review & Editing, Funding acquisition. **Fabian Weber**: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition.

Summary

The importance of polyphenols in the context of red wine quality is well established, whereby their composition in the finished wine is determined by various factors such as compound class, winemaking protocols, and grape maturity. Grape ripening is associated with softening of the grape skin due to the enzymatic degradation of cell wall material (CWM) like pectin among others (Nunan et al., 1998). Together with the polyphenols, de-esterified and depolymerized pectin fragments are extracted during winemaking (Gao et al., 2015). Driven by hydrophobic interactions, hydrogen bonding, and electrostatic forces they can interact with polyphenols in the wine (Weber, 2022). Due to these interactions, polysaccharides were found to play a modulating role in color and color stability (Mazzaracchio et al., 2004; Padayachee et al., 2012; Fernandes et al., 2020). Additionally, they were reported to disrupt and/or enhance tannin precipitability possibly impacting wine astringency perception (de Freitas et al., 2003; Vidal, Francis, Williams, et al., 2004; Carvalho et al., 2006; Watrelot et al., 2017). Because of the high complexity of red wines, these previous studies used model wines and isolated reactions between polyphenols, polysaccharides, and proteins to investigate the influence of polysaccharides on the polyphenolic composition of red wines (González-Muñoz et al., 2022). However, it is necessary to evaluate the implications of this in finished wines to fully understand which polysaccharide structures are desirable for red wine quality.

To address the relation of polysaccharides and polyphenols, the aim of this study was to characterize pectic polysaccharides in commercially available Cabernet Sauvignon wines. To investigate the influence of the structural features of these pectic polysaccharides on the polyphenolic composition of the wines, polysaccharide-free reconstituted wines were composed, and polyphenols were determined and compared in all samples.

Six commercially available Cabernet Sauvignon wines of the 2018 vintage were selected. The wines were from the following wineries: Weinbiet and Emil Bauer (Bundschuh) from the Palatinate region in Germany, Adentu and Las Mulas from Central Valley, Chile, and Beringer and Canyon Road from California, USA. Polysaccharides were removed using SPE to obtain polysaccharide-free extracts and polyphenolic compositions were photometrically characterized. From all samples, total soluble polysaccharides (TSP) were extracted by ethanolic precipitation and characterized including monomer composition, molecular weight (MW) distribution and the degree of methylation (DM) and acetylation (DA).

In most of the wines, polysaccharides increased the anthocyanin readings in the photometric assay compared to the corresponding polysaccharide-free extracts, which may be attributed to a copigmentation-like effect resulting from the adsorption of anthocyanins to pectin

fragments followed by their self-association (Mazzaracchio et al., 2004; Padayachee et al., 2012; Fernandes et al., 2020).

The wines can be divided into two groups according to the precipitability of condensed tannins. In the presence of pectic polysaccharides, the Adentu, Las Mulas, and Weinbiet wines showed higher concentrations of protein precipitable tannins than their corresponding extracts, while the Beringer, Bundschuh, and Canyon Road wines showed the opposite. Similarly, the precipitability of polymeric pigments seemed to be influenced by polysaccharides. All wines contained less np-PP and more p-PP than their corresponding polysaccharide-free extracts, with Adentu, Las Mulas, and Weinbiet wines showing the greatest differences. No conventional p-PP, but pigmented, protein precipitable polysaccharide-polyphenol aggregates were formed in the latter two wines. The lack of pigmented tannins indicates that certain pectic polysaccharides lead to an increased precipitation of anthocyanins, condensed tannins, and np-PP, which may result in an impaired incorporation of anthocyanins into condensed tannins.

The MW distribution and monomer composition of the TSP showed that the Adentu, Las Mulas, and Weinbiet wines contain high proportions of large homogalacturonan (HG), rhamnogalacturonan I (RG-I), arabinogalactans (AG), and arabinogalactan-proteins (AGP) fragments. On the other hand, the Beringer, Bundschuh, and Canyon Road wines contain high proportions of rhamnogalacturonan II (RG-II) and low molecular weight HG fragments. While AGs and AGPs are considered neutral pectin fragments (Vernhet et al., 1996; Carvalho et al., 2006), the polarity of HG depends on its DM. The Adentu, Las Mulas, and Weinbiet wines showed that their high MW HG fragments are highly esterified, whereas the other wines contained small, less esterified HG fragments, which may be described as polygalacturonic acids (de Freitas et al., 2003).

Altogether, specific polysaccharide compositions led to interactions with polyphenols, which altered the tannin and pigment compositions of Cabernet Sauvignon red wines. While large and neutral pectic polysaccharides increased tannin and pigment precipitation, small and acidic pectin fragments decreased tannin precipitation. The former led to the impaired formation of p-PP and the development of pigmented, protein precipitable polysaccharide-polyphenol aggregates which may contribute to red wine color. Unlike conventional p-PP, these complexes are non-covalently bound making their formation reversible (Weber, 2022), which could possibly lead to a lower long-term color stability. Furthermore, pigmented tannins were associated with the attenuation of astringency during red wine aging; hence, the altered protein precipitability of condensed tannins and pigments in the presence of pectic polysaccharides could also affect red wine astringency perception.

Chapter 4

Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines

This chapter was submitted for publication as
Weilack, I., Mehren, L., & Weber, F. Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines.

The revised article will be published in Food Hydrocolloids in December 2024, 157, Weilack, I., Mehren, L., Schieber, A., & Weber, F. (2024). Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines, 110402. *
DOI: 10.1016/j.foodhyd.2024.110402

The final and fully citable article was published online on 09 July 2024 (open access).

The research presented in this chapter was reprinted in Appendix E with permission from the authors.

Author contributions: Ingrid Weilack: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Lea Mehren**: Methodology, Investigation, Formal analysis. **Fabian Weber**: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition.

Summary

Anthocyanins, condensed tannins, and polymeric pigments, which are formed during red wine aging, are arguably the most important phenolic constituents of red wine because they provide color, color stability, and mouthfeel properties like astringency. The extraction of polyphenols is accompanied with the extraction of grape-derived pectic polysaccharides in the must and wine (Gao et al., 2015), where both compound classes can interact with each other during fermentation and wine aging (Weber, 2022). As shown earlier (de Freitas & Mateus, 2001; Carvalho et al., 2006; Watrelot et al., 2017), these interactions can affect tannin and pigment precipitability, altering their composition and ultimately red wine quality. The implications for red wine quality depend on the structural features of the polysaccharides. While large and neutral pectic polysaccharides showed an increase in tannin and pigment precipitation, small and acidic pectin fragments decreased tannin precipitation (Weilack et al., 2023). Therefore, polysaccharides can be categorized according to their colloidal stability. Polysaccharides with the ability to stabilize wine color, prevent tannin (self-) aggregation and precipitation are referred to as protective/stable colloids (Riou et al., 2002; Guadalupe et al., 2007; Alcalde-Eon et al., 2014). In contrast, the enhanced precipitability of condensed tannins and pigments in the presence of certain polysaccharides is due to the formation of unstable colloids (Guadalupe et al., 2007; Alcalde-Eon et al., 2014).

This study is based on the previous work presented in **chapter 3**, where the implications of the interactions between pectic polysaccharides and polyphenols on the composition of the latter were investigated. **Chapter 3** showed that the complexation of condensed tannins and pigments by large and neutral polysaccharides might have led to an impaired formation of precipitable polymeric pigments (p-PP). Instead, pigmented protein precipitable polysaccharide-polyphenol complexes emerged from these interactions (**Chapter 3**). Because p-PP were reported to ensure long-term color stability and play a role in the attenuation of red wine astringency during aging (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013; Weilack et al., 2021), the present study aimed at revealing the impact of pectic polysaccharides on the astringency perception and aging potential of red wines.

To investigate the effect of red wine aging on the interactions between polysaccharides and polyphenols, polyphenolic and pectic polysaccharide composition of commercially available Cabernet Sauvignon wines which were subjected to forced aging were characterized. The wines included six commercially available Cabernet Sauvignon wines of the 2018 vintage, which were from the following wineries: Weinbiet and Emil Bauer (Bundschuh) from the Palatinate region in Germany, Adentu and Las Mulas from Central Valley, Chile, and Beringer

and Canyon Road from California, USA. To trigger age-related compositional changes, the wines were stored at 35 °C for 10 weeks. The aging effect was examined by comparing the aged samples with reference wines, which were stored at 10 °C for the same amount of time. Polysaccharides were removed using SPE to obtain polysaccharide-free extracts and polyphenolic compositions of both sample types were photometrically characterized. From all samples, total soluble polysaccharides (TSP) were precipitated and characterized including monomer composition, molecular weight (MW) distribution and the degree of methylation (DM) and acetylation (DA). Sensory profiling of the attributes “sour”, “bitter”, “overall astringency”, “unripe” and “dry” was conducted on all samples to examine the implications of polysaccharide-polyphenol interactions on the astringency perception of red wines.

Polysaccharides showed a shift of MW towards smaller pectin fragments together with an increase in galacturonans (HG, RG-I, and RG-II) indicating that polysaccharides were degraded and re-solubilized from the wine sediment. Despite the changes in polysaccharide composition, the analyzed wines can still be classified into the same two groups based on their colloidal stability, with group 1 (Adentu, Las Mulas, and Weinbiet “aged” wines) showing an increased precipitability of tannins, while group 2 tannins (Beringer, Bundschuh, and Canyon Road “aged” wines) are prevented from protein precipitation.

Similar to the previous study (Weilack et al. 2023), the “aged” Las Mulas and Weinbiet extracts contained hardly any p-PP, whereas the corresponding “aged” wines showed an increase in p-PP. This gives rise to two conclusions: On the one hand, the incline in p-PP measured in the wines is due to the ongoing pigmentation of polysaccharide-polyphenol complexes. On the other hand, these complexes lead to an impaired age-related incorporation of anthocyanins into tannins. In contrast, the “aged” Bundschuh wine showed an actual increase in conventional p-PP.

Sensory analysis revealed that the perception of the astringency sub-qualities “unripe” and “dry” are related to the interactions between pectic polysaccharides and polyphenols, whereas “overall astringency” was related to the aging of the samples. Depending on the polysaccharide composition, the astringency of the wines increased or decreased during aging. The astringency of group 1 wines, which showed an increase in precipitable and pigmented polysaccharide-polyphenol complexes, attenuated during aging. On the contrary, the astringency of the group 2 wines, which showed an incline in conventional p-PP, increased during aging.

Altogether, the results indicate that the age-related attenuation of red wine astringency may not only be due to pigmented tannins but also non-covalently bound polysaccharide-polyphenol-pigments. Moreover, the polysaccharide composition at the beginning of the aging

process appears to be decisive for the colloidal stability of the wines as well as the development of the polyphenolic composition, especially the formation of p-PP, and ultimately the astringency of aging wines.

1 Introduction

It is generally accepted that anthocyanins and condensed tannins are two of the major red wine components which determine product quality by providing color and mouthfeel to red wines. Anthocyanins and condensed tannins are extracted from the grape berry to the must during winemaking, but the extractability of these compounds is strongly influenced by various inherent and external factors like the type of polyphenol, winemaking techniques, and grape maturity (Hanlin et al., 2010; Hernández-Hierro et al., 2012; Hensen et al., 2022). The latter also determines the composition of cell wall polysaccharides, as the ripening process of grapes is associated with a balance between biosynthesis and enzymatic degradation of polysaccharides like hemicellulose, cellulose, and in particular pectin (Nunan et al., 1998). This includes the de-esterification of methylated galacturonic acid units followed by the breakdown of unesterified polygalacturonans and (1→4)-β-galactans of pectic polysaccharides (Nunan et al., 1998; Minic & Jouanin, 2006). These alterations in polysaccharide composition affect the interactions with polyphenols and ultimately the extractability of condensed tannins and anthocyanins as they constantly de- and adsorb on grape cell material (Hanlin et al., 2010; Medina-Plaza et al., 2020). Together with polyphenols, pectin fragments end up in the wine where interactions driven by hydrogen bonding, hydrophobic and electrostatic forces take place (Gao et al., 2015; Weber, 2022).

The interactions with polyphenols impact their protein precipitability, whereby prevention and enhancement of protein precipitation were both reported depending on the type of pectic polysaccharide (de Freitas & Mateus, 2001; Carvalho et al., 2006; Watrelot et al., 2017). Polysaccharides with the ability to stabilize wine color, prevent tannin (self-) aggregation and precipitation are referred to as protective/stable colloids (Riou et al., 2002; Guadalupe et al., 2007; Alcalde-Eon et al., 2014). The enhanced precipitability of condensed tannins and pigments in the presence of certain polysaccharides is due to the formation of unstable colloids, which consist of pigmented protein precipitable polysaccharide-polyphenol complexes (Guadalupe et al., 2007; Alcalde-Eon et al., 2014; Weilack et al., 2023). This phenomenon was reported to impair the covalent incorporation of anthocyanins into tannins to form pigmented tannins during red wine aging (Weilack et al., 2023). Earlier research (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013; Weilack et al., 2021) suggests that the formation of pigmented tannins plays a role in the attenuation and softening of astringency associated with wine aging due to changes in physico-chemical properties; hence, the impaired formation of pigmented tannins could possibly impact red wine astringency perception. This highlights the necessity to examine the relationship between the structural features of pectic polysaccharides and the colloidal stability of wines.

In general, polysaccharides can alter red wine astringency in the finished wines either by changing its texture or interacting with polyphenols and/or salivary proteins (Luck et al., 1994; Vidal, Francis, Williams, et al., 2004; Brandão et al., 2017). According to Gawel et al. (2000), red wines induce not only the perception of overall astringency, but also astringency related sensations, which can be classified as astringency sub-qualities. Due to the heterogeneous and complex composition of red wines, the exact triggers of different sub-qualities are difficult to unravel but have received increased attention in recent years (Sáenz-Navajas et al., 2020; Wang et al., 2020; Ferrero-del-Teso et al., 2024). However, most studies are based on model wines and isolated reactions between polyphenols, polysaccharides, and proteins (González-Muñoz et al., 2022).

The present work is a follow-up of a previous study (Weilack et al., 2023), which investigated the implications of the interactions between pectic polysaccharides and polyphenols on the composition of the latter in young wines. For the present study, the same six commercially available Cabernet Sauvignon wines were subjected to forced aging to trigger age-related changes in the polyphenolic composition like the formation of polymeric pigments. Besides the characterization of pectic polysaccharides, polysaccharide-free reconstituted wines were composed, and polyphenols were determined and compared in all samples. Additionally, all samples were subjected to sensory profiling including taste attributes “sour” and “bitter” and mouthfeel attributes “overall astringency”, “unripe” and “dry” to examine the influence of pectic polysaccharides on the astringency perception and the changes thereof during aging.

2 Materials and Methods

2.1 Materials

Maleic acid, ferric chloride, triethanolamine (TEA), and tartaric acid were sourced from Alfa Aesar (Kandel, Germany). Urea, bovine serum albumin fraction V, and (+)-catechin were acqired from Carl Roth (Karlsruhe, Germany). Acetic acid, hydrochloric acid (HCl), ethanol, and potassium bisulfite were sourced from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide and sodium nitrate were obtained from Honeywell Fluka (Offenbach, Germany) and Acros Organics (Geel, Belgium), respectively. Propionic acid, n-propanol, and sodium azide were purchased from Merck KGaA (Darmstadt, Germany). Sodium chloride, sulfuric acid, and methanol (HPLC grade) were acquired from Th. Geyer GmbH & Co. KG (Renningen, Deutschland). Food-grade sodium hydroxide, ethanol, and acetic acid were obtained from Emprove Essential (Merck KGaA, Darmstadt, Germany), Brennerei Kessler (Bad Peterstal-Griesbach, Germany), and Macron Fine Chemicals (VWR International GmbH, Darmstadt, Germany), respectively. Food grade adsorbent resin (Resinex AD3300) was provided by Jacobi Carbons Group (Frankfurt am Main, Germany). For the model wines, food-grade sucrose (Südzucker AG, Mannheim, Germany), tartaric acid (Otto Fischar GmbH, Saarbrücken, Germany), potassium bitartrate (Natuurlijk, Ede, Netherlands) and lactic acid (Otto Fischar GmbH, Saarbrücken, Germany) were used.

2.2 Wine samples

This study was carried out on commercially available Cabernet Sauvignon wines, which were subjected to forced aging. This was achieved by storing the wines at 35 °C for 10 weeks. Originally, the six different Cabernet Sauvignon wines were of the 2018 vintage from three wine-growing regions. At the time of the study, the wines were 3 years old. The wines were made in the following wineries and regions: Weinbiet (14 % v/v ethanol) and Emil Bauer (Bundschuh, 13.5 % v/v ethanol) from the Palatinate region in Germany, Adentu and Las Mulas (each 13.5 % v/v ethanol) from Central Valley, Chile, and Beringer and Canyon Road (each 13 % v/v ethanol) from California, USA. The wines were chosen to reflect a broad variability of geographical origins. The sample coding references the respective winery with the prefixes “fresh reference” wines for the reference wines, which were kept at 10 °C for 10 weeks, hence, did not undergo aging, and “aged” for the aged wines.

The general composition of the “aged” wines was assessed by Fourier-transform mid-infrared (FT-IR) spectroscopy, including the appropriate calibration method (WineScan FT120 Basic,

Foss, Hilleroed, Denmark) (Table 1). Free and total SO_2 contents were determined by titration and are included in Table 1. All bottles were closed with screw caps. The "fresh reference" wines were object of the previous study (Weilack et al. 2023), in which their general, phenolic, and pectic polysaccharide composition have been investigated.

Table 4-1 General composition of the "aged" Cabernet Sauvignon samples after storage at 35 °C for 10 weeks determined by Fourier-transform mid-infrared (FT-IR) spectroscopy and titration for total and free SO_2. Due to method robustness, analysis was conducted in single determination.

Wine	Glycerol [g/L]	Residual sugars [g/L]	Titratable acidity [g/L TAE[a]]	Tartaric acid [g/L]	Lactic acid [g/L]	pH	Total SO_2 [mg/L]	Free SO_2 [mg/L]
Adentu (CHL)	8.4	2.4	4.7	1.7	1.4	3.7	56	n.d.[b]
Beringer (USA)	9.5	8.0	4.9	1.2	0.9	3.8	99	5
Bundschuh (GER)	9.3	5.7	5.3	1.3	2.0	3.8	90	4
Canyon Road (USA)	9.1	11.2	4.6	1.8	0.9	3.9	72	7
Las Mulas (CHL)	9.5	1.8	4.5	1.2	1.4	3.8	69	5
Weinbiet (GER)	10.5	2.9	4.4	1.1	1.0	3.9	73	23

[a]Titratable acidity is expressed in g/L tartaric acid equivalents (TAE)

[b]n.d. = not detected

2.3 Removal of wine polysaccharides by using solid phase extraction

Solid phase extraction was performed to obtain polysaccharide-free phenolic extracts of the "aged" red wines using a food-grade adsorbent resin and food-grade chemicals. The applied extraction protocol was published by Weber et al. (2013) with a few modifications as described by Weilack et al. (2023). The extracts of two bottles of each wine were combined, concentrated under vacuum, consecutively lyophilized, and yields were determined gravimetrically.

2.4 Polyphenol composition of the wines and polyphenolic extracts

For polyphenol analyses, the lyophilized extracts were combined and 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). The determined phenolic characteristics of the "fresh reference" and "aged" wines and corresponding extracts included total anthocyanins, non-precipitable polymeric pigments (np-PP), precipitable polymeric pigments (p-PP), and were assessed

following the photometric assays described by Harbertson et al. (2002, 2003, 2009, 2015) using a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany). Anthocyanins are expressed as malvidin-3-glucoside equivalents (Mal-3-glu E) based on an empirical factor and tannins were expressed as catechin equivalents (CE) according to an external calibration curve. The “fresh reference” wines and corresponding extracts have been analyzed and results have been published (Weilack et al. 2023). The analyses were conducted in triplicates.

2.5 Precipitation of total soluble polysaccharides

Total soluble polysaccharides (TSP) were obtained from red wines and polyphenol-rich extracts using ethanolic precipitation following the protocol published by Watrelot et al. (2017) with some adjustments outlined by Weilack et al. (2023). The extraction process was carried out twice and yields were determined through gravimetric measurements.

2.6 Characterization of total soluble polysaccharides

2.6.1 Degree of methylation (DM) and degree of acetylation (DA)

According to Larsen et al. (2019), the determination of the degree of methylation (DM) and the degree of acetylation (DA) employed headspace solid-phase dynamic extraction gas chromatography (HS-SPDE-GC) with flame ionization detection (FID) after saponification. The SPDE equipment (Chromtech, Idstein, Germany) was integrated in a CTC-Combi-PAL-Autosampler (Bender and Hobein, Zurich, Switzerland) and connected to a GC FID system (Agilent Technologies model 6890). DM and DA were quantified as mol of methyl/acetyl groups per 100 mol of galacturonic acid (GalAc), as previously outlined (Levigne et al., 2002), and are expressed in percentage. The analysis was conducted in triplicates.

2.6.2 Determination of monosaccharide composition (galacturonic acid, L-rhamnose, and L-fucose)

The quantification of the monomer composition of the total soluble polysaccharides was conducted using the methodology established by Larsen et al. (2019). Sample hydrolysis was carried out as indicated in the enzyme kits from Megazyme (Wicklow, Ireland) using sulfuric acid (2 M) at 100 °C (6 h) for the determination of GalAc and hydrochloric acid (2.4 M) at 100 °C (1 h) for contents of rhamnose and fucose, respectively. After centrifugation at 10947*g*

for 10 minutes, the specific monosaccharides were measured in the supernatant by absorbance readings at 340 nm. The analysis was conducted in triplicates.

2.6.3 Molecular weight distribution of total soluble polysaccharides

The molecular weight (MW) distribution was assessed through high-performance size exclusion chromatography (HPSEC) equipped with a Smartline HPLC system with a RI detector 2300 (Knauer, Berlin, Germany) and two SEC-Diol columns (300 and 120 Å, 3 µm; YMC, Kyoto, Japan) following the method published by Larsen et al. (2019). Samples were dissolved in water (50 °C) and dialyzed against demineralized water (MWCO 12 - 14 kDa). Elution of polysaccharides was achieved using water containing 50 mM sodium nitrate and 0.25 % sodium azide. MWs were determined using eight pullulan standards ranging from 0.504 to 708 kDa (ReadyCal-Kit Pullulan, PSS- Polymer Standards, Mainz, Germany). Chromatograms were segmented into three representative fractions: high molecular weight fraction (15–708 kDa), medium molecular weight fraction (5.5–15 kDa), and low molecular weight fraction (< 5.5 kDa). The proportions of each fraction were calculated relative to the total area. The analysis was conducted in triplicates.

2.6.4 Protein content

The proportion of proteins of the total soluble polysaccharide (TSP) precipitates was quantified using the combustion method on a Euro EA - CHNSO Elemental Analyzer (HEKAtech GmbH, Wegberg, Germany) following the instructions of the manufacturer. Of the samples, 2 mg were weighed into a sample cup and directly analyzed. Acetanilide was used as external standard. Using 6.25 as nitrogen to protein conversion factor, the percentage of proteins was calculated. The analysis was conducted in duplicates.

2.7 Sensory profiling of samples

To determine the effects of interactions between polysaccharides and polyphenols on red wine astringency, the “fresh reference” and “aged” wines and their polysaccharide-free extracts were evaluated by panel tasting. The protocol of the sensory profiling was oriented on the optimized descriptive profiling (ODP) established by Rita de Cássia dos Santos Navarro da Silva et al., (2012), which was shown to be suitable for the correlation of sensory and instrumental measurements. The wines were subjected to a bench tasting to identify the most

important attributes and astringency sub-qualities of the wines. The astringency sub-qualities were selected based on the mouth-feel wheel by Gawel et al. (2000). The attributes that were agreed upon were “sour” and “bitter” as taste attributes and “astringency”, “unripe” and “dry” as mouthfeel attributes. After the pre-selection of the judges, the sensory panel consisted of 19 judges, of which nine were female and 10 were male with ages ranging from 23 to 60 years (mean 41 years). Panelists were recruited form the professional and private environment of the study leader on a strict voluntary basis. The pre-selection of the panel was composed of two sessions. During the first session panelists were familiarized with the attributes and their differentiation. Solutions of caffeine (1.5 g/L; Siegfried Pharma Chemikalien, Minden, Germany), tartaric acid (2.5 g/L; Otto Fischar GmbH, Saarbrücken, Germany), aluminum sulfate (2.5 g/L; Euro OTC Pharma GmbH, Bönen, Germany), catechin (3 g/L; Sigma Aldrich Chemie GmbH, Steinheim, Germany) and tannic acid (2 g/L; Omikron GmbH, Neckarwestheim, Germany) were presented in a Pinot noir wine from 2018 used as basic wine to train “bitter”, “sour”, “astringency”, “unripe” and “dry”, respectively. This was achieved by advising the panel to assign the attributes to the corresponding solutions, whereby the matching had to be 80 % correct. The second session was dedicated to the recognition of various concentrations of these solutions. Solutions of the reference materials were presented containing zero, 33 %, 66 %, and 100 % of the standard concentrations used in the first session, whereby only two of the consecutive samples were allowed to be mixed up. Afterwards, the judges were familiarized with the intensity scale of the final tasting, which was a continuous scale from 0 to 10 for “very low intensity” and “very high intensity”, respectively, and reference material. Two differently concentrated reference solutions were presented and their position on the scale was discussed. Trial tastings were held in two sessions, each presenting four wines and one reference solution. For the profiling tasting, the extract samples were dissolved in model wine solutions prepared for each wine sample according to their general composition determined by FT-IR (Table 1; compare Weilack et al. (2023) for composition of “fresh reference” wines) except for the SO_2 addition and their yield after extraction (Table A.1). Wine samples were presented in a balanced random order in clear, coded glasses and were tasted in duplicate. One reference solution for each attribute was provided in every session. The panelists were advised to taste 10 mL of the samples, keep them in the mouth for 10 seconds and rank intensities after spitting. They were advised to neutralize the oral cavity with water and bread and to wait 3 minutes before tasting the following sample. All sessions were conducted as home use tests using the sensory tool RedJade 2021 (RedJade Sensory Solutions, LLC., Martinez, CA).

The study design was submitted to the Ethics Committee of the Rheinische Friedrich-Wilhelms-Universität Bonn, which approved this study (reference number 460/20). Informed consent was obtained from each panelist as part of the submitted request.

2.8 Statistical analysis

The results were statistically analyzed using XLSTAT (Version 2019.1.1, Addinsoft Technologies, Paris, France). For pairwise comparisons, an ANOVA with a selected significance level of $p < 0.05$ was used. The phenolic composition of the “fresh reference” wines and corresponding extracts, which were previously published by Weilack et al. (2023), were statistically reanalyzed considering the comparison with the results of the “aged” wines and corresponding extracts.

3 Results and Discussion

3.1 Removal of polysaccharides and gravimetric determination of total soluble polysaccharides (TSP)

Table 4-2 presents the yields of the polyphenolic extracts obtained by solid phase extraction and the TSP concentrations of the wines and the extracts, whereby the results for the “fresh reference” wines have been published earlier (Weilack et al., 2023). The TSP concentrations of the polyphenolic extracts were determined by referencing to the corresponding extract concentration in the wine. The results (**Table 4-2**) show that the polyphenolic extracts contain only negligible amounts of TSP compared to the high yields of the wines; hence, the wine polysaccharides were successfully removed ensuring that the differences seen between wines and polysaccharide-free extracts can be assigned to the presence, respectively absence, of soluble polysaccharides.

Because the alcoholic precipitation of TSP causes the co-precipitation of proteins (Selvendran, 1975), the protein concentrations in the precipitates were determined to rule out that these differences are due to interactions between polyphenols and proteins. The protein concentrations ranged from 0.9±0.1 % for the “fresh reference” Adentu wine to 6.3±0.2 % for the “fresh reference” Beringer wine (**Table 4-2**) indicating that the majority of compounds in the precipitates were wine polysaccharides. However, after the aging, the TSP yields of the “aged” Beringer and Canyon Road wines are significantly higher. While the “aged” Canyon Road wine shows no significant difference in protein concentration when compared to the “fresh reference” wine, the protein concentration of the “aged” Beringer wine TSPs is even significantly lower than the one of the “fresh reference” wine. This is why the increase in TSP of these samples is not related to an increased co-precipitation of proteins due to the storage (**Table 4-2**). According to previous studies (Doco et al., 1999; Guadalupe & Ayestarán, 2007; Quijada-Morín et al., 2014), the amount of wine TSP remains unchanged or declines over various periods of storage, but the polysaccharide composition shifts towards smaller pectin fragments. This shows that the precipitation and solubilization of TSP are subject to constant change.

Table 4-2 Yields of the Cabernet Sauvginon polyphenol-rich extracts that are poor in polysaccharides obtained by solid phase extraction, total soluble polysaccharides (TSP) contents of the wine and extract samples obtained by alcoholic precipitation, and protein concentrations of TSP. Means having the same letters are not significantly different at $p \leq 0.05$. Means presented with standard deviation; n = 2-3.

	Wine	Yield Extraction [g/L]	Wine TSP [g/L]	Extract TSP [g/L]	Protein [%]
Adentu	"Fresh reference"	4.32±0.11	3.75±0.23 C,D,E	0.01±0.01 F	0.93±0.06 H
	"Aged"	4.45±0.19	4.99±0.31 A,B,C,D	0.10±0.01 F	3.98±0.07 E
Las Mulas	"Fresh reference"	3.76±0.07	3.24±0.14 D,E	0.03±0.00 F	2.81±0.05G
	"Aged"	4.09±0.52	4.44±0.08 B,C,D,E	0.02±0.02 F	3.44±0.02 F
Weinbiet	"Fresh reference"	4.63±0.38	4.34±0.27 B,C,D,E	0.07±0.02 F	4.55±0.07 D
	"Aged"	4.61±0.33	5.00±0.11 A,B,C,D	0.02±0.02 F	4.78±0.11 C,D
Beringer	"Fresh reference"	3.99±0.20	4.68±0.12 B,C,D,E	0.04±0.01 F	6.32±0.21 A
	"Aged"	3.90±0.01	6.55±0.45 A	0.04±0.01 F	4.59±0.01 D
Bundschuh	"Fresh reference"	5.15±0.07	5.05±0.38 A,B,C,D	0.08±0.00 F	5.76±0.16 B
	"Aged"	5.29±0.29	5.40±0.22 A,B,C	0.20±0.32 F	5.04±0.06 C
Canyon Road	"Fresh reference"	4.24±0.08	3.18±0.07 E	0.01±0.00 F	4.89±0.06 C,D
	"Aged"	4.20±0.03	6.12±0.21 A,B	0.01±0.03 F	4.70±0.03 C,D

3.2 Polyphenol characterization of the wines and the corresponding polysaccharide-free counterparts

Anthocyanins, tannins, and the ratio of protein precipitable polymeric pigments (p-PP) and non-precipitable polymeric pigments (np-PP) of the "fresh reference" and "aged" wines and corresponding polysaccharide-free counterparts were determined (**Figure 4-1**). To display and discuss the compositional changes the wines underwent during aging and the removal of polysaccharides, the results of the "fresh reference" wines and their corresponding extracts, which have been published earlier (Weilack et al. 2023), are included in **Figure 4-1**.

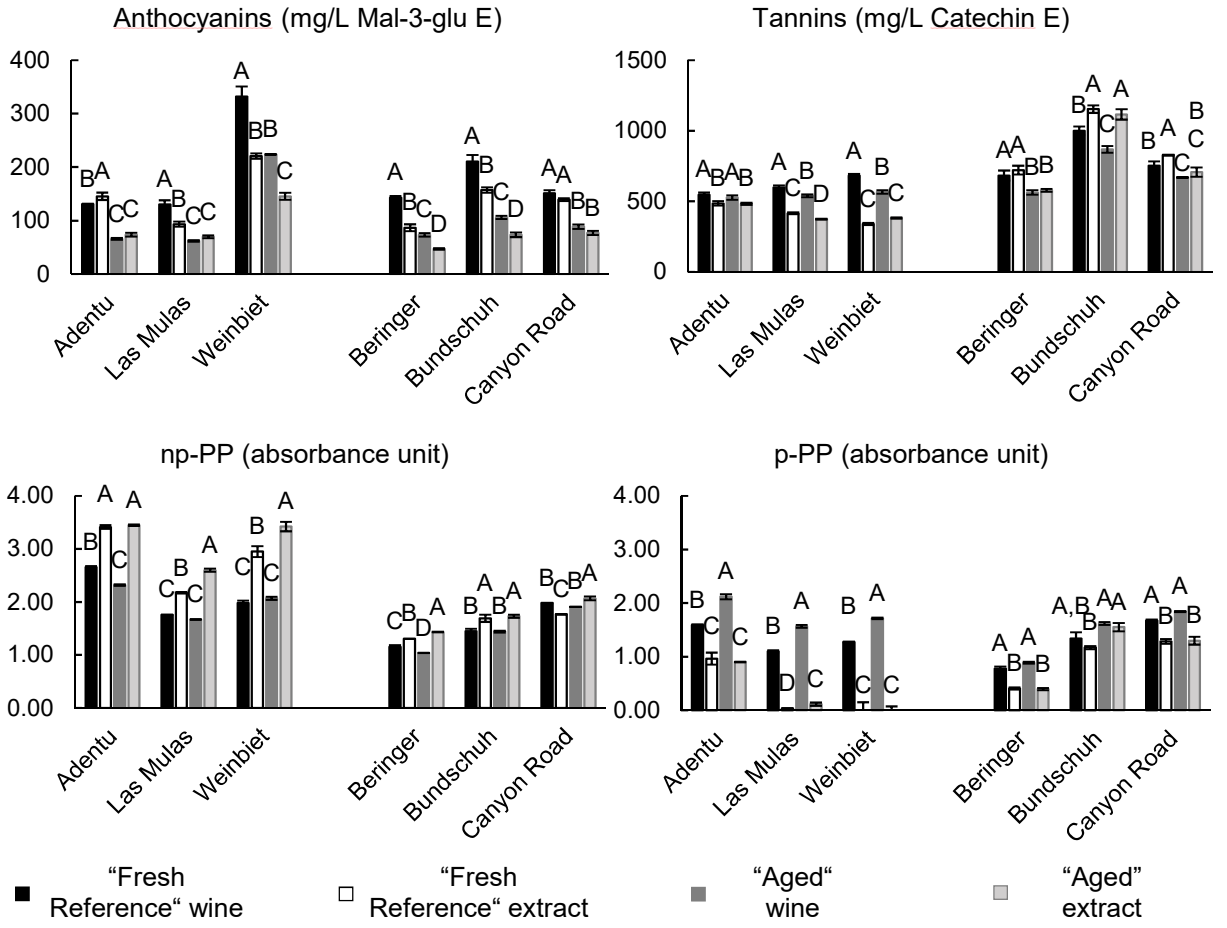


Figure 4-1 Phenolic composition of commercially available Cabernet Sauvignon wines, which were subjected to forced aging (35 °C for 10 weeks; "aged"), "fresh reference" wines (10 °C for 10 weeks), and their corresponding polysaccharide-free extracts. Analyses included total anthocyanins, non-precipitable polymeric pigments (np-PP), precipitable polymeric pigments (p-PP), and total tannins and were obtained by photometric assays (Harbertson et al. 2002, 2003, 2009, 2015). Weilack et al. (2023) have previously published the results of the "fresh reference" wines and extracts. Means having different letters show a significant difference ($p \leq 0.05$) within each wine; n = 3.

While the general compositions of the wines were not altered during the aging (**Table 4-1**; compare with Weilack et al., 2023), **Figure 4-1** shows that the polyphenolic composition of the wines changed due to age-related reactions indicating that the "aged" samples can be seen as a new set of samples. During aging, anthocyanin concentrations generally decreased in wine and extract samples, which was expected due to degradation, conversion, and incorporation of anthocyanins into pyranoanthocyanins and polymeric pigments, respectively (Fulcrand et al., 2006; McRae et al., 2012). Besides the anthocyanins of the "aged" Weinbiet wine, which declined by 33 %, the other "aged" wines show a decline of around 50 % in anthocyanins. Comparing the results of the "fresh reference" extracts with the "aged" extracts reveals that the actual decline of Las Mulas and Weinbiet anthocyanins amounts to 26 % and

34 %, respectively, while the decline in the other extract samples stays at around 50 %. This indicates that the anthocyanins found in these two wines were less affected by the age-related changes in polyphenol composition. The “aged” Beringer, Bundschuh and Weinbiet wines show higher anthocyanin concentrations than their corresponding extracts probably due to copigmentation-like effects resulting from the interactions between wine polysaccharides and anthocyanins (Padayachee et al., 2012; Weilack et al., 2023). These effects lead to batho- and hyperchromic shifts in the absorbance spectra of anthocyanins (Mazzaracchio et al., 2004; Fernandes et al., 2020) and to seemingly higher anthocyanin concentrations in the wines, as assessed by the photometric assay. However, data shows that these copigmentation-like effects became less pronounced during the aging.

Tannin concentrations decreased during aging in all wine samples but the Adentu wine, which shows no changes in tannin concentrations. In contrast, the tannin readings of the polysaccharide-free extracts with the exception of Beringer and Canyon Road show negligible changes in tannin concentrations. According to Weilack et al. (2023) the differing tannin readings, which base on the protein precipitation of tannins, result from the modulation of tannin precipitability by pectic polysaccharides. Thereby, the composition of polysaccharides determines whether tannin precipitability is enhanced or protected. Small, more polar and acidic polysaccharides, like RG-II and polygalacturonic acids, prevent tannin precipitability, whereas large, more neutral and esterified polysaccharides, like AGP, AG and HG fragments, increase tannin precipitability (Vernhet et al., 1996; de Freitas et al., 2003; Mateus et al., 2004; Carvalho et al., 2006; Weilack et al., 2023). As seen earlier (Weilack et al., 2023), this allowed the categorization of the “fresh reference” wines into two distinct groups. Comparing the tannin concentrations of the “aged” wines and corresponding “aged” extracts, it appears that the “aged” samples still divide into the same two groups. Group 1 includes the Adentu, Las Mulas and Weinbiet wines, which show an increased protein precipitability, whereas in group 2 (Beringer, Bundschuh and Canyon Road wines) tannin precipitability decreases in the presence of the respective polysaccharides.

The aging process led to a slight decline in non-precipitable polymeric pigments (np-PP) in the “aged” Adentu and Beringer wines, while they stagnated in the other wines. On the other hand, precipitable polymeric pigments (p-PP) increased significantly in the “aged” group 1 wines. This is supported by previous publications (Merrell et al., 2018; Weilack et al., 2023), which postulated that the formation of np-PP would reach a plateau during red wine aging either due to balanced formation and degradation of np-PP or favored formation of p-PP. However, this study shows that np-PP increased in the “aged” polysaccharide-free extracts while p-PP level in all “aged” extracts but the Bundschuh extract, which shows an increase in p-PP. In general, the “aged” wines contain less np-PP and more p-PP than the “aged” polysaccharide-free

counterparts do, whereas the “aged” polysaccharide-free extracts show higher np-PP proportions and lower p-PP proportions of. Thereby, group 1 wines show the bigger differences; hence, in the presence of pectic polysaccharides np-PP are apparently measured as p-PP indicating a higher protein precipitability (Weilack et al., 2023). Ultimately, this leads to the conclusion that certain structural features of pectic polysaccharides result in the formation of protein precipitable pigments, which are measured as p-PP, but are composed of pigmented polysaccharide-polyphenol complexes (Weilack et al., 2023). The differences in polymeric pigment proportions between wines and extracts become more pronounced with the aging of the wines suggesting the ongoing formation or pigmentation of these protein precipitable pigments. The “fresh reference” Las Mulas and Weinbiet extracts did not contain p-PP, which led Weilack et al. (2023) to hypothesize that their pectic polysaccharides could affect the formation of p-PP. The present study shows that the “aged” Las Mulas and Weinbiet extracts contain very few to no p-PP. Together with the fact that anthocyanins were seemingly less affected by age-related reactions, this confirms that the aggregation of polysaccharides and pigments can even impair the incorporation of anthocyanins into tannins hindering the formation of conventional, covalently bound p-PP. In contrast, the increase in p-PP of the “aged” Bundschuh polysaccharide-free extract suggests that conventional p-PP were actually formed in this wine during aging. The proceeding incorporation of anthocyanins into tannins renders the tannins more polar, which may lead to an increased interaction with polar RG-II molecules (Vernhet et al., 1996). This may result in an enhanced protective effect of RG-II towards the precipitation of tannins in the “aged” Bundschuh wine (Watrelot et al., 2017).

Earlier research (Siebert et al., 1996; Riou et al., 2002; Guadalupe et al., 2007; Alcalde-Eon et al., 2014) reported that some polysaccharides may act as protective colloids in red wine, stabilizing anthocyanins, anthocyanin-derived pigments, and tannins preventing them from aggregating with proteins and self-aggregation. At the same time, Siebert et al. (1996) and Guadalupe et al. (2007) observed the precipitation of unstable colloidal polyphenol protein polysaccharide complexes. Therefore, the altered protein precipitability of tannins and pigments due to polysaccharides can be assigned to the presence and formation of protective/stable or unstable colloids, respectively. Accordingly, the categorization of the wines into two groups can be based on the stability of the colloidal system, being unstable for group 1 (Adentu, Las Mulas and Weinbiet) and stable for group 2 (Beringer, Bundschuh and Canyon Road). In the following, total soluble polysaccharides will be characterized to investigate age-related changes of the structural features of (un-)stable colloids.

3.3 Composition of total soluble polysaccharides (TSP)

To characterize the soluble polysaccharides of the “aged” wines, the molecular weight (MW) distribution, the monomeric sugar composition, and the degree of methylation (DM) and acetylation (DA) were determined using size exclusion chromatography (SEC), photometric assays, and GC-FID (**Table 4-3** and **Figure 4-2**). The polysaccharide composition of the Cabernet Sauvignon wines that were not subjected to accelerated aging, following referred to as “fresh reference” wines, was subject of an earlier work and is described by Weilack et al. (2023).

Figure 4-2 shows the MW distribution of the polysaccharides of the wines, which were stored at elevated temperatures for 10 weeks to simulate red wine aging, following referred to as “aged” wines. The polysaccharides show a high polydispersity across all samples and are split into three fractions: the high MW fraction (>15 kDa) comprising substances such as arabinogalactans (AG), arabinogalactan-proteins (AGP), mannans, mannoproteins (MP), as well as small quantities of homo- (HG) and rhamnogalacturonan I (RG-I) (Ayestarán et al., 2004; Guadalupe & Ayestarán, 2007; Gao et al., 2015); the medium MW fraction (5.5 – 15 kDa) and the low MW fraction (<5.5 kDa), including rhamnogalacturonan II (RG-II) dimers and monomers (~10 kDa and ~4.6 kDa; Pellerin et al. 1996), respectively. Furthermore, these fractions contain fragments of homogalacturonan (HG), rhamnogalacturonan I (RG-I), AGP, and MP with low molecular weights (Guadalupe & Ayestarán, 2007). Except for the “aged” Bundschuh and Beringer wines, the MW distributions of the “aged” wines shift slightly from the high MW to the medium and small MW fractions (**Table 4-3** and **Figure 4-2**). All “aged” wines show a distinct peak in the low MW fraction at around 4.6 kDa, suggesting the presence of RG-II monomers and low MW fragments of other pectic polysaccharides, like polygalacturonic acids. While the proportions of fractions are constant for the Bundschuh wines, the “aged” Beringer wine shows a higher proportion of high MW and a lower proportion of low MW polysaccharides after the aging of the wine. Like the “fresh reference” wines, the “aged” Adentu and Weinbiet wines show the highest proportions of the high MW fraction (**Table 4-3**) suggesting high proportions of AG, AGP, MP, HG and RG-I. In contrast, the “aged” Bundschuh, Beringer, Canyon Road and Las Mulas wines show high proportions of the medium MW fraction, hence, considerable amounts of RG-II molecules and other low MW fragments.

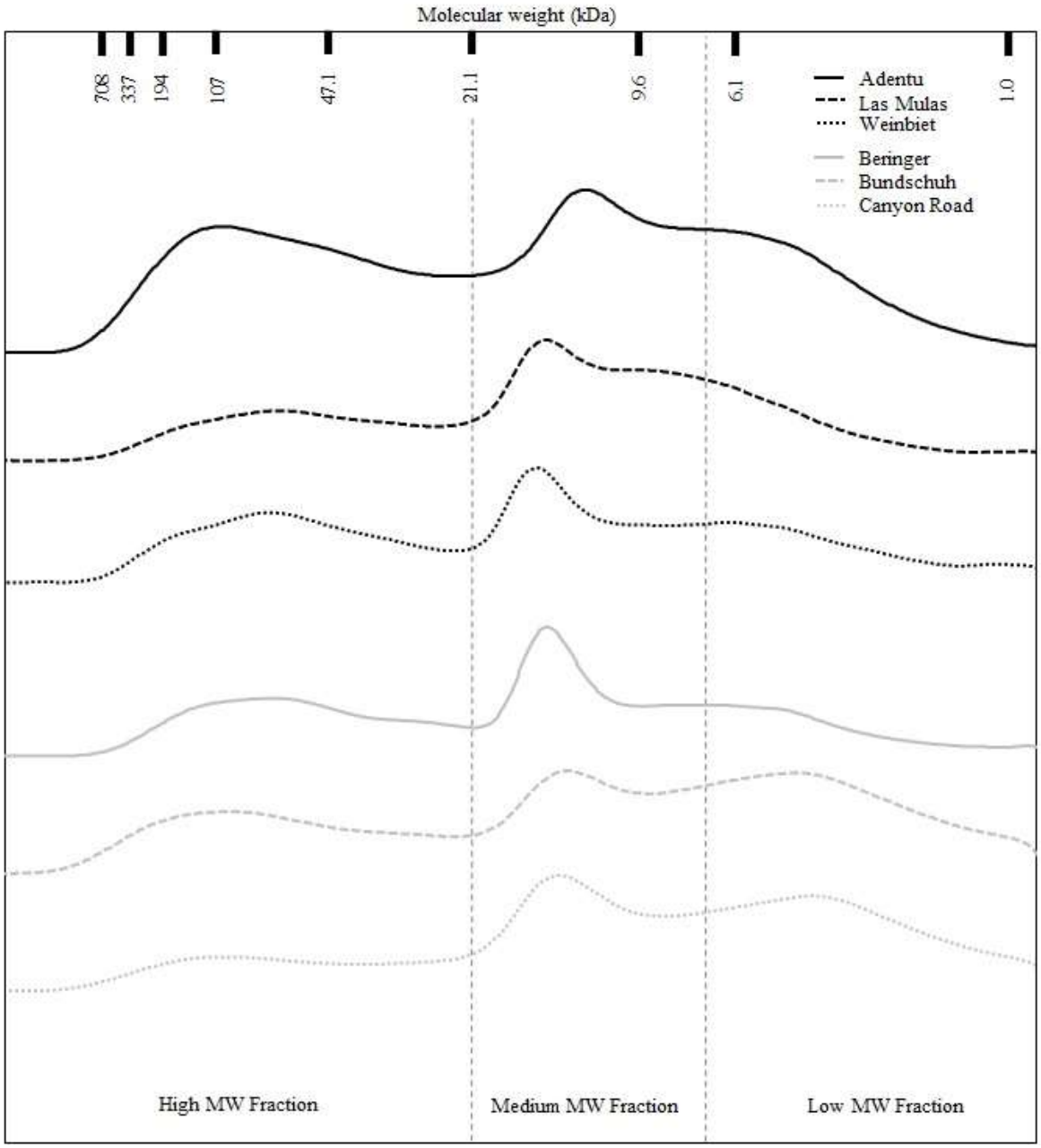


Figure 4-2 Size exclusion chromatograms of the pectic polysaccharides of the "aged" Cabernet Sauvignon wine samples after storage at 35 °C for 10 weeks including the high (> 15 kDa), medium (15 – 5.5 kDa) and low (< 5.5 kDa) molecular weight (MW) fractions. Chromatograms of the "fresh reference" wine samples were presented earlier (Weilack et al., 2023). Column was calibrated with standards illustrating the peak molecular weight distribution.

To further characterize the composition of pectic polysaccharides, galacturonic acid (GalAc), rhamnose (Rha) and fucose (Fuc) concentrations were determined, which can be correlated with the relative proportions of HG, RG-I and RG-II fragments, respectively, due to their well-defined occurrence in the pectin structure (Larsen et al., 2019). While the "fresh reference" wines showed a wide range of GalAc concentrations (16.0±0.6 mg/g for the Adentu wine to

47.6±0.6 mg/g for the Bundschuh wine; Weilack et al. 2023), the aging of the wines led to the approximation of the GalAc concentrations ranging from 31.1±0.1 to 37.0±1.5 mg/g for the "aged" Las Mulas and Adentu wines, respectively. In contrast to the "aged" Bundschuh and Weinbiet wines, which showed lower GalAc concentrations after aging, the GalAc concentrations of the other wines increased. The shift of the MW distribution of the polysaccharides towards smaller molecules, together with an increased TSP yield indicates the degradation of larger HG chains to smaller oligomers during the simulated aging, which resulted in the enhanced solubility and solubilization from the wine sediment.

Table 4-3 Characterization of the pectic polysaccharides of the "aged" Cabernet Sauvignon wine samples after storage at 35 °C for 10 weeks including the distribution of the high and medium molecular weight (MW) fractions, the degree of methylation (DM) and acetylation (DA) and the concentrations of the sugar moieties galacturonic acid (GalAc), rhamnose (Rha), and fucose (Fuc). Means having the same letters are not significantly different at $p \leq 0.05$. Means presented with standard deviation; n = 3.

Wine	High MW fraction (15-708 kDa) [%]	Medium MW fraction (5.5-15 kDa) [%]	DM [%]	DA [%]	GalAc [mg/g]	Rha [mg/g]	Fuc [mg/g]
Adentu	55.3±0.6 A	44.7±0.6 D	8.3±0.1 E	0.1±0.1 D	37.0±1.5 A	9.1±0.5 A	1.7±0.1 A
Beringer	47.4±2.4 B	52.6±2.4 C	24.3±1.1 C	8.2±0.1 A	32.9±3.1 A	3.2±0.1 B	1.3±0.1 B
Bundschuh	49.5±0.6 B	50.5±0.6 C	30.7±0.8 B	3.2 ±0.6 B,C	35.2±0.6 A	2.8±0.1 B,C	1.7±0.1 A
Canyon Raod	34.4±0.6 D	65.6±0.6 A	13.4±0.1 E	3.5±0.2 B,C	33.0±1.8 A	3.3±0.1 B	1.4±0.1 B
Las Mulas	39.7±0.5 C	60.3±0.5 B	19.3±1.0 D	1.9±0.4 C,D	31.1±0.1 A	2.4±0.1 C	1.3±0.1 B
Weinbiet	50.6±1.6 B	49.4±1.6 C	48.5±2.6 A	4.3±0.4 B	34.9±1.0 A	2.3±0.1 C	1.4±0.1 B

Table 4-3 and **Figure 4-2** show that the high MW polysaccharides proportions of the "aged" Adentu wine shows the highest loss during the aging process together with a considerable increase of low MW polysaccharides. Together with the increase in Fuc and Rha this suggests a release of RG-II residues and low MW RG-I fragments from the wine sediment during storage, whereby the former can also be observed in the "aged" Bundschuh wine. On the other hand, the "aged" Beringer wine appears to have lost RG-I fragments, which may be assigned to their precipitation during storage, whereas the increase of the high MW polysaccharides may be due to increased solubilization from the wine sediment. These changes show that precipitation and solubilization of polysaccharides can occur simultaneously and form an equilibrium, which is supported by the other "aged" wines, which show no significant changes in the Rha and Fuc concentrations.

The degree of methylation (DM) indicates the proportion of GalAc units within the HG and RG-I chains that undergo esterification with methanol. Alongside methylation, acetylation of GalAc and Rha units is also observed, which is described with the degree of acetylation (DA). The number of free hydroxyl groups and in particular the number of free and dissociated carboxylic acid groups significantly influences the polarity and hydrophobic nature of pectic polysaccharides. During the aging of the wines, the DM and DA decreased in all samples apart from the “aged” Beringer wine, in which the esterification increased. The decrease of DM and DA together with the increase in GalAc concentrations and the shift to lower MW render the resulting polysaccharides more polar, which impacts the interactions with polyphenols and proteins. As the changes of the polysaccharide composition led to smaller proportions of the large, neutral polysaccharides in the “aged” Adentu, Las Mulas and Weinbiet wines and an increase in smaller, less esterified, thus more acidic, pectin fragments, the differences of tannin concentrations between “aged” wines and corresponding polysaccharide-free extracts become less pronounced. This indicates that the structural changes of pectic polysaccharides result in a slightly less enhanced precipitation of tannins, thus, less unstable colloids. On the other hand, the “aged” Bundschuh wine shows a significantly higher RG-II concentration than the “fresh reference” wine, thus the Bundschuh polysaccharides show a higher masking effect for tannins.

Overall, as described earlier, the “aged” wines can still be categorized into two groups according to the precipitability of tannins and pigments, thus, the colloidal stability. Group 1 wines mainly contained large, neutral, and more esterified, HG and RG-I fragments, which formed unstable colloids with polyphenols and proteins, whereas group 2 polysaccharides appear to be protective colloids, which were composed of a larger proportion of small, acidic, more polar, and less esterified pectin fragments including RG-II. This indicates that the polysaccharide composition of the wines at the beginning of the aging process is decisive for the development of wine polyphenols and particularly for essential compounds like polymeric pigments.

3.4 Sensory analysis

3.4.1 Implications of polyphenol and polysaccharide interactions on the astringency perception of “fresh reference” Cabernet Sauvignon wines

Astringency and bitter perception of red wines are associated with tannin concentrations and their composition, which includes the degree of polymerization, galloylation and the number of trihydroxylated monomers (Noble, 1998; de Freitas & Mateus, 2001; Vidal, Francis, et al.,

2003). To ensure that the results of the sensory analysis can be reasoned by the presence or the lack of polysaccharides rather than tannin composition in the samples, the polysaccharide-free polyphenolic extracts were dissolved in model wines that were composed according to their yields and the general composition of the corresponding wines. This way, differences caused by ethanol content, residual sugar, acidity, and pH value are eliminated.

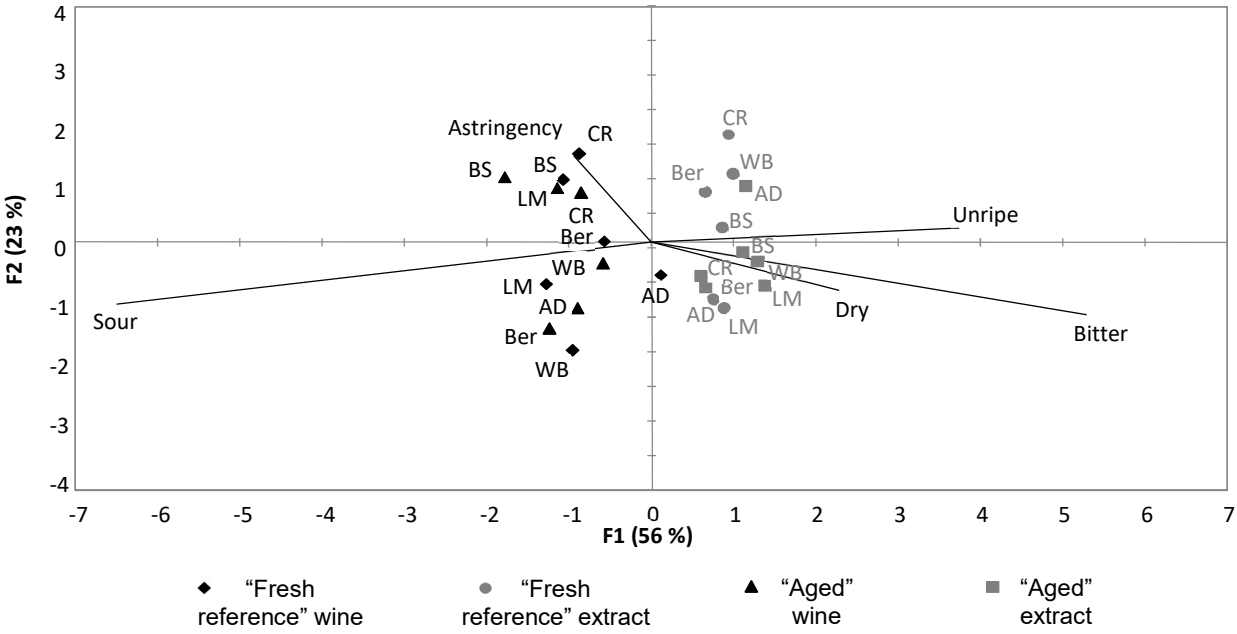


Figure 4-3 Principal component analysis (PCA) of the sensory profiling of all Cabernet Sauvignon wines (AD: Adentu, Ber: Beringer, BS: Bundschuh, CR: Canyon Road, LM: Las Mulas, WB: Weinbiet) and corresponding polysaccharide-free extracts, including the attributes "sour", "bitter", "astringency", "unripe", and "dry".

Figure 4-3 shows the principal component analysis (PCA) of the sensory profiling of all Cabernet Sauvignon samples, the wines and the corresponding polysaccharide-free extracts, including the attributes "sour", "bitter", "astringency", "unripe", and "dry". Gawel et al. (2000) defined the astringency sub-qualities "unripe" and "dry" as "a negative hedonic grouping consisting of an astringent feel associated with excessive acidity and associated green flavor notes" and "feelings of lack of lubrication or desiccation in the mouth", respectively. These definitions were presented to the panel during the tastings. The detailed ratings of the attributes for the wines and extracts are presented in **Figure 4-4**.

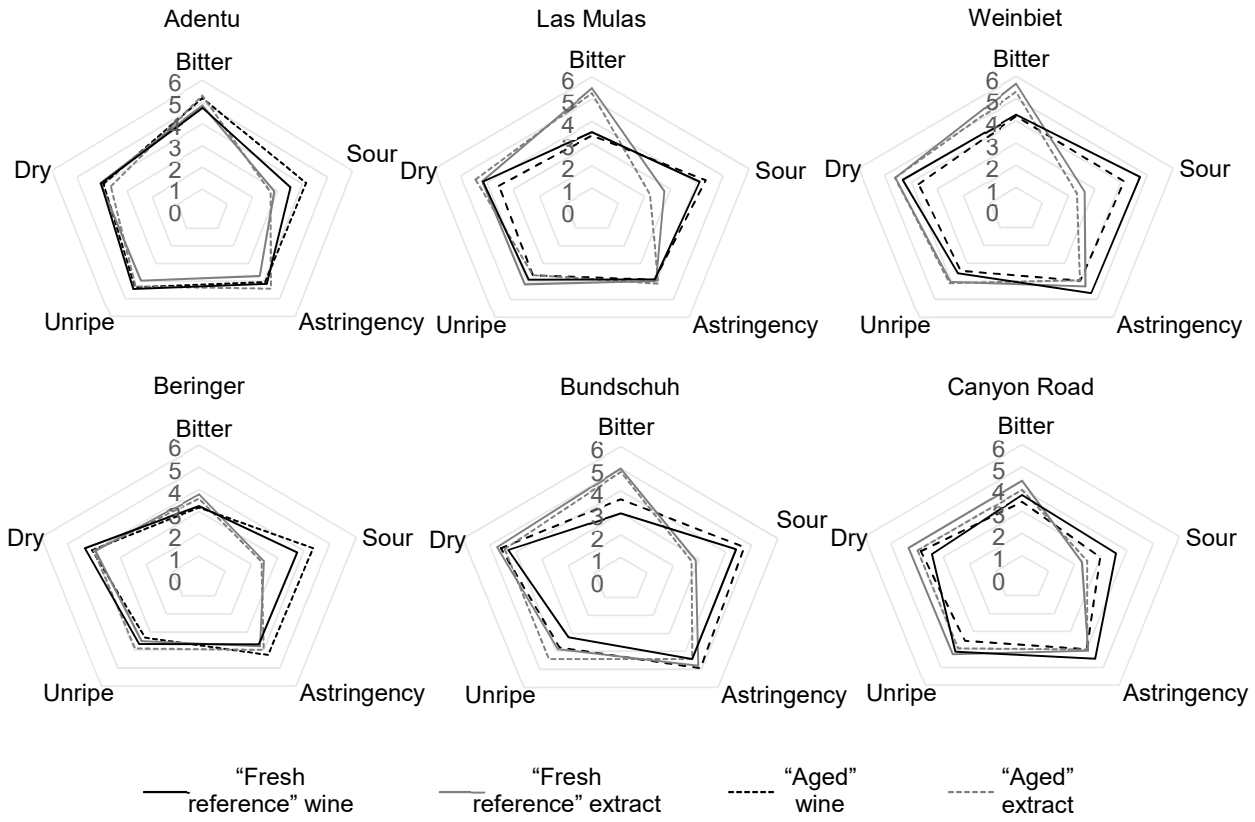


Figure 4-4 Ratings of the sensory profiling of all Cabernet Sauvignon wines and corresponding polysaccharide-free extracts including the attributes "sour", "bitter", "astringency", "unripe", and "dry".

The attribute "sour" correlates negatively with the attributes "bitter", "unripe", and "dry", while "astringency" hardly correlates with any of the other attributes. The samples are split into two groups along the first PC showing that the wines tend to be rated more sour than the extracts, whereas the extracts appear to be more bitter, unripe and dry; hence, the samples can be differentiated by whether or not they contain polysaccharides. The sourness of the "fresh reference" wines correlates with the GalAc concentration (R^2=0.792); thus, the sourness perception may be related to the proportion of HG fragments, which were extracted into the wine. Therefore, the wines taste more sour in the presence of acidic pectic polysaccharides. This is supported by Chong et al. (2019), who reported that sourness of Cabernet Sauvignon wines was related to HG concentrations.

While the astringency sub-qualities, "unripe" and "dry", are related to the polysaccharide content of the samples, the overall "astringency" appears to be correlated rather to the aging of the samples, as the "fresh reference" and "aged" samples are spread along the second PC. Therefore, overall astringency and sub-qualities seem to be perceived independently from each other which was also observed by Wang et al. (2020). The PCA shows a higher variance (56 %) between the wines and their polysaccharide-free extracts than between the "fresh reference" and "aged" samples (23 %). According to Sáenz-Navajas et al. (2020), tannin

concentrations were correlated to the general dryness and dryness on palate perception of red wines, whereas tannin activity, which is driven by hydrophobic interactions with proteins, was solely related to the dryness on palate. However, the authors could not link other mouthfeel perceptions to the studied chemical parameters highlighting the need of extending the investigations beyond tannin composition (Sáenz-Navajas et al., 2020). Since hydrophobic interactions play a role in the interactions of tannins and polysaccharides, mouthfeel perceptions are very likely to be also related to polysaccharide composition.

The “fresh reference” Adentu, Canyon Road and Weinbiet wines are more astringent than their polysaccharide-free extracts, whereas the “fresh reference” Bundschuh wine shows lower “astringency” ratings than the extract (**Figure 4-4**). This is more or less in line with the tannin concentrations (**Figure 4-2**), as the Adentu and Weinbiet wines show higher and the Bundschuh wine lower tannin readings than their polysaccharide-free counterparts. However, the higher tannin readings of the Adentu and Weinbiet wines result from an increased tannin precipitability due to interactions between tannins and larger, neutral polysaccharides with a high DM (Weilack et al., 2023). This leads to the assumption, that such tannin-polysaccharide aggregates, which are measured as tannins, contribute to the astringency perception of the wines. Furthermore, not only tannins show a higher precipitability, but also precipitable polymeric pigments are more precipitable. Previous studies (Weber et al., 2013; Watrelot et al., 2017; Weilack et al., 2021) suggested that the formation of pigmented tannins, which are determined as precipitable polymeric pigments, play a role in the attenuation of red wine astringency. The comparison of the “fresh reference” Adentu and Weinbiet wines with their corresponding polysaccharide-free extracts shows that in these wines little to no p-PP were formed, which may also be adding to the increased astringency perception of the wines.

In contrast, the Bundschuh wine contains a higher proportion of small, acidic polysaccharides and a high concentration of RG-II molecules that lead to the prevention of tannin precipitability (Weilack et al., 2023) and consequently to a lowered astringency perception in the wine when compared to the polysaccharide-free extract. The Canyon Road wine appears to be out of line because the “fresh reference” wine is rated more astringent than the corresponding extract while having a lower tannin precipitability due to its polysaccharide composition (Weilack et al. 2023). According to Carvalho et al. (2006), RG-II molecules can have different effects on the protein-tannin aggregation depending on the type of protein. In the presence of the globular protein α-amylase, RG-II appeared to prevent tannin precipitability while in the presence of IB8c, a representative of the proline-rich proteins (PRPs), RG-II increased protein-tannin aggregation (Carvalho et al. 2006). Since BSA used for tannin precipitation is a globular protein, the lower tannin readings of the “fresh reference” Bundschuh and Canyon Road wines cannot simply be transferred to the astringency perception that is assigned to the interactions

between tannins and PRPs (Charlton et al., 2002). However, Vidal et al. (2004) showed that acidic and neutral polysaccharides alone can decrease the astringency perception of a model wine indicating that they can interact with salivary proteins. Besides the highest RG-II concentration, the “fresh reference” Bundschuh wine contains the highest amount of GalAc and the second highest DM indicating the presence of a high proportion of methylated HG fragments. Due to the methylation, these fragments are more hydrophobic and therefore they may take part in hydrophobic interactions with salivary PRPs comparable to the first step of astringency elucidation (Charlton et al. 2002). Consequently, this interaction may lead to a lack of PRPs that could precipitate tannins, thus, to a lower astringency perception of the “fresh reference” Bundschuh wine than of the polysaccharide-free counterpart.

In the “fresh reference” wines and the corresponding extracts, the “unripe” and “dry” perceptions of the wines are significantly correlated with Rha concentrations (“unripe”: R^2=0.625, “dry”: R^2=0.634), GalAc (“unripe”: R^2=-0.725) concentrations and the Rha/GalAc (“unripe”: R^2=0.817) ratio indicating a relationship between these mouthfeel attributes and the HG and RG-I proportions of the wine polysaccharides. The higher proportions of the HG fragments and the lower concentrations of the RG-I fragments of the “fresh reference” Bundschuh, Canyon Road, Las Mulas and Weinbiet wines are related to lower “unripe” and “dry” perceptions of the wines when compared to the corresponding polysaccharide-free extracts (**Figure 4-4**). Vice versa, the “fresh reference” Adentu and Beringer wines with the highest Rha/GalAc ratio appear to be perceived as more unripe than the polysaccharide-free counterparts (**Figure 4-4**). Wang et al. (2020) hypothesized that polysaccharides could lower saliva precipitation by competing with polyphenols in aggregating saliva proteins indicating that polysaccharides can reduce the amount of saliva proteins that could precipitate polyphenols. They used maltodextrin as a model polysaccharide, which is a linear oligo-/polysaccharide consisting of a variable number of glucose units, to prevent saliva precipitation. As HG is a linear chain of repeating GalAc units, which are also partly methylated leading to a higher hydrophobicity, it may be possible that HG fragments, too, could interact directly with the saliva proteins (Einhorn-Stoll et al., 2021; Rodrigues et al., 2021). This may lead to a lack of the latter to precipitate tannins resulting in a protective effect of HG fragments against an unripe and dry perception of red wine. RG-I, on the other hand, is a branched polysaccharide that may be sterically hindered to align and interact with the salivary proteins. A high number of RG-I fragments in the wines would therefore lead to the loss of the protective effect of HG fragments, resulting in an increased unripe and dry astringency.

3.4.2 Implications of polyphenol and polysaccharide interactions on the astringency perception of bottle aged Cabernet Sauvignon wines

As mentioned before, the PCA of the sensory analysis showed a clear separation of the sample types, wine and polysaccharide-free wine, according to their polysaccharide content. The variance between the samples based on aging is not as pronounced. Like shown before, the polysaccharide composition of the wines changes during the aging and the degradation and de-esterification of the pectic polysaccharides into smaller molecules and HG fragments could increase the sourness of the wines (Chong et al. 2019). However, the sourness does not correlate with either the GalAc concentration, or the DM of the acidic pectin fragments after forced aging as it did before.

In contrast to the “fresh reference” wines, the “aged” Adentu, Las Mulas and Weinbiet wines (group 1) are less “astringent”, “unripe” and “dry” than their polysaccharide-free counterparts (**Figure 4-4**). This is accompanied with decreasing tannin concentrations, while the proportions of p-PP increase during aging. As described before, these changes in polyphenol composition are not reflected in the results of the corresponding polysaccharide-free extracts, which stagnate at lower concentrations for both parameters; hence, while still having an increased tannin precipitability, tannins of the “aged” group1 wines are less precipitable after aging. Therefore, the prevented tannin precipitability together with the higher proportions of pigmented polysaccharide aggregates formed during aging may have a positive effect on the astringency development of the wines. Considering that these pigmented polysaccharide aggregates are measured as p-PP because of their protein precipitability and ability to absorb light at 520 nm, this supports the hypothesis that the formation of p-PP during red wine aging may be beneficial for astringency perception and quality. Yet, this study shows that this may not only be due to pigmented tannins, but also non-covalently bound polysaccharide-polyphenol-pigments may contribute to the attenuated astringency of aged red wines.

In contrast, the “aged” group 2 wines (Beringer, Bundschuh and Canyon Road) show higher ratings in the astringency-associated attributes than the “fresh reference” wines, and the “aged” wines are perceived as more astringent than their polysaccharide-free counterparts suggesting an opposing astringency development to the one described before. Thereby, the “aged” Bundschuh wine shows the highest difference in astringency perception between wine and corresponding polysaccharide-free extract. Contrary to the expectation, that astringency would decrease in the presence of pigmented tannins (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013; Weilack et al., 2021), the “aged” Bundschuh wine shows an increased astringency after aging despite the formation of p-PP. As the “aged” Bundschuh wine contains the highest proportion of RG-II and pigmented tannins, the increased astringency perception

may be assigned to enhanced polar interactions between polysaccharides, pigmented tannins and proteins. This suggests that the soluble complexes formed between the polymeric pigments, tannins, and the acidic, low molecular weight pectin fragments, including RG-II molecules, of the “aged” group 2 wines may still add to the astringency perception. This is supported by Brossard et al. (2021), who showed that both, soluble and insoluble, protein-tannin aggregates can modulate red wine astringency and sub-qualities.

Based on the polysaccharide composition of the wines, the Bundschuh wine appears to have undergone an enzyme treatment, while the Weinbiet wine is considered not treated (Weilack et al., 2023). Similar to the results presented in this study, Kuhlman et al. (2022) showed that Cabernet Sauvignon wines which were not treated with enzymes contained higher concentrations of large, neutral pectic polysaccharides and were described as being soft, fine, and velvety, whereas enzyme treated wines were more astringent with hard, chalky, grippy, grainy, and dry sub-qualities. The enzyme treatment was accompanied with higher amounts of polymerized and galloylated polyphenols (Ducasse et al., 2010; Kuhlman et al., 2022), which could lead to a coarser astringency perception (Vidal, Francis, et al., 2003). Overall, astringency appears to emerge from the combination of the respective polysaccharide and polyphenol compositions, whereby the polysaccharide composition can be modulated by using pectolytic enzymes during the winemaking.

It should be mentioned that the astringency attributes are examined individually in this study, but it is unlikely that the reactions described are triggered independently of each other, since many different molecules and structures come together at the same time during red wine consumption (González-Muñoz et al., 2022).

4 Conclusion

The results of the sensory analysis together with the polyphenolic characterization of the wines and corresponding polysaccharide-free extracts show that the samples can be divided into two groups according to the polysaccharide composition of the “fresh reference” wines and their colloidal stability. On the one hand, the polysaccharides of the “fresh reference” Beringer, Bundschuh and Canyon Road wines consisted of a larger proportion of small, acidic, more polar, and less esterified, thus less hydrophobic pectin fragments, which formed stable colloids with polyphenols and prevented precipitation. On the other hand, the “fresh reference” Adentu, Las Mulas, and Weinbiet wines contained a greater portion of large, neutral, and more esterified, thus, more hydrophobic pectin fragments, which form unstable colloids and promote protein precipitation of polyphenols. Moreover, the latter appears to impair the formation of covalent protein precipitable polymeric pigments, which is associated with red wine aging due to the complexation of anthocyanins, tannins, and non-precipitable polymeric pigments and keeping them from further reactions. Instead, pigmented protein precipitable polysaccharide-polyphenol aggregates were formed during aging, which contribute to red wine astringency perception and possibly also to color stability. This suggests that the polysaccharide composition of the wines at the beginning of the aging process may be decisive for the development of the polyphenolic composition and astringency of aging wines. Using enzymes with certain pectolytic activities can modulate the composition of pectic polysaccharides during winemaking. This can influence wine style and its potential for aging, which is why further research on the influence of pectic polysaccharides on the polyphenolic composition and astringency perception of red wine is necessary.

Chapter 5

Concluding remarks

Besides red wine color and mouthfeel, color stability is one of the quality characteristics that determines the potential of the wine for aging. Color stability is obtained by the reactions between flavanols and anthocyanins to form pigments, which provide red color independently of wine pH and age. Not only do these polymeric pigments ensure stable color, but also play a role in the astringency perception of aged red wines as they were associated with its softening (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013). However, the mechanisms of astringency in general and specifically its attenuation during aging are not yet fully understood. This is partly due to the difficulties to characterize polymeric flavanols and pigments and to assign certain astringency perceptions unambiguously to individual structural features of condensed tannins. Moreover, grape-derived polysaccharides were shown to interfere with the interactions between condensed tannins and proteins, which limits the analytical tools even more (Garrido-Bañuelos et al., 2022).

The study presented in **Chapter 2** aimed at establishing the relationship between the structural features of condensed tannins and polymeric pigments and two physico-chemical properties, polarity, and hydrophobicity. These parameters determine the interactions between condensed tannins and polysaccharides/proteins; hence, the goal was to shed light on the mechanism behind the age-related decrease in astringency. This was achieved by combining forced aging, suitable analytical techniques, and the astringency assessment of two commercially available Cabernet Sauvignon wines.

The extracted polyphenols were separated according to their size and polarity on a normal phase (silica gel) column using a ternary gradient, which resulted in three distinct fractions. Their composition was analyzed by UHPLC-DAD-MS and photometric assays and their hydrophobicity was determined by the octanol-water partitioning coefficient (K_{OW}). Whereas the non-polar and hydrophobic fraction contained hydroxybenzoic and hydroxycinnamic acids as well as monomeric and oligomeric flavonoids, the two polar and hydrophilic fractions comprised of anthocyanins, condensed tannins, and polymeric pigments. The elution pattern together with the K_{OW} show that monomeric and oligomeric non-anthocyanin polyphenols are non-polar and hydrophobic and that the polarity and hydrophilicity of condensed tannins and

polymeric pigments increases with size. Therefore, the correlation of astringency and mDP may not only be due to the increase in binding sites (de Freitas & Mateus, 2001; McRae et al., 2013), but also due to a higher polarity and hydrophilicity, which strengthens the binding between condensed tannins and salivary proteins.

Overall, the combination of all analytical techniques together with the simulated aging achieved to visualize the complex structural transformations of condensed tannins and polymeric pigments. Because small and large polymeric pigments were found in both polar fractions with different molecular sizes, their precipitability is not only determined by their size, which is insinuated by their categorization into SPP and LPP. Polymeric pigments have a wide variety of structural features, which is based on differences in subunits, chain length, and pigmentation (Weilack et al., 2021). Therefore, the categorization of polymeric pigments into small and large polymeric pigments is misleading and is replaced by non-precipitable (np-PP) and precipitable (p-PP) polymeric pigments.

Decreasing tannin concentrations of the aged wines correlated partially with their decreasing astringency perception, indicating that tannin concentrations indeed influence the latter. However, comparing the concentrations of the wines and the polyphenolic extracts, which are free from polysaccharides, reveals stagnating rather than decreasing tannin concentrations during aging. Therefore, the alleged decrease in tannin concentrations was rather due to an increasing masking effect of polysaccharides, which protect condensed tannins from precipitating. The enhanced masking effect might be attributed to the structural changes undergone by condensed tannins, which include the polymerization of flavanols and the incorporation of anthocyanins. Because the incorporation of anthocyanins into condensed tannins leads to a positively charged polymer, the higher polarity of pigmented tannins may strengthen the interaction with dissociated pectin fragments and favor ionic interactions. Two mechanisms causing the protective effect of polysaccharides are plausible: First, polysaccharides might complex tannin-protein aggregates, keeping them in solution, or second, polysaccharides might compete with proteins to bind condensed tannins (Mateus et al., 2004). Therefore, the enhanced interaction between p-PPs and polysaccharides may lead to an increased protective effect or a lack in condensed tannins that could precipitate saliva proteins, resulting in a decreased astringency perception.

The astringency development follows the pattern of the polymeric composition and the hydrophobicity of the medium polar fraction, hence, the fraction with smaller condensed tannins and p-PPs. The correlation between the astringency and the physico-chemical properties of the medium polar fraction is either due to its high yield, which exceeds the other yields, or to its composition. Weber et al. (2013) reported that the polymeric pigment fraction

comprising of smaller pigmented tannins had the lowest astringency perception. Together with the study presented in **Chapter 2**, the results suggest that the charge density of polymeric pigments may play an important role in the astringency attenuation of red wines.

The following studies (**Chapter 3** and **Chapter 4**) were designed to further address the impact of ongoing pigmentation of condensed tannins on their interactions with pectic polysaccharides. Therefore, the polysaccharide composition of six commercially available Cabernet Sauvignon wines was characterized and their polysaccharide-free extracts were reconstituted according to their general composition. In both sample types, wines and polysaccharide-free extracts, polyphenols were analyzed using the above-mentioned photometric assays (**Chapter 3**). The wines were subjected to simulated aging and the characterization and reconstitution was repeated with the aged wines. Sensory profiling of all samples, fresh and aged wines, and extracts, was conducted to assess overall astringency. Additionally, the two astringency sub-qualities “unripe” and “drying” were determined to investigate whether the age-related attenuation of astringency is due to a decrease in overall astringency or rather to a change in quality (**Chapter 4**).

All wines contained high MW pectin fragments including AGPs, AGs, RG-I, and HG, as well as low MW fragments including RG-II and polygalacturonic acids. However, they showed a wide range of proportions in which they were present. Moreover, the DM and DA varied from high to very low esterification. These variations in composition indicate different stages of polysaccharide degradation due to endo- or exogenous enzyme activities (Nunan et al., 1998; Minic & Jouanin, 2006). Because no information was available on grape maturity or the use of maceration enzymes, these activities can only be assumed. However, to reveal the impact of different pectin structures on the composition and precipitability of condensed tannins and polymeric pigments is utterly helpful in determining desirable or detrimental effects of enzyme treatments.

The comparison of fresh wines and corresponding polysaccharide-free extracts showed that the pectin fragments had several effects on the composition of the wines. A copigmentation-like effect led to an overestimation of anthocyanins, which was due to the increased absorbance at 520 nm. While p-PP were also overestimated, np-PP appeared to be underestimated, indicating that the presence of the pectin fragments resulted in an increased precipitation of np-PP and probably anthocyanins. However, not all wines showed the same effects, which becomes even clearer when the tannin concentrations were compared. While half of the wines showed a decrease in tannin precipitability due to pectic polysaccharides, in the other wines an increase in tannin precipitability was observed. The same distribution is visible in the proportions of polymeric pigments, where the first half showed only small

differences in polymeric pigment precipitability, while the differences of the second half were much higher. Moreover, two of the polysaccharide-free extracts did not contain any p-PPs at all.

The differences in polymer precipitability can be explained by the polysaccharide composition of the wines, which also separates the wines in two groups. The first group contained high proportions of small, acidic pectin fragments like polygalacturonic acids and RG-II, which led to a protective effect on tannin precipitation, while the second group contained high proportions of large, neutral polysaccharides like AGPs, AGs, and highly esterified HG leading to increased tannin and polymeric pigment precipitation.

Altogether, this study (**Chapter 3**) showed that the colloidal stability of the complexes formed by red wine polyphenols, polysaccharides, and/or proteins depends on the composition of pectic polysaccharides. Moreover, the presence of certain polysaccharide structures appears to prevent the reaction of anthocyanins and condensed tannins, hindering the incorporation of the latter and the formation of polymeric pigments. Instead, the pigmented complexes of polysaccharides and polyphenols mimic p-PPs, which still contribute to the color of red wines. As a result, not only the measurability of polymeric pigments is impaired but also their composition is affected by the interactions of polysaccharides and polyphenols.

Because **chapter 2** showed that pigmented tannins may be important for their astringency perception, **chapter 4** focused on the implications of the mentioned observations on the astringency development of aging red wines.

The aged wines could still be divided in two groups according to the precipitability of condensed tannins and polymeric pigments (**Chapter 3**), indicating that the initial composition of pectic polysaccharides determines the development of the polyphenolic composition of aging red wines. Similar to the results of the first study (**Chapter 2**), tannin concentrations decreased in the wines and stagnated in the corresponding polysaccharide-free extracts during the aging of the wines. However, the comparison of p-PP in wines and extracts showed that the increased p-PP readings in the wines were only due to the ongoing formation or pigmentation of pigmented polysaccharide-polyphenol complexes. Therefore, this complexation was shown to impair the incorporation of anthocyanins into condensed tannins, which can leave some wines with no conventional p-PP at all. Only one wine is excluded from this observation, as it showed an actual increase in p-PP and a decrease in the precipitability of condensed tannins. These results support the hypothesis that higher pigmentation of condensed tannins leads to an enhanced protective effect of polysaccharides.

One of the most significant findings of the study presented in **Chapter 4** is the unequivocal relation between the astringency sub-qualities and the presence of pectic polysaccharides.

While all wines were rated more sour, the polysaccharide-free extracts were characterized by a bitter taste and an “unripe” and “drying” astringency. Moreover, the overall astringency was related to the aging of the wines, which can not simply be explained by an age-related increased pigmentation of condensed tannins because some aged wines did not contain any pigmented tannins.

The astringency perception of the fresh wines and corresponding polysaccharide-free extracts were in line with the tannin concentrations. However, it must be highlighted that in the group of wines with large and neutral polysaccharides the tannin concentrations measured derive from the protein precipitation of tannin-polysaccharide complexes. Therefore, these complexes seem to contribute to the astringency perception of the wines. Keeping this in mind, it is not surprising that the pigmented polysaccharide-polyphenol aggregates which were found in the wines containing large and neutral polysaccharides can also alter astringency. Brossard et al. (2021) proposed that the astringency intensity is driven by the number of aggregates formed, whereby the lowest astringency was perceived with the highest proportion of aggregates. Similarly, in this study the increased aggregation of polysaccharides and pigmented polyphenols was correlated with a decrease in all astringency-related attributes. Overall, the age-related attenuation of astringency may be assigned to an increase in pigmented polymers, which is not only referring to conventional pigmented tannins but also to aggregates of polysaccharides and pigmented polyphenols.

In contrast, the wines that contained small and acidic polysaccharides showed the opposing astringency development as all astringency-associated attributes increased during the aging. One of them was the only wine in which pigmented tannins were formed, indicating that the protection of condensed tannins from precipitation does not necessarily result in a decrease in astringency perception. Due to the composition of polysaccharide structures, the higher amounts of small and acidic pectin fragments are believed to have resulted from enzymatic degradation (Nunan et al., 1998; Minic & Jouanin, 2006). Enzyme treatment during winemaking was reported to result in a higher extraction of highly polymerized and galloylated condensed tannins, which elicit a coarse astringency (Vidal, Francis, et al., 2003; Ducasse et al., 2010). As discussed before, the polymerization of condensed tannins leads to an increase in their polarity and hydrophilicity and is correlated with increased astringency, maybe due to stronger binding to salivary proteins (**Chapter 2**). Furthermore, the pigmentation of galloylated condensed tannins is faster (Bindon, Kassara, et al., 2014), which may lead to a higher solubility of complexes formed between these condensed tannins and polysaccharides. Because both soluble and insoluble aggregates can modulate the astringency perception (Brossard et al., 2021), the formation of soluble complexes may result in the undesirable development of astringency as reported in **Chapter 4**. Overall, astringency appears to emerge

from the combination of the respective polysaccharide and polyphenol compositions, whereby the polysaccharide composition can be modulated using pectolytic enzymes during winemaking.

Conclusion

Red wine polyphenols and their impact on color and mouthfeel have been the subject of investigations for decades. Therefore, phenolic compositions and some structural features are well studied. However, information on compositional changes of polymeric flavonoids during red wine aging and the correlation with astringency development is limited. The mechanistic approach of this thesis provided insights in the complex structural changes red wine polymers are subject to during aging and, therefore, extends existing knowledge. A special role can be assigned to the reactions between anthocyanins and condensed tannins with respect to both color stabilization and alteration of astringency perception.

While the influence of tannin mDP, degree of galloylation, and hydroxylation are well studied, recent reports suggested that structural polysaccharides like pectin interfered with the protein precipitation of condensed tannins and possibly with astringency perception. The present thesis could connect the precipitation of condensed tannins and polymeric pigments with specific structural characteristics of grape-derived pectic polysaccharides and could unambiguously link astringency perception with the presence of these polysaccharides. Moreover, it revealed that some pectin fragments prevent the pigmentation of condensed tannins and instead pigmented polysaccharide-polyphenol aggregates are formed. These aggregates are partly involved in the astringency perception as they correlate with the age-related attenuation of astringency, which indicates that specific pectin fragments are quite beneficial for red wine astringency and especially for the potential for aging.

This thesis sheds new light on the importance of grape-derived pectic polysaccharides for red wine polyphenolic composition and ultimately its astringency perception. Since the use of maceration enzymes can modulate the composition of red wine pectic polysaccharides, it is a powerful tool to achieve a distinct, favorable polysaccharide profile. Therefore, future research should focus on the impact of specific enzyme activities on the polysaccharide composition. Combined with the aging of red wines and assessment of astringency, this approach provides a holistic image of desirable effects on the composition of red wine polyphenols, which ensure long-term color stability and balanced astringency. Furthermore, because polyphenolic compositions depend on grape variety and maturity, experiments should expand to include a wider range of red wine cultivars of different maturities.

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Acknowledgement

To mark the end of this dissertation, I would particularly like to thank the people who have accompanied and supported me during my doctorate at the Institute of Nutritional and Food Sciences at the University of Bonn.

First and foremost, I would like to express my gratitude to Prof. Dr. Andreas Schieber for the opportunity to work at the institute and conduct the experiments that led to the composition of this thesis. I highly appreciate the freedom and support he gave me during my studies.

I would like to thank Prof. Dr. Matthias Wüst for reviewing this dissertation, as well as Prof. Dr. Sarah Egert and Prof. Dr. Karl G. Wagner for the participation in the examination committee.

A special thanks goes to Prof. Dr. Fabian Weber for the consistent guidance, support, and encouragement throughout the years. Thank you for the long discussions about the individual pieces of the puzzle, which should bring us a little closer to the truth hidden in red wine.

Furthermore, I appreciate the work of all the students, who contributed to this dissertation with their enthusiastic work and data analysis. Particularly, I want to thank Christina and Lea who played an important role in the preparation of the mentioned publications.

I would like to express my sincere thanks to all my colleagues for all collaborations and conversations of both professional and private nature. Above all, I would like to thank my office colleague and project partner Jan for all the knowledge and advice he shared with me. I would like to extend my highest appreciation to Rita, Sandra, and Timo, who provided me with so much valuable work in the lab and everything around it. Finally, I would like to thank Maike and Nadine who always had an open door for me, supporting me with their honest and experienced words.

In addition to the support from the professional community, support outside of work played a very important role during the years of my doctoral studies. I would like to wholeheartedly thank my family and friends for all the wonderful moments that have motivated and inspired me, especially in challenging times. The greatest thanks go to my partner Jens, who accompanied me through all the ups and downs for so many years now and has always had my back. I am so very grateful to have you in my life.

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Appendix C

Effect of structural transformations on precipitability and polarity of red wine phenolic polymer

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

Ingrid Weilack,[1] Christina Schmitz,[1] James F. Harbertson,[2,3] and Fabian Weber[1]*

Abstract: Condensed tannins and polymeric pigments are essential red wine components that contribute to color stability, taste, and mouthfeel. Phenolic polymers in red wine consist of flavan-3-ol monomers and anthocyanins and cause the perception of astringency. Due to the chemical heterogeneity of proanthocyanidin polymers, analytical tools to determine the polymers' structural features are limited. Incorporation of anthocyanins increases the structural complexity even more and makes it almost impossible to assess the influence of structure on the perceived astringency. To better understand the structural diversity of red wine polymers, this study combines forced aging and 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 using polarity, the octanol-water partitioning coefficient, protein precipitation assay, ultra high-performance liquid chromatography-mass spectrometry, and color. Tannin concentrations in wine decreased during forced aging and were constant in the corresponding extracts, suggesting alteration of the precipitation behavior. A simultaneous increase in precipitable polymeric pigments leads to the assumption that incorporating anthocyanins into tannin molecules alters their interactions with red wine polysaccharides and proteins, lowering tannin readings. Finding tannins and polymeric pigments in different FLASH-fractions indicates that precipitability of polymers is affected by their 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, increasing understanding of astringency mechanisms.

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

Phenolic compounds are essential components of wine. Anthocyanins and flavan-3-ols are arguably of utmost importance for red wine quality since they contribute to color, stability, taste, and mouthfeel 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 and the number of trihydroxylated monomers, are the driving forces for the intensity and quality of astringency perception, which is due to the loss of lubrication when polyphenols precipitate 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 winemaking. They have a key role in the modulation of color and mouthfeel properties during red wine aging.

Anthocyanins are transformed into more stable pigments, which is accompanied by a loss in wine color density (Bindon et al. 2014). Together with some low molecular weight 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 called polymeric pigments (Remy et al. 2000).

An age-related decrease in tannin concentrations and mean degree of polymerization (mDP) was accompanied by a decline in perceived astringency (Chira et al. 2012). A conflicting study 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 (McRae et al. 2012). 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, incorporation of anthocyanins may affect astringency perception even more than the concomitant increasing polymer length.

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Acknowledgments: This research project was financially supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 20024 N.

Supplemental data is freely available with the online version of this article at www.ajevonline.org.

Manuscript submitted Oct 2020, revised Jan 2021, accepted Feb 2021

doi: 10.5344/ajev.2021.20064

Due to similar chemical structures and the chemical heterogeneity of proanthocyanidin polymer length, subunit composition, and constitution, analysis of these phenolics has proved difficult. Reversed-phase high-performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS) is commonly used to identify and quantify low molecular weight polyphenols, but this approach is limited for tannin analysis since tannins elute as a polydisperse hump (Ma et al. 2018). Methods used to partly characterize red wine polymers include tannin precipitation, either by proteins in combination with bisulfite bleaching (Harbertson et al. 2002, 2003) or by 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 could not analyze pigmented tannins sufficiently (Vidal et al. 2004a), leaving the manifold structures of polymeric pigments still undefined. Consequently, the complex composition of, and alterations in, red wine polymers and their impact on astringency perception remain important topics for study.

This study used normal-phase FLASH-chromatography to fractionate red wine polyphenols by size and polarity. The fractions were chemically characterized, including the determination of their octanol-water partitioning coefficients (K_{OW}) to measure hydrophilicity. K_{OW} is influenced by tannin composition and red wine maturity (Merrell et al. 2018). Combining forced aging and fractionation of polyphenolic wine extracts clarified the relationships between polymeric pigments, tannins, and two physicochemical properties. Polarity and hydrophilicity were investigated to better understand 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. Ethanol, bovine serum albumin fraction V, and (+)-catechin were purchased from Carl Roth. Silica gel 60 Å (particle size 0.063 to 0.2 mm, 70-230 mesh) and sodium hydroxide were purchased from Honeywell Fluka. Urea, maleic acid, ferric chloride, triethanolamine (TEA), and octanol were purchased from Alfa Aesar. Sodium chloride and Amberlite XAD7 were purchased from Labochem Int. and Sigma-Aldrich, respectively.

Wine samples. Six bottles each of two different commercially available wines were analyzed: 2018 Cabernet Sauvignon from the Trapiche winery (Maipú, Mendoza, Argentina) and 2016 Cabernet Sauvignon from the Salentein winery (Tunuyán, Mendoza, Argentina). The wines were assessed in advance by Fourier-transform mid-infrared spectroscopy (FTIR) 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 during forced aging in an older wine than in a younger wine. The 2018 wine had 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, and 1935 mg/L catechin equivalents total phenolic content. The 2016 wine had 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, and 2117 mg/L catechin equivalents total phenolic content. Except for the phenolic content, these parameters were obtained using FTIR, with appropriate calibration (WineScan FT120 Basic, Foss). The total phenolic contents of the wines were not 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 screwcaps 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 loaded onto an Amberlite XAD7 column (65 mm × 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 water. After elution of the wine, the column was washed with 2 L water (1.3 × the bed volume) to remove sugars and organic acids. The polyphenols were eluted with ~3 L ethanol acidified with acetic acid (29:1 v/v) at a gravity flow rate of ~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 × 460 mm; 0.5 L bed volume) using a low-pressure chromatography pump (C-605 pump with C-615 pump manager, Büchi Labortechnik GmbH). 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 min, then preconditioned with solvent A for another 10 min. Subsequently, 5 mL extract dissolved in solvent B was loaded onto the column at a concentration of 75 g/L. Solvents A, B, and C were successively applied to the column for 10 min each and changed manually. Elution was monitored at 280 nm and 520 nm with a Knauer BlueShadow 50D detector and the ClarityChrom Software (Knauer). 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 min. 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 of undiluted wines and sample solutions between 300 and 800 nm were determined using a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH) and a 1 mm path-length glass cuvette (Hellma GmbH & Co. KG). 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) according to OIV recommendations (OIV 2006).

Chemical characterization. Anthocyanins were analyzed as described (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 described (Harbertson et al. 2015). To quantify total iron reactive phenolics, an aliquot of the sample was diluted with the previously mentioned resuspension buffer to a total volume of 875 µL and incubated for 10 min. Absorbance at 510 nm was measured before and after addition of 125 µL ferric chloride solution. Tannins and total iron reactive phenolics were expressed as catechin equivalents (CE) according to an external calibration curve.

Octanol-water partitioning coefficient. One mL of the sample solution was thoroughly mixed with 1 mL octanol and vortexed for 10 sec. For faster separation of the phases, the samples were centrifuged at 9600*g* for 10 min. Subsequently, an aliquot of both phases was injected into the Shimadzu Nexera X2 ultra high-performance liquid chromatography (UHPLC)-DAD system (two Nexera X2 LC-30AD high-pressure gradient pumps, a Prominence DGU-20A5R degasser, a Nexera SIL-30AC autosampler [15°C, injection volume 2 µL], a CTO-20AC Prominence column oven [40°C], and an SPD-M20A Prominence diode array detector; Shimadzu) using an Acquity HSS T3 column (50 mm × 2.1 mm, 1.8 µm; Waters). At a flow rate of 0.5 mL/min, samples were eluted using the following gradient: 0 min, 50% B; 2 min, 100% B; 3.3 min, 100% B; 4 min, 50% B; 7 min, 50% B, with A being water/formic acid (97/3; v/v) and B being acetonitrile/formic acid (97/3; v/v). The partitioning coefficient was formed by the ratio of the samples' total peak area in the octanol phase and the water phase, respectively, according to the chromatogram at 280 nm.

UHPLC-ESI-MS/MS. UHPLC-MS analysis of the fractions was performed on an Acquity UPLC I-Class system (Waters) consisting of a binary pump, an autosampler cooled at 10°C, a column oven set at 40°C, and a DAD scanning from 190 to 800 nm. An Acquity HSS-T3 RP18 column (150 × 2.1 mm; 1.8 µm particle size) combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 × 5 mm, 1.8 µm), both from Waters, was used for 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 UHPLC was coupled to an LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc.) equipped with an electrospray interface (ESI) 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 to 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 measurements using one scan event.

Sensory analysis. To determine the effects of altered 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 who participated in three training sessions prior to the final tasting. The first session was dedicated to 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 2018 Pinot noir base wine were presented to train astringency, bitterness, and sourness perception. The second session was dedicated to 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 wine in individual booths while wearing a blindfold. They were advised to neutralize their oral cavity with water and bread and to wait 3 min before tasting the following sample.

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

Results

Wine samples and storage. The two wines chosen for this study had a similar initial composition and were stored at elevated temperature to accelerate reactions that occur normally during red wine aging. Two bottles of each wine were subjected to forced aging for three or six weeks. FTIR analysis revealed only negligible changes in the wines' general composition after storage. The color, assessed by the CIELab parameters h° and C* (Table 1), showed that the 2018 wines had greater color intensities than the 2016 wines. 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 greater ΔE value of the 2018 wines may be explained by a faster loss of anthocyanins in younger wines due to an exponential decline of anthocyanins during aging (McRae et al. 2012).

Since color intensity correlates with anthocyanin concentration and red wine maturity, the loss of color is consistent

with the fast decline in anthocyanin concentrations during storage (Figure 1A). This development can be explained by the degradation, conversion, and incorporation of anthocyanins into pyranoanthocyanins and polymeric pigments. Figure 1B and 1C indicate higher proportions of polymeric pigments in the 2016 wines than in the 2018 wines, whereby both contain more non-precipitable than precipitable polymeric pigments (PP). While the proportion of precipitable PP increased in both samples, the amount of non-precipitable PP increased in the 2018 wine only. 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 FTIR data, only wine astringency was assessed further by sensory analysis. Sensory evaluation of perceived astringency revealed that the 2016 wine appears to induce higher but still moderate, astringency (Table 2). A four-way ANOVA of the astringency rating including vintage, storage, panelist, and replicate is presented (Supplemental Table 1). The astringency of the wines declined slightly with aging, consistent with the findings for tannin concentrations (Figure 1D). Interestingly, the astringency of the 2018 wine stored for three weeks dropped to 3.5, but increased during another three weeks of storage. This coincides only partially with the tannin concentrations, as tannin concentration declined constantly over time.

Isolation of a polyphenol-rich extract and fractionation using silica gel. The yields of polyphenol-rich extracts obtained by solid phase extraction using Amberlite XAD7

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

Sample/ weeks	Wine		Fraction 1		Fraction 2		Fraction 3	
	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	17.60	30.10	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

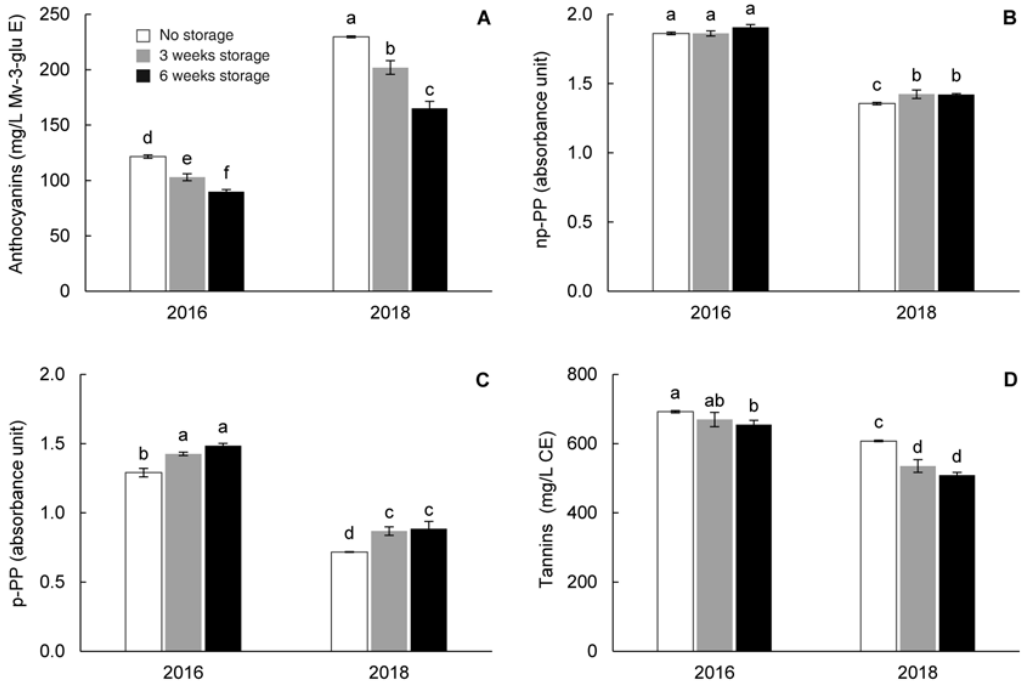

Figure 1 Phenolic composition, including total anthocyanins (**A**), non-precipitable polymeric pigments (np-PP; **B**), precipitable polymeric pigments (p-PP; **C**), and total tannins (**D**), of Cabernet Sauvignon wines after various lengths of storage at 35°C. Results were obtained by photometric assays as described (Harbertson et al. 2002, 2003, 2009, 2015). Means are presented with standard deviation; n = 3. Means having the same letters are not significantly different at $p \leq 0.05$. CE, catechin equivalents.

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 six to eight times to produce enough material for analysis. The separation with silica gel works primarily 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 distribution are shown (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 ~70, fractions 1 had a light orange to yellow color, indicating a limited amount of red pigments. With color hues of 28 and 35, respectively, fractions 2 and 3 of the 2018 wine appeared closer to a blueish red color than fractions 2 and 3 of the 2016 wine, which had values of 37 and 41, respectively.

The protein precipitation assay showed that the highest number of anthocyanins were found in fraction 2 of the 2018 wine (Figure 2A). In all fractions, the amount of non-precipitable PP (Figure 2B) is greater 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 K_{OW} of the fractions. A K_{OW} greater than 1 implies that the fraction is lipophilic, while values below 1 show 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 had hydrophobic properties, while fractions 2 and 3 were both hydrophilic. The greatest hydrophilicity was found in fraction 3 of both vintages. Merrell et al. (2018) determined the K_{OW} of young and aged Cabernet Sauvignon wines and defined coefficients of ~0.19 for young wines. This is comparable to the values found here for the wine extracts (Figure 3A).

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

Changes in the fractions during wine storage. 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 did not lead to a loss in color intensity (chroma), but is consistent with a change in hue that indicates structural changes of pigments rather than a mere loss. Non-precipitable PP (Figure 2B) in the 2018 wine increased in fraction 2 and decreased in fraction 3. Since a less polar solvent elutes fraction 2, these changes in the non-precipitable PP fractions 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, which showed a slight decrease, indicating that the amount of less polar tannins 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 were small, with only fraction 3 of the 2018 wine undergoing considerable changes (Figure 3). Fraction 1 of the 2016

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

	Wine		Extract
Sample/ weeks	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

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 to 8).

	Yield (mg/g) (Proportion [%])					
	2016 sample			2018 sample		
	0 weeks	3 weeks	6 weeks	0 weeks	3 weeks	6 weeks
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)

wine became more hydrophilic, while fraction 1 of the 2018 wine was more hydrophobic after storage. Hydrophilicity increased in fraction 2 of the 2016 wine and fraction 3 of the 2018 wine, while in fraction 3 of the 2016 wine and fraction 2 of the 2018 wine there was no change after six weeks' storage. Nevertheless, after three weeks storage, fraction 3 of the 2016 wine had greater water solubility and fraction 2 of the 2018 wine had less.

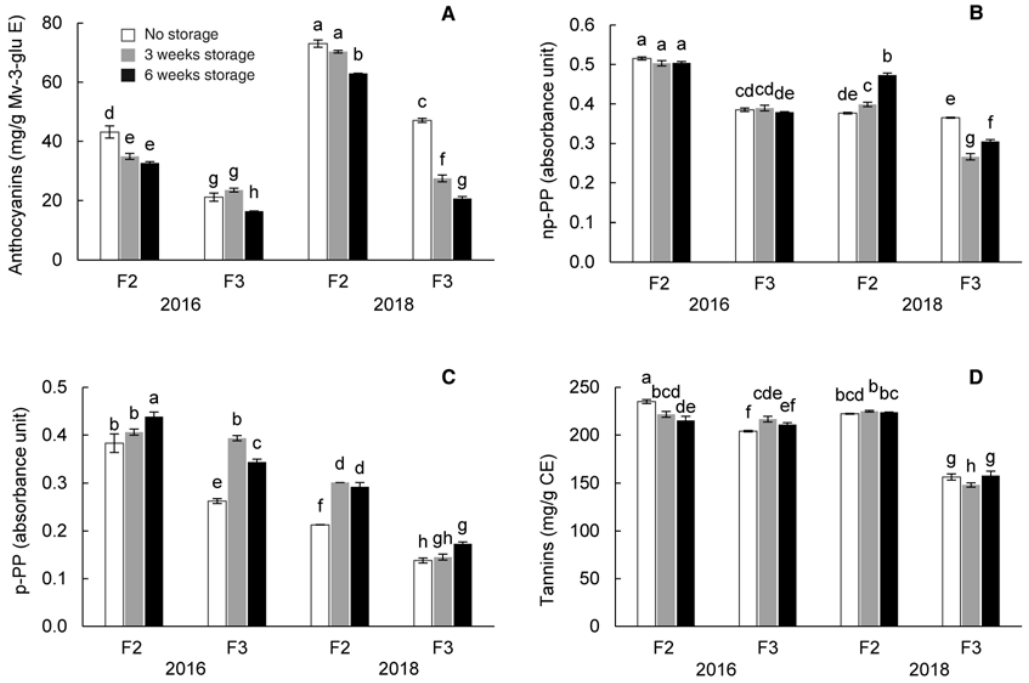

Figure 2 Phenolic composition, including total anthocyanins (**A**), non-precipitable polymeric pigments (np-PP; **B**), precipitable polymeric pigments (p-PP; **C**), and total tannins (**D**) of silica gel chromatography fraction 2 (F2) and fraction 3 (F3) of Cabernet Sauvignon XAD7 extracts after three lengths of storage at 35°C. Results were obtained by photometric assays as described (Harbertson et al. 2002, 2003, 2009, 2015). Means are presented with standard deviation; n = 3. Means having the same letters are not significantly different at $p \leq 0.05$. CE, catechin equivalents.

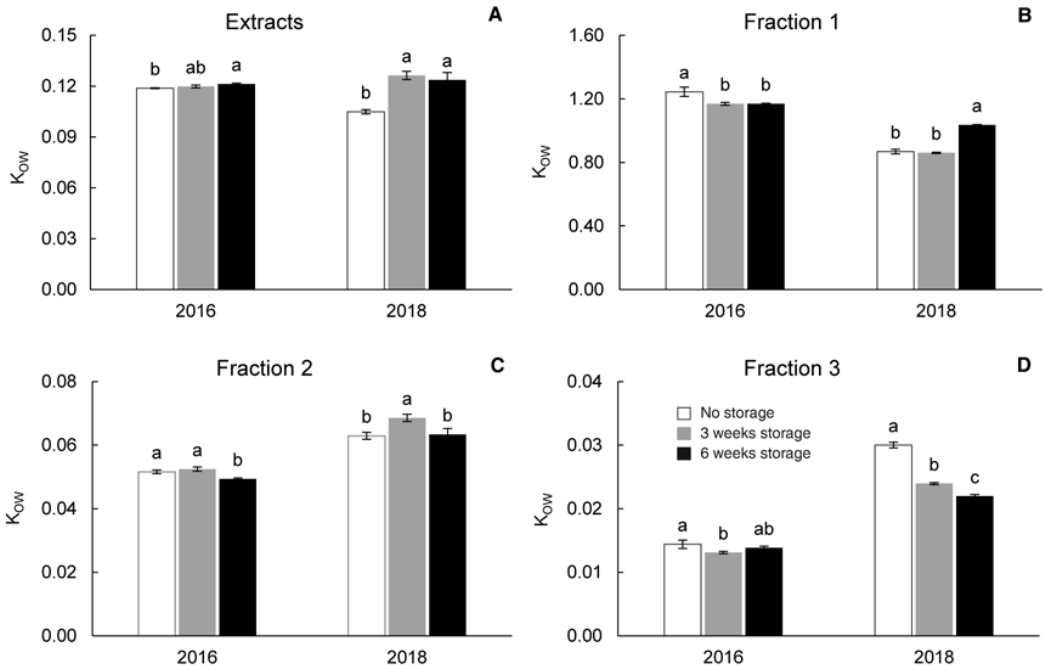

Figure 3 Octanol-water partitioning coefficients (K_{OW}) of XAD7 extracts (**A**) and of silica gel chromatography fractions 1 (**B**), 2 (**C**), and 3 (**D**) of Cabernet Sauvignon wines after three lengths of storage at 35°C. Means are presented with standard deviation; n = 3. Means within columns having the same letters are not significantly different at $p \leq 0.05$.

In contrast to the anthocyanin concentrations, UHPLC-MS showed no changes in the concentration of anthocyanin-derived pigments like pyranoanthocyanins or anthocyanin-flavanol oligomers (Supplemental Tables 2 and 3). Likewise, monomeric flavanols, benzoic acids, hydroxycinnamic acids, and 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 associated red wine astringency with tannin concentration and wine vintage (Boselli et al. 2004, Landon et al. 2008, Chira et al. 2011). Accordingly, 2018 wine was expected to be more astringent than the 2016 wine and both 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 subunits; in particular, their degree of galloylation and trihydroxylation on the B-ring (Vidal et al. 2003). Roughness of astringency increases with proceeding galloylation and decreases with the number of epigallocatechin subunits (Vidal et al. 2003). To compare tannin concentrations in the wines and extracts, the values obtained for the extracts were referenced to the corresponding volumes of the wines considering the respective yield (Table 3). In contrast to the results obtained for the wines, significantly greater tannin concentrations and no significant changes in 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 bovine serum albumin (BSA) (Mateus et al. 2004) used to quantify tannins, which results in lower tannin readings. Since the differences in tannin concentrations between wines and extracts increased, these interactions may become more pronounced when the wine is subjected to forced aging, probably due to structural changes in 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. An investigation of haze formation in red wines treated with carboxymethyl cellulose (CMC) 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 (Sommer et al. 2016). Accordingly, incorporation of anthocyanins into tannin molecules changes the interaction of tannin subunits with polysaccharides and 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 overall astringency intensity, which was compared to the drying mouthfeel evoked by aluminum sulfate. Future research should look at the perception of different astringency sub-qualities to investigate whether decreased astringency rather represents a change in sub-qualities toward a less harsh mouthfeel. These results show that the tannin concentration may not be the only factor that should be considered to evaluate astringency and the sensory quality of the wine in general. Gel permeation chromatography fractions with the most polymeric pigments and rather small tannin concentrations elicited the lowest astringency and green and dry tannins intensity (Weber et al. 2013). A continuously increasing precipitable PP/tannin ratio in the wines may have favored perception of a softer astringency.

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

The ratio of the concentration of lipophilic to hydrophilic compounds in the fractions is reflected by the K_{OW}. The generally greater anthocyanin concentrations in fraction 2 of all samples raised the expectation of greater hydrophilicities of this fraction compared with fraction 3. Since this was not the case, other compounds, like polymeric pigments and tannins, must contribute more to the overall hydrophilicity of the fractions. Tannins with higher degrees of polymerization had lower K_{OW} than their corresponding flavan-3-ol subunits (Hagerman et al. 1998). Hence, a greater degree of polymerization results in greater hydrophilic properties and precipitability. The hydrophobic character of fraction 1 is the result of the presence of monomeric flavan-3-ols, oligomeric procyanidins, and benzoic and hydroxycinnamic acids.

The leveling concentrations of non-precipitable PP in fractions 2 and 3 of the 2016 wine lead to the assumption that the wines reached a maximum non-precipitable PP, as previously reported (Merrell et al. 2018), and which may have two explanations. Either the formation and degradation processes of non-precipitable PP reached an equilibrium or formation of polymeric pigments in the older red wine that was subjected to forced aging favored development of high-molecular weight pigments that are not included in the non-precipitable PP measurement. Precipitation with BSA increased with tannin polymer size, indicating that polymeric pigments that are resistant against SO_2 bleaching and not precipitated with BSA include oligomeric anthocyanin adducts in addition to pyranoanthocyanins (Harbertson et al. 2014). The UHPLC-MS

results showed no considerable changes in the concentration of pyranoanthocyanins and anthocyanin-flavanol dimers (Supplemental Tables 2 and 3). Hence, the protein precipitation assay indicates that anthocyanins are incorporated into existing polymeric structures to form polymeric pigments, rather than forming new oligomeric pigments that grow in size. This is supported by earlier studies that demonstrated that direct adducts of tannins and anthocyanins are formed after the preceding acid-catalyzed cleavage of procyanidins (Haslam 1980, Salas et al. 2003, 2004). The products formed during this reaction may still be regarded as polymeric structures, although they may be of lower molecular weight due to the breakdown process.

The decline of tannins in fraction 2 of the 2016 wine, together with a rise of precipitable PP, results in increased hydrophilicity. This indicates that tannins initially found in fraction 2 of the 2016 wine are rather small and, therefore, non-polar and hydrophobic, while the proceeding incorporation of anthocyanins during forced aging leads to more water-soluble polymeric pigments (Singleton and Trousdale 1992, Merrell et al. 2018). Since the tannin concentration of fraction 3 of the 2016 wine remains constant, the corresponding partitioning coefficients follow the development of precipitable PP, showing that fraction 3 of the 2016 wine contains large and polar tannins that were progressively pigmented during storage. In the 2018 wines, tannin concentrations in fractions 2 and 3 showed no changes over time and accordingly, hydrophilicity seems to be affected by the compositional changes in precipitable PP and non-precipitable PP. As the determination of polymeric pigments is based on their absorption at 520 nm, the protein-precipitation assay does not distinguish among polymers with different intramolecular compositions (Weber et al. 2013). Hence, no conclusion can be drawn about the exact size of the molecules and the proportion of anthocyanins incorporated. The chemical composition of red wine polymers obtained by gel permeation chromatography, based on separation of molecules by size and polarity, has been examined by Weber et al. (2013). Combining several analytical techniques, they showed that early-eluting fractions were composed of large and less pigmented polymers. Further retention on the column eluted polymers of decreasing molecular size and increasing anthocyanin incorporation, followed by less-pigmented, proanthocyanidin-like oligomers. Together with the results of the present study, the changes in hydrophilicity and distribution of polymeric pigments between fractions visualize the compositional transformations of red wine polymers. The hydrophilicity of fraction 2 of the 2018 wine decreased during the first three weeks, while the precipitable PP increased. Because fraction 2 contains less-polar, smaller polymers than fraction 3, this suggests an increase in the amount of smaller precipitable PP, rather than an increase in the proportion of incorporated anthocyanins, i.e., the degree of pigmentation.

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

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

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 perceived astringency (McRae et al. 2013). Our study and others argue for the concept of pigmented tannins being less astringent than non-pigmented tannins (McRae et al. 2013, Weber et al. 2013). Accordingly, a greater degree of pigmentation does not necessarily result in lower hydrophobicity, since other structural features also contribute to overall hydrophobicity of tannins. The greater hydrophobicity of the butanol tannins may be due to greater oxidation and an increased amount of intramolecular bonds, possibly leading to fewer binding sites and reduced astringency (McRae et al. 2013). The interim decline in astringency of the 2018 wine stored for three weeks may be the consequence of the considerably higher non-precipitable PP in fraction 2 and the increased hydrophobicity of this fraction at this point in forced aging, while further alterations of the tannins lead to increased astringency after six weeks of storage.

Finding tannins and PP in both fractions 2 and 3 indicates that size is not solely determinant of protein precipitation by these polymers, it is also affected by physicochemical properties, which in turn depend on tannin molecule size and the ratio of incorporated anthocyanins, among other factors. 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 differences in subunits, chain length, and ratio of incorporated

anthocyanins, and leads to polymers with different physicochemical properties that can be visualized by the K_{OW} and FLASH fractionation. The change in polarity of polymeric pigments in turn alters their ability to interact with wine polysaccharides and saliva proteins. Since the presumed proceeding incorporation of anthocyanins into tannin molecules, which can be assumed by the presented increase in precipitable PP, appears to reduce the measurability of precipitable tannins during forced aging, a special role may be assigned to the interactions of precipitable PP with polysaccharides and proteins. The formation of precipitable PPs during forced red wine aging and their putative enhanced interactions with wine polysaccharides obviously play a key role in the perception of red wine astringency. In particular, the perception of different sub-qualities of astringency seems to be related to the proportion of precipitable PP and polysaccharides, which should be clarified during further research.

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Supplemental Data for:
Weilack I, Schmitz C, Harbertson JF and Weber F. 2021.
Effect of structural transformations on precipitability and polarity of red wine phenolic polymers.
Am J Enol Vitic 72:230-239. doi: 10.5344/ajev.2021.20064.

Supplemental Table 1 Four-way analysis of variance 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

Supplemental Table 2 Heat map of the low molecular weight phenolic composition of 2016 Cabernet Sauvignon wine fractions determined with ultra high-performance liquid chromatography/tandem mass spectrometry after various stages of storage at 35°C. Means are presented with mean standard deviation (mSD) for substance classes; n = 3.

	Fraction 1			Fraction 2			Fraction 3		
Substance (mg/g)	**0 weeks**	**3 weeks**	**6 weeks**	**0 weeks**	**3 weeks**	**6 weeks**	**0 weeks**	**3 weeks**	**6 weeks**
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-(6-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-(6-acetyl)glucoside	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-(6-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-(6-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-(6-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	n.d.	n.d.	n.d.	0.05	0.05	0.05	0.04	0.04	0.04
Peonidin-3-glucoside pyruvate	n.d.	n.d.	n.d.	0.05	0.05	0.05	n.d.	n.d.	n.d.
Malvidin-3-glucosid pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.23	0.20	0.20	0.10	0.11	0.11
Malvidin-3-acetyglucoside pyruvate	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.
Malvidin-3-glucoside-4-vinylcatechol (Pinotin A)	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.
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.

Supplemental Data for:
Weilack I, Schmitz C, Harbertson JF and Weber F. 2021.
Effect of structural transformations on precipitability and polarity of red wine phenolic polymers.
Am J Enol Vitic 72:230-239. doi: 10.5344/ajev.2021.20064.

Supplemental Table 3 Heat map of the low molecular weight phenolic composition of 2018 Cabernet Sauvignon wine fractions determined using ultra high-performance liquid chromatography/tandem mass spectrometry after various times of storage at 35°C. Means are presented with mean standard deviation (mSD) for substance classes; n = 3.

	Fraction 1			Fraction 2			Fraction 3		
Substance (mg/g)	**0 weeks**	**3 weeks**	**6 weeks**	**0 weeks**	**3 weeks**	**6 weeks**	**0 weeks**	**3 weeks**	**6 weeks**
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-(6-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-(6-acetyl)glucoside	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-(6-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-(6-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-(6-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	n.d.	n.d.	n.d.	0.04	0.04	0.03	0.13	0.04	0.03
Peonidin-3-glucoside pyruvate	n.d.	n.d.	n.d.	0.04	0.05	0.04	n.d.	n.d.	n.d.
Malvidin-3-glucosid pyruvate (Vitisin A)	n.d.	n.d.	n.d.	0.22	0.19	0.19	0.09	0.09	0.08
Malvidin-3-acetyglucoside pyruvate	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.
Malvidin-3-glucoside-4-vinylcatechol (Pinotin A)	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.
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.

Appendix D

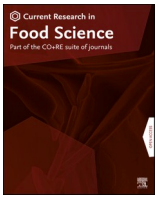
Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines

Current Research in Food Science 6 (2023) 100506

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science



Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines

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ARTICLE INFO

Handling Editor: Professor A.G. Marangoni

Keywords:
Pectic polysaccharides
Tannins
Anthocyanins
Pigments
Interactions
Precipitability

ABSTRACT

Tannins, anthocyanins, and polymeric pigments are essential phenolic constituents of red wine because they provide color, color stability, and mouthfeel properties like astringency. The behavior of these compounds is significantly affected by pectic polysaccharides, whereby the extent of their influence on red wine quality depends on their structural features and their interactions with the polyphenols. In the present study, the composition of the pectic polysaccharides of commercially available Cabernet Sauvignon wines and their impact on anthocyanin, tannin, and polymeric pigment analyses was characterized. This was accomplished by preparation of polysaccharide deprived wines and comparison of the polyphenolic composition of both, the wines and their corresponding polysaccharide-free counterparts. The results show that the cell wall fragments enhance the spectral absorbance of anthocyanins by facilitating anthocyanin self-association, leading to a co-pigmentation-like effect. Low molecular weight pectins like rhamnogalacturonan II and polygalacturonic acids with a low degree of esterification are assumed to form soluble complexes with anthocyanins and also prevent protein precipitation of tannins, which was reduced by 6–13%. High molecular weight pectins with a high degree of esterification lead to the increased precipitability of pigments and tannins by a factor of 1.3 to 32.4 and 1.1 to 1.9, respectively, seemingly impairing the incorporation of anthocyanins in tannins to form precipitable polymeric pigments that are responsible for the longevity of red wine color. The increased precipitability of the pigments due to the interactions with the polysaccharides may indicate the formation of pigmented yet non-covalent aggregates that show comparable properties to the covalently formed precipitable pigments. The formation of those non-covalent structures may affect red wine color stability and astringency.

1. Introduction

While the presence and composition of polyphenols in red wine are undisputed of prime importance for red wine quality, it has become evident that polysaccharides play a modulating role for color and color stability but also for mouthfeel properties like astringency. During fermentation, these compounds are extracted from the grapes to the must or wine, whereby their extractability depends on numerous intrinsic and extrinsic factors like the class of polyphenols, fermentation protocols, and grape maturity. The very polar anthocyanins are readily extracted at the beginning of the fermentation, whereas tannins or procyanidins are extracted later due to the increasing alcohol content. Besides the changes in solubility, the constant desorption and adsorption of phenolic compounds on grape cell materials are of at least the same importance (Hensen et al., 2022). The ripening process of grapes is accompanied by the softening of the grape skin, which is associated with the enzymatic degradation of the polysaccharides in the cell walls like hemicellulose, cellulose, and in particular pectic compounds. This degradation process encompasses overall depolymerization, the loss of arabinogalactans, and the decrease of the degree of methylation, and consequently to an increased solubility of the pectin molecules (Nunan et al., 1998). The change of polysaccharide composition influences the potential interactions with the polyphenols, which increases the extractability of tannins and anthocyanins (Hanlin et al., 2010; Hernández-Hierro et al., 2012). Previous studies (Guadalupe et al., 2007; Ducasse et al., 2010) showed that this can have both positive and negative effects on red wine quality. Wines treated with enzymes displayed higher color intensity and color stability but also higher tannin

Abbreviations: p-PP, precipitable polymeric pigments; np-PP, non-precipitable polymeric pigments.

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https://doi.org/10.1016/j.crfs.2023.100506
Received 30 January 2023; Received in revised form 11 April 2023; Accepted 20 April 2023
Available online 23 April 2023

concentrations. These tannins presented a higher degree of polymerization and galloylation, which was shown to elicit a coarser astringency (Vidal et al., 2003a).

While all these processes occur during maceration, they vastly modify the composition of the resulting wine as most of the de-esterified and de-polymerized pectin fragments find their way into the wine (Gao et al., 2015), where they can interact with the wine polyphenols. These interactions are driven by various mechanisms like hydrophobic interactions, hydrogen bonding, and electrostatic forces, resulting in the formation of non-covalent binding and complexation (Weber, 2022). As a result, the interactions between polysaccharides and polyphenols influence red wine quality characteristics like color and mouthfeel. Several authors (Mazzaracchio et al., 2004; Padayachee et al., 2012; Fernandes et al., 2020) observed that the binding of anthocyanins and polysaccharides results in an intensified red color due to a co-pigmentation-like effect, which can increase the anthocyanin stability. The protein precipitability of tannins can be enhanced or impaired by polysaccharides depending on their structure. De Freitas et al. (2003) showed a disrupting effect for both neutral and acidic polysaccharides, whereas others (Carvalho et al., 2006; Watrelot et al., 2017) reported an increased protein precipitation in the presence of arabinogalactan proteins and rhamnogalacturonan II (RG-II) molecules. Consequently, the changed tannin precipitability alters the astringency perception (Luck et al., 1994; Vidal et al., 2004). Because of the high complexity of red wines, these studies used model experiments to investigate the mechanisms of the underlying interactions between certain pectin fragments and polyphenolic compounds. However, it is necessary to understand which of the before-mentioned interactions actually occur in finished red wines to understand which structural features of pectic polysaccharides are desirable in red wines. This will help to make informed decisions during the winemaking to ensure red wine quality with the potential for ageing. The present study addresses this lack of knowledge by characterizing pectic polysaccharides in commercially available Cabernet Sauvignon wines and investigating their influences on the polyphenolic composition. This was achieved by composing polysaccharide-free counterparts from the wines and comparing the phenolic composition of wines and the corresponding reconstituted wines.

2. Materials and methods

2.1. Materials

Acetic acid, hydrochloric acid (HCl), ethanol, and potassium bisulfite were obtained from VWR International GmbH (Darmstadt, Germany). Urea, bovine serum albumin fraction V, and (+)-catechin were sourced from Carl Roth (Karlsruhe, Germany). Sodium hydroxide and sodium nitrate were acquired from Honeywell Fluka (Offenbach, Germany) and Acros Organics (Geel, Belgium), respectively. Sodium chloride, sulfuric acid, and methanol (HPLC grade) were purchased from Th. Geyer GmbH & Co. KG (Renningen, Deutschland). Maleic acid, ferric chloride, triethanolamine (TEA), and tartaric acid were obtained from Alfa Aesar (Kandel, Germany). Propionic acid, *n*-propanol, and sodium azide were acquired from Merck KGaA (Darmstadt, Germany). Food-grade sodium hydroxide, ethanol, and acetic acid were sourced from Emprove Essential (Merck KGaA, Darmstadt, Germany), Brennerei Kessler (Bad Peterstal-Griesbach, Deutschland), and Macron Fine Chemicals (VWR International GmbH, Darmstadt, Germany), respectively. Food grade adsorbent resin Resinex AD3300 was provided by Jacobi Carbons Group (Frankfurt am Main, Germany).

2.2. Wine samples

Experiments were carried out with six different commercially available Cabernet Sauvignon wines of the 2018 vintage from three wine-growing regions. The wines were from the following wineries and regions: Weinbiet (14% v/v ethanol) and Emil Bauer (Bundschuh, 13.5% v/v ethanol) from the Palatinate region in Germany, Adentu and Las Mulas (each 13.5% v/v ethanol) from Central Valley, Chile, and Beringer and Canyon Road (each 13% v/v ethanol) from California, USA. The wines were chosen to reflect a broad variability of geographical origins. The vintage that the wines were made of assured enough time for initial polymeric pigment formation while still holding an ageing potential. When the experiments were conducted, the wines were three years old. The general composition of the wines was assessed by Fourier-transform mid-infrared (FT-IR) spectroscopy, including the appropriate calibration method (WineScan FT120 Basic, Foss, Hilleroed, Denmark) (Table 1). Free and total SO_2 contents were determined by titration and are included in Table 1. All bottles were closed with screw caps.

2.3. Separation of wine polyphenols and polysaccharides by using solid phase extraction

To obtain polysaccharide-free phenolic extracts from the wines, solid phase extraction using a food-grade adsorbent resin and food-grade chemicals was performed following the protocol published by Weber et al. (2013) with a few modifications as follows. Each wine sample (750 mL) was diluted with water (3:5) and was loaded onto a column filled with Resinex AD3300 (65 mm × 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. The loaded column was washed with 2 L of water (1.3 fold of the bed volume) 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. Extractions were conducted in duplicate, and yields were determined gravimetrically. Prior to further chemical analyses, the lyophilized extracts were pooled and 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).

2.4. Polyphenol characterization of the wine and polyphenolic extracts

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 was diluted with the previously mentioned resuspension buffer to a total volume of 875 μL and incubated for 10 min. Absorbance at 510 nm was measured before and after the addition of 125 μL of ferric chloride solution using the Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany). Tannins and total iron reactive phenolics were expressed as catechin equivalents (CE) according to an external calibration curve.

2.5. Precipitation of total soluble polysaccharides

The total soluble polysaccharides (TSP) were extracted from red wines and polyphenol-rich extracts by ethanolic precipitation according to Watrelot et al. (2017) with some modifications as follows. Ethanol was evaporated from 180 mL of wine and wine was concentrated to dryness by lyophilization. The residue was dissolved in 18 mL of water, obtaining a 10-fold concentration of the wine, and 90 mL of cold ethanol acidified with hydrochloric acid (0.1 M) was added. Samples were kept at 4 °C for 18 h on an orbital shaker at a speed of 150 rpm. Subsequently, the samples were centrifuged at 4816*g* for 20 min. Pellets were washed three times with 80% ethanol, then dissolved in water, and finally lyophilized. To precipitate the TSP from the extract samples, 300 mg of

Table 1
General composition of red wine samples determined by Fourier-transform mid-infrared (FT-IR) spectroscopy and titration for total and free SO_2.

Wine	Glycerol [g/L]	Residual sugars [g/L]	Titratable Acidity [g/L TAE[a]]	Tartaric acid [g/L]	Lactic acid [g/L]	pH	Total SO_2 [mg/L]	Free SO_2 [mg/L]
Adentu	8.4	2.4	4.7	1.9	1.4	3.7	32	n.d.[b]
Beringer	9.5	9.2	4.9	1.3	1.0	3.8	111	25
Bundschuh	9.4	5.4	5.3	1.3	2.1	3.8	90	12
Canyon Road	9.1	11.1	4.4	1.3	0.9	3.9	70	3
Las Mulas	9.5	1.9	4.6	1.3	1.4	3.8	64	6
Weinbiet	10.8	2.8	4.5	1.2	1.1	3.9	78	24

[a] Titratable acidity is expressed in g/L tartaric acid equivalents (TAE).
[b] n.d. = not detected.

extracts was dissolved in 7.5 mL of water and 37.5 mL of cold acidified ethanol (0.1 M) was added. After the precipitation process at the conditions described before, the samples were centrifuged at 10 947*g* for 20 min. Pellets were washed and lyophilized as described before. The extraction was conducted in duplicate, and yields were determined gravimetrically.

2.6. Characterization of the soluble polysaccharides

2.6.1. Determination of the degree of methylation and the degree of acetylation

The degree of methylation (DM) and the degree of acetylation (DA) were determined according to Larsen et al. (2019) using headspace solid-phase dynamic extraction gas chromatography (HS-SPDE-GC) with flame ionization detection (FID) after saponification. The SPDE equipment (Chromtech, Idstein, Germany) was installed in a CTC-Combi-PAL-Autosampler (Bender and Hobein, Zurich, Switzerland) to a GC FID system (Agilent Technologies model 6890). DM and DA were calculated as mol of methyl/acetyl groups per 100 mol of galacturonic acid (GalAc) as described earlier (Levigne et al., 2002) and are given in percentage.

2.6.2. Quantification of galacturonic acid, L-rhamnose, and L-fucose

The monomer composition of the soluble polysaccharides was analyzed following the protocol of Larsen et al. (2019). Hydrolysis of the samples was carried out according to the enzyme kits from Megazyme (Wicklow, Ireland) using sulfuric acid (2 M) at 100 °C (6 h) for the determination of GalAc and hydrochloric acid (2.4 M) at 100 °C (1 h) for contents of rhamnose and fucose, respectively. Specific monosaccharides were analyzed in the supernatant after centrifugation at 10 947*g* for 10 min. Absorbance was measured at 340 nm.

2.6.3. Determination of the molecular weight distribution of soluble polysaccharides

High-performance size exclusion chromatography (HPSEC) on a Smartline HPLC system with a RI detector 2300 (Knauer, Berlin, Germany) equipped with two SEC-Diol columns (300 and 120 Å, 3 μm; YMC, Kyoto, Japan) was used to determine the molecular weight (MW) distribution of the soluble polysaccharides as described by Larsen et al. (2019). Samples were dissolved in water (50 °C) and dialyzed against demineralized water (MWCO 12–14 kDa). Polysaccharides were eluted using water with 50 mM sodium nitrate and 0.25% sodium azide. MWs were calculated with eight pullulan standards ranging from 0.504 to 708 kDa (ReadyCal-Kit Pullulan, PSS- Polymer Standards, Mainz, Germany). The chromatograms were divided into three representative fractions: High molecular weight fraction (15–708 kDa), medium molecular weight fraction (5.5–15 kDa), and low molecular weight fraction (<5.5 kDa). The proportions of the fractions relative to the total area were calculated. Raw data of the MW distribution determined with SEC is shown in table A1 in the supplemental data.

2.7. Statistical analysis

Statistical analysis of the results was performed using XLSTAT (Version 2019.1.1, Addinsoft Technologies, Paris, France). For pairwise comparisons, an ANOVA with a selected significance level of $p < 0.05$ was used.

3. Results and discussion

3.1. Solid phase extraction of polyphenols and gravimetric determination of total soluble polysaccharides (TSP)

The yields of the polysaccharide-free polyphenolic extracts obtained by solid-phase extraction are presented in the supplementary material (Table A2). To verify that the polysaccharides were successfully separated from the polyphenols, the concentrations of the TSP (Table A2) of the polyphenolic extracts were determined and referenced to the corresponding wine concentrations. As the precipitation of the TSP entails the co-precipitation of proteins and polyphenols (Selvendran, 1975), the amounts of proteins and polyphenols in the precipitate were determined. The protein concentration ranged from 0.9 ± 0.1% for the Adentu wine to 6.3 ± 0.2% for the Beringer wine, and the proportions of iron reactive polyphenols ranged from 5.9 ± 1.0% to 16.8 ± 3.4% for the Las Mulas and Bundschuh wines, respectively, indicating that the majority of compounds in the precipitate were wine polysaccharides. The TSP yields of the polyphenolic extracts (Table A2) show that the extracts contain negligible amounts of TSP compared to the high values of the wines and that the wine polysaccharides were successfully removed.

3.2. Polyphenol characterization of the wines and polyphenolic extracts

Fig. 1 presents the results of the photometric assay including the anthocyanin, non-precipitable (np-PP) and precipitable polymeric pigments (p-PP), and tannin measurements of the wines and polyphenolic extracts, respectively. To ensure that no phenolic subclasses were discriminated by the extraction protocol, the total phenolic contents of the samples were determined. The total phenolic contents ranged from 2000 mg/L to 3085 mg/L catechin equivalents in the Las Mulas and Bundschuh wines and from 1667 mg/L to 3217 mg/L catechin equivalents in the Las Mulas and Bundschuh extracts, respectively. The total phenolics of the wines were not significantly different ($p \leq 0.05$) from the corresponding extracts. As expected, there are obvious differences in the composition of the polyphenols between the wine samples that can be caused by regional and enological differences. Since the subject of this study was the evaluation of the interactions between wine polyphenols and polysaccharides, only the differences between the wine samples and the corresponding polysaccharide-free extracts will be discussed in depth. The Weinbiet wine and extract contain the highest concentrations of anthocyanins and show the highest difference between wine and extract (Fig. 1). The differences in the other samples are comparatively low. The Adentu, Las Mulas, and Weinbiet extracts contain fewer tannins than the corresponding wines, whereas the tannin

Table 2
Characterization of the pectic polysaccharides of the wine samples including the distribution of the high and medium molecular weight (MW) fractions, the degree of methylation (DM) and acetylation (DA) and the concentrations of the sugar moieties galacturonic acid (GalAc), rhamnose (Rha), and fucose (Fuc). Means having the same letters are not significantly different at $p \leq 0.05$. Means presented with standard deviation; n = 3.

Sample	High MW fraction (15–708 kDa) [%]	Medium MW fraction (5.5–15 kDa) [%]	DM [%]	DA [%]	GalAc [mg/g]	Rha [mg/g]	Fuc [mg/g]
Adentu	63.9 ± 0.3 A	36.1 ± 0.3 F	27.2 ± 0.5 C	1.8 ± 0.1 C	16.0 ± 0.6 D	5.5 ± 0.8 B	1.3 ± 0.1 C
Beringer	41.9 ± 1.5 E	58.1 ± 1.5 B	12.3 ± 0.1 D	2.9 ± 1.6 B,C	27.3 ± 1.2 C	11.0 ± 0.4 A	1.4 ± 0.1 B,C
Bundschuh	49.0 ± 0.3 C	51.0 ± 0.3 D	38.4 ± 1.5 B	2.8 ± 0.2 B,C	47.6 ± 0.6 A	2.8 ± 0.1 C,D	1.5 ± 0.1 A,B
Canyon Road	37.3 ± 0.7 F	62.7 ± 0.7 A	17.8 ± 0.1 D	9.2 ± 0.7 A	26.7 ± 1.2 C	3.3 ± 0.1 C	1.4 ± 0.1 B,C
Las Mulas	45.4 ± 1.4 D	54.6 ± 1.4 C	34.6 ± 2.7 B	4.3 ± 0.4 B	25.4 ± 1.5 C	2.3 ± 0.1 C,D	1.3 ± 0.1 B,C
Weinbiet	54.7 ± 0.3 B	45.3 ± 0.3 E	53.1 ± 2.4 A	3.8 ± 0.3 B,C	38.4 ± 1.7 B	2.2 ± 0.1 D	1.3 ± 0.1 C

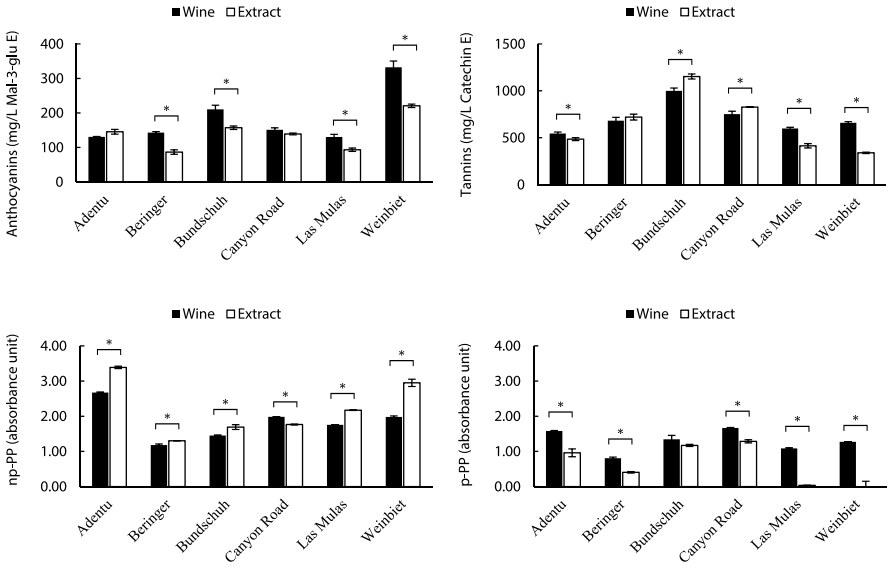

Fig. 1. Phenolic composition including total anthocyanins, non-precipitable polymeric pigments (np-PP), precipitable polymeric pigments (p-PP), and total tannins of the wine and extracts. Results obtained by photometric assays (Harbertson et al., 2002, 2003, 2009, 2015). Means having an asterisk (*) show a significant difference ($p \leq 0.05$) between wine and extract samples.

concentrations of the other extracts are higher than the ones in the wines. The latter was already observed in an earlier study (Weilack et al., 2021). Accordingly, the wines can be classified into two groups, with one group that comprises more tannins in the wines, whereas the other group shows more tannins in the extracts. Interestingly, in most samples, the removal of the polysaccharides results in an increase in np-PP and a concomitantly decrease in the p-PP, with the Canyon Road samples being the only exceptions. The Adentu, Las Mulas, and Weinbiet present the highest differences in polymeric pigment composition between wines and extracts, whereby the Las Mulas and Weinbiet extracts seem to have no p-PP at all. The differences in p-PP proportion and tannin concentrations between wines and extracts correlate statistically significant (Pearson correlation with $\alpha = 0.05$; $R^2 = 0.981$) supporting the classification of the wines into these two groups.

3.3. Composition of the total soluble polysaccharides

To characterize the soluble polysaccharides of the wines, different parameters including molecular weight (MW) distribution, monomeric sugar composition, and degrees of methylation and acetylation (DM and DA) of the pectin remnants were determined using size exclusion chromatography (SEC), photometric assays, and GC-FID (Table 2 and Fig. 2). The molecular weights of the soluble polysaccharides show a broad distribution across all samples with characteristic peaks at around 150, 50, 10, and 4.6 kDa (Fig. 2). According to the literature (Ayestarán et al., 2004; Guadalupe and Ayestarán, 2007; Gao et al., 2015), the high MW fraction (>15 kDa) contains arabinogalactans (AG), arabinogalactan-proteins (AGP), mannans, mannoproteins (MP), and in small amounts homo- (HG) and rhamnogalacturonan I (RG-I), whereas the main constituent of the medium (15–5.5 kDa) and low MW fractions (<5.5 kDa) are rhamnogalacturonan II (RG-II) dimers and monomers (~10 kDa and ~4.6 kDa; Pellerin et al., 1996), respectively. Besides the RG-II molecules, these MW fractions also include low molecular weight fragments of homogalacturonan (HG), rhamnogalacturonan I (RG-I), AGP, and MP (Guadalupe and Ayestarán, 2007). The Adentu and Weinbiet wines present the highest proportions of the high MW fraction (Table 2), indicating high proportions of AG, AGP, and MP. Accordingly, these wines have the lowest proportions of the medium MW fraction. The Beringer and Canyon Road wines show the opposite ratio, indicating high proportions of RG-II. Although the Bundschuh wine contains statistical significantly more high MW polysaccharides than the Las Mulas wine, the differences between the two wines of the high and medium MW polysaccharides are relatively low. The chromatograms in Fig. 2 indicate that the Las Mulas wine has a similar polysaccharide profile as the Weinbiet wine, which is more accentuated in the high MW fraction. Besides the Adentu wine, all wines possess a considerable amount of low MW fraction of the pectic polysaccharides, whereby the Bundschuh wine shows the most pronounced peak followed by the Beringer and Canyon Road wines, which indicates the presence of RG-II monomers and other low MW fragments.

To determine the relative proportions of the pectic structures in the wines, the monosaccharide composition including galacturonic acid (GalAc), rhamnose (Rha), and fucose (Fuc) was analyzed (Table 2). Due to their well-defined occurrence in the pectin structure, this allows for

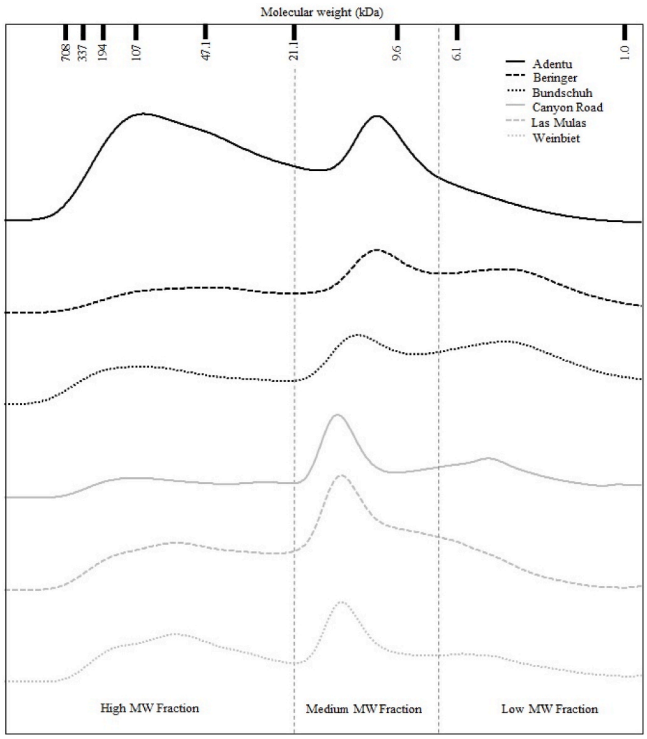

Fig. 2. Size exclusion chromatograms of the pectic polysaccharides of the wine samples including the high (>15 kDa), medium (15–5.5 kDa) and low (<5.5 kDa) molecular weight (MW) fractions. Column was calibrated with standards illustrating the peak molecular weight distribution.

correlating the GalAc, Rha, and Fuc concentrations to the relative amount of HG, RG-I, and RG-II molecules, respectively (Larsen et al., 2019). The ratio of Rha to GalAc provides information on the proportion of the RG-I main chain, and hence, on the relative amount of GalAc that is incorporated in the RG-I backbone (Ma et al., 2016). The GalAc concentrations vary widely from 16.0 ± 0.6 mg/g for the Adentu wine to 47.6 ± 0.6 mg/g for the Bundschuh wine. The Adentu wine has the highest proportion of high MW polysaccharides, whereas the Bundschuh wine possesses less high MW molecules and a pronounced low MW fraction (Fig. 2), suggesting that the HG chain was degraded to a different extent during ripening and winemaking. This can be assigned to different polygalacturonase activities, which in turn depend on grape maturity and/or the use of pectolytic enzymes (Vidal et al., 2001). The Rha concentrations were generally lower than the GalAc concentrations and ranged from 2.2 ± 0.1 mg/g for the Weinbiet wine to 11.0 ± 0.4 mg/g for the Beringer wine (Table 2), showing that the wines contain small proportions of RG-I. This finding is supported by Vidal et al. (2003b), who stated that RG-I is a minor component of wine pectic polysaccharides and determined it to be of around 4%, which may be due to its poor solubility or its fragmentation by glycanases during winemaking (Vidal et al., 2001). However, the pectin fragments of the Adentu and Beringer wines still comprise higher ratios of Rha to GalAc, indicating that 34.6% and 40.4% of the GalAc found in the wines, respectively, derive from the RG-I backbone. This is supported by a characteristic peak in the SEC chromatograms at around 50 kDa, which may be assigned to RG-I (Vidal et al., 2003b).

RG-II is the most abundant pectic polysaccharide in wines (Guadalupe et al., 2014), thus, the Fuc concentrations of the wines are quantified to characterize their RG-II content. While the Adentu, Las Mulas, and Weinbiet wines have the lowest Fuc concentration, therefore proposedly the lowest RG-II proportion, the Bundschuh wine exhibits the highest RG-II proportion. This coincides only partially with the size distribution of the polysaccharides that assigned the highest proportion of RG-II to the Canyon Road wine. However, as reported earlier, some wines still contain high GalAc and Rha contents, indicating the presence of HG and RG-I fragments, which can also be found in the medium MW fraction.

The degree of esterification of the pectic polysaccharides, being the degree of methylation (DM), describes the proportion of GalAc units that is esterified with methanol. Besides that, GalAc and Rha can be bound to acetyl residues, which are determined by the degree of acetylation (DA). Accordingly, the remaining GalAc monomers carry a free carboxy and hydroxy group, whereby the first one can be dissociated depending on the pH of the wine, both of which have a large impact on polarity and hydrophobicity of the pectic polysaccharides. The DMs range from 12.3 ± 0.1% for the Beringer wine to 53.1 ± 2.4% for the Weinbiet wine, whereas the DAs of the wines are smaller and range from 1.8 ± 0.1% for the Adentu wine and 9.2 ± 0.7% for the Canyon Road wine (Table 2). Since a higher degree of esterification increases the hydrophobicity of the pectic molecules, the pectin fragments found in the Weinbiet wine can be considered more hydrophobic, whereas the polysaccharides of the Beringer wine are more polar. During the ripening process of grapes, several enzymes play a role in the degradation of the cell wall polysaccharides and the accompanied softening of the grape skin. The main acting enzymes of this process are polygalacturonase (PG), pectin methylesterase (PME), and β-galactosidase. They are responsible for the cleavage of unesterified polygalacturonans, the de-esterification of methylated galacturonic acid units, and the de-polymerization of (1 → 4)-β-galactan constituents of pectic polysaccharides, respectively (Nunan et al., 1998; Minic and Jouanin, 2006). Nunan et al. (1998) stated that at the end of the maturation process, the degree of esterification of pectin is at around 48% and that the polygalacturonase can only cleave bonds of de-esterified GalAc units, making their preceding de-esterification inevitable. Together with the high amount of high MW pectic polysaccharides and a degree of esterification of 53.1 ± 2.4% of the Weinbiet sample, this gives rise to the assumption that the grapes of this wine, in contrast to the other wines, may have been harvested earlier and not treated with any macerating enzymes.

3.4. Interactions between wine polysaccharides and polyphenols

Due to the comparison of the wines with their corresponding extracts, the differences observed in the polyphenolic composition between them (Fig. 1) can be attributed to the presence or absence of the polysaccharides in wine or extracts, respectively. These polysaccharides can interact directly with the wine polyphenols and/or with the BSA that is used to precipitate polymeric pigments and tannins during analysis (de Freitas et al., 2003; Mateus et al., 2004). Both interactions, also in synergy, can lead to differences in the assay readings.

In most samples, the presence of the polysaccharides increase the anthocyanin readings in the wines compared to the extracts. Since the determination of the anthocyanins is a photometric measurement, this enhancing effect may be assigned to the adsorption of the anthocyanin molecules to the pectin fragments followed by the self-association of other anthocyanin molecules (Padayachee et al., 2012). This anthocyanin stacking results in the bathochromic and hyperchromic shift of the absorbance spectra of the anthocyanins, which occurs in the presence of certain polysaccharide structures (Mazzaracchio et al., 2004; Fernandes et al., 2020). Due to their pH dependent structural equilibria, part of the anthocyanins exists as the flavylium cation at wine pH, which allows ionic interactions between the anthocyanins and dissociated GalAc units (Holzwarth et al., 2012). The free hydroxy groups of the HG and RG backbone can form hydrogen bonds with the hydroxy groups of polyphenols. However, as the major anthocyanin malvidin-3-glucoside carries two methoxy groups on its B-ring, hydrophobic interactions with methylated GalAc units also play a role in the stabilization of anthocyanins (Mazzaracchio et al., 2004; Fernandes et al., 2020). The Weinbiet, Las Mulas, and Bundschuh wines show the highest degrees of esterification and the biggest differences in anthocyanin concentrations.

The higher hydrophobicity of these high methoxylated pectic polysaccharides may lead to stronger interactions with the predominant malvidin-3-glucoside. Despite the lower degree of esterification of the Beringer polysaccharides, they still cause an increase in the anthocyanin concentrations, indicating that other forces are also involved in the self-association of the anthocyanins.

The differences in tannin and polymeric pigment concentrations demonstrate that the pectic polysaccharides not only contribute to the stacking of anthocyanins but also alter the precipitability of other polyphenols. As described above, the Adentu, Weinbiet, and Las Mulas wines have higher proportions of the high MW polysaccharides AGPs, AGs, and MPs, whereas the Beringer, Bundschuh, and Canyon Road wines possess high proportions of smaller polysaccharides like RG-II and fragments of HG, RG-I, AGPs, and MPs. While the exclusion of these polysaccharides leads to a decrease in tannin concentrations of the Adentu, Las Mulas and Weinbiet wines by 11%, 31% and 49%, respectively, the tannin concentrations of the other wines increase by 6–13%, indicating that the respective pectic polysaccharides of the wines enhance or prevent tannin precipitation and accordingly lead to altered tannin readings. Attenuated tannin precipitation results in lower tannin readings, whereas enhanced precipitation will increase the apparent tannin concentration. A strong correlation of the differences in tannin concentrations with the Fuc concentrations ($R^2 = 0.834$) supports the theory that the precipitability of tannins is affected by the polysaccharide composition, more specifically by the proportion of RG-II. The disruptive effect of pectins on the aggregation of tannins and proteins has been shown to increase with the polarity of the polysaccharide (de Freitas et al., 2003; Mateus et al., 2004; Carvalho et al., 2006), whereby the polarity depends on the amount of GalAc units and the degree of esterification. According to Vernhet et al. (1996), RG-II has the highest charge density when compared to AGP fractions and may therefore be more effective in the prevention of aggregation. In this study, the Beringer, Bundschuh, and Canyon Road wines show the highest inhibiting effect, which may be assigned to their higher proportions of RG-II. Additionally, the pectins found in the Bundschuh wine have the highest amount of GalAc units and the most pronounced fraction of low MW polysaccharides, leading to the assumption that they contain a higher concentration of polygalacturonic acids that were shown to solubilize protein-tannin aggregates due to their ionic character (de Freitas et al., 2003). Altogether, this results in an effective prevention of precipitation in the Beringer, Bundschuh, and Canyon Road wines. In contrast, some AGPs and AGs are considered neutral carbohydrates that not only have a small or no preventive effect on protein-tannin aggregation but even enhance protein precipitation by co-aggregation of these polysaccharides with proteins and tannins, leading to even bigger complexes (Vernhet et al., 1996; Carvalho et al., 2006). As the Adentu, Las Mulas, and Weinbiet wines have high proportions of the high MW polysaccharides that consist of AGPs and AGs, besides others, this may explain the increased precipitability of the tannins found in the wines. Furthermore, the pectic polysaccharides of the Weinbiet wine are the highest esterified, which reduces their polarity and may lead to the strongest enhancement of the precipitation. This indicates that the tannins, that were additionally found, in the Adentu, Las Mulas, and Weinbiet wines when compared to the according extracts may not be actually present but are the result of the interactions and co-aggregation with wine polysaccharides.

The differences between the non-precipitable polymeric pigments (np-PP) and the precipitable polymeric pigments (p-PP) correlate strongly ($R^2 = -0.746$), which might be explained by an increased (1.3- to 32.4-fold) precipitability of polymeric pigments as a result of their interaction with polysaccharides. Additionally, the complexation of the anthocyanins by the polysaccharides may lead to the formation of precipitable pigments as shown by Sommer et al. (2016), which depends on the polysaccharide composition. The polysaccharides of the Beringer and Bundschuh wines exhibit a relatively strong co-pigmenting effect on anthocyanins, but small differences in the np-PP and p-PP proportions comparing wine and polysaccharide deprived extracts. The increased concentration of p-PP may be explained by the higher precipitability of parts of the np-PP due to their interactions with the relatively low proportions of AGPs and AGs. However, as anthocyanins can interact even more strongly with the high proportion of RG-II and polygalacturonic acids due to their cationic character and the high electron density of these polysaccharides, the complexes between the anthocyanins and polysaccharides appear to remain soluble, which is supported by earlier studies (Ducasse et al., 2010; Larsen et al., 2019). In the Adentu, Las Mulas, and Weinbiet wines, greater differences in anthocyanin and polymeric pigment concentrations were observed, suggesting that not only np-PP but also anthocyanins may form precipitable pigments with the large AGP and AG molecules of these wines. Guadalupe et al. (2007) reported that most of the neutral pectic polysaccharides, like AGPs and AGs, precipitated post maceration due to their size, but acidic pectic polysaccharides remained constant. This raises the question whether the increased amount of p-PP can be reasoned only by the enhanced protein precipitability of the pigments and the tannins, or whether these complexes would also precipitate without the addition of BSA during the centrifugation of the samples. Since no p-PP were found in the Las Mulas and Weinbiet wines, the complexation of the anthocyanins by the polysaccharides may have resulted in the formation of insoluble pigments, which may lead to an impaired incorporation of anthocyanins into tannin molecules. This is further supported by the fact that the sum of the polymeric pigments is higher in these wines than in their extracts. It was also observed for anthocyanin-polysaccharide complexes in previous studies that attributed this behavior to the size of the polysaccharides (Larsen et al., 2019; Hensen et al., 2022).

3.5. Implications for red wine quality

Despite the precipitation of some anthocyanins, the co-pigmentation of the anthocyanins by wine polysaccharides stabilizes the color of young wines and provides long-term stabilization of the anthocyanins against degradation (Cheynier et al., 2006; Holzwarth et al., 2012; Larsen et al., 2019). The complexes that are formed by non-covalent bonds between anthocyanins and neutral, high molecular weight polysaccharides appear to have the same properties as p-PP as they absorb light at 520 nm. Complexation limits discoloration by pH changes and SO_2-bleaching, suggesting that the polysaccharides prevent additions of sulfite and water probably by steric hindrance. Furthermore, these complexes are seemingly protein precipitable, hence, the resulting aggregates are measured as p-PP. This shows that they are colored complexes that may continue to contribute to the color of red wines. However, their structure differs from the original definition of polymeric pigments as tannins with incorporated anthocyanins (Somers, 1971). Unlike the pigmented tannins, which are based of covalent bonds, the formation of the polysaccharide-pigment complexes is largely reversible (Weber, 2022), which could lead to a lower long-term stability of the wine color. Moreover, depending on the polysaccharide composition, the complexes formed seem to impair the formation of p-PP in the original sense, being tannins with covalently incorporated anthocyanins, which could affect not only color stability but also red wine astringency.

Red wine astringency is the result of the interactions of tannins with saliva proteins, leading to the precipitation of the tannins and a loss of lubrication in the oral cavity (Charlton et al., 2002), whereby the extent of this reaction is influenced by tannin concentration and composition (Gawel, 1998). Additionally, the present study shows that the protein precipitability of tannins may be enhanced or inhibited in the presence of pectic polysaccharides depending on the polarity of the polysaccharides, which may result in a different astringency perception. The polarity of the tannins, in turn, may influence their interactions with the polysaccharides, and the pigmentation of tannins is believed to increase their polarity due to the cationic character of the anthocyanins (Salas

et al., 2003; Weber et al., 2013). Consequently, the formation of p-PP during red wine aging will affect the interactions with both the saliva proteins and the pectic polysaccharides. While the Beringer, Bundschuh, and Canyon Road wines contain p-PP that may further decrease tannin precipitation due to their enhanced interaction with the RG-II molecules, the lack of p-PP in the Las Mulas and Weinbiet wines may support the increased precipitation of tannins or the formation of bigger aggregates due to their hydrophobic interactions with the high MW pectic polysaccharides. Therefore, this study partly supports the previously stated hypothesis (Weilack et al., 2021) that a change in perceived astringency of aged wines may be due to increased interactions between pigmented tannins and wine polysaccharides, as precipitation of tannins was prevented in some wines. However, another study showed different behaviors of RG-II molecules depending on the structure of the protein that was used for the precipitation of tannins (Carvalho et al., 2006). This indicates that the effects of the pectic polysaccharides may differ comparing saliva proteins and BSA and accordingly red wine astringency, which is part of ongoing investigations.

4. Conclusions

The interactions between wine polyphenols and pectic polysaccharides were investigated by comparing their composition in red wines and corresponding polysaccharide-free extracts. The results show that the composition of the grape pectic polysaccharides, that are extracted and modified during fermentation, can alter the properties of tannins and pigments by stabilizing anthocyanins through co-pigmentation like effects and changing their precipitability. This precipitability is used as an analytical measure on the one hand but is also the driving force for the perception of astringency, which leads to the assumption that the observed effects can also change mouthfeel properties of the wines. The color of aged red wines is mainly attributed to the presence of polymeric pigments; however, this study reveals that polysaccharides can form complexes with anthocyanins and np-PP, which affect the formation and measurement of precipitable polymeric pigments as they have comparable properties. This leads to the conclusion that it is necessary to reconsider the established concept of polymeric pigments, which until now mainly refers to tannins with incorporated anthocyanins. As certain structural characteristics of the pectin molecules enhance or attenuate tannin precipitation, it may be very likely that they influence the perception of astringency and in particular the perception of astringency sub-qualities. Altogether, this study provides continued information on the impact of certain cell wall polysaccharides and their degradation products on the tannin and pigment composition of red wines. Since these hypotheses result from the study of Cabernet Sauvignon wines, it is plausible that the results of other wines may be different given the substantial varietal differences in composition of polysaccharides and polyphenols. This emphasizes the necessity of further research in this area.

Funding

This work was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 20024N.

CRediT authorship contribution statement

Ingrid Weilack: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Lea Mehren:** Methodology, Investigation, Formal analysis. **Andreas Schieber:** Resources, Supervision, Writing – review & editing, Funding acquisition. **Fabian Weber:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank Sandra Feifel from the Wein Campus Neustadt for supporting us with the data on the general composition of the wines.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100506.

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Supplementary Data

Table A.1 Areas of the high and medium molecular weight (MW) fractions and total area of both fractions of the wine TSP determined with size-exclusion chromatography. Due to many factors that may influence the concentration of the polysaccharides along the sample preparation, absolute areas should be considered and compared cautiously. Means having the same letters are not significantly different at $p \leq 0.05$. Means presented with standard deviation; n = 3.

Sample	Total area of High and medium MW fractions	Area High MW fraction (15-708 kDa)	Area Medium MW fraction (5.5-15 kDa)
Adentu	1352547±79148 A	864333±52619 A	488214±26718 A
Beringer	536392±84923 C,D	224015±27376 C,D	312377±57714 B
Bundschuh	603096±45365 C	295381±20503 B,C	307715±24862 B
Canyon Road	410650±762 D	153091±3108 D	257560±2345 B
Las Mulas	808924±57492 B	366382±15636 B	442542±42109 A
Weinbiet	597279±12625 C	326550±6082 B	270730±6877 B

Table A.2 Yields of the polyphenol-rich extracts that are poor in polysaccharides and the total soluble polysaccharides (TSP) of the wine and extract samples. Means having the same letters are not significantly different at $p \leq 0.05$. Means presented with standard deviation; n = 2-3.

Sample	Yield Extraction [g/L]	Wine TSP [g/L]	Extract TSP [g/L]
Adentu	4.32±0.11	3.75±0.23 C, D	0.01±0.01 E
Beringer	3.99±0.20	4.68±0.12 A	0.04±0.01 E
Bundschuh	5.15±0.07	5.05±0.38 A, B	0.08±0.00 E
Canyon Road	4.24±0.08	3.18±0.07 D	0.01±0.00 E
Las Mulas	3.76±0.07	3.24±0.14 D	0.03±0.00 E
Weinbiet	4.63±0.38	4.34±0.27 B, C	0.07±0.02 E

Appendix E

Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines

Food Hydrocolloids 157 (2024) 110402

Contents lists available at ScienceDirect

Food Hydrocolloids

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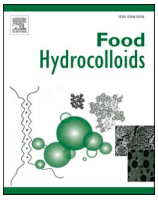

Pectic polysaccharides modulate colloidal stability and astringency perception of bottle aged Cabernet Sauvignon wines

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ARTICLE INFO

Keywords:
Pectic polysaccharides
Tannins
Anthocyanins
Pigments
Precipitability
Astringency

ABSTRACT

Anthocyanins, tannins, and polymeric pigments, which are formed during red wine aging, are arguably the most important phenolic constituents of red wine, because they provide color, color stability, and mouthfeel properties like astringency. The extraction of polyphenols is accompanied with the extraction of grape-derived pectic polysaccharides leading to interactions between the two compound classes during fermentation and wine aging affecting tannin and pigment precipitability. Thereby, the implications for red wine quality are dependent on the structural features of the polysaccharides. In this study, polyphenolic and pectic polysaccharide composition of commercially available Cabernet Sauvignon wines, which were subjected to forced aging, were characterized to investigate the effect of red wine aging on the interactions between polysaccharides and polyphenols. This was accomplished by removing polysaccharides from the wines and comparing the polyphenolic composition of the wines and the reconstituted polysaccharide-free wines. Descriptive sensory analysis including "overall astringency", "unripe" and "dry" was conducted on all samples to examine the implications of polysaccharide-polyphenol interactions on the astringency perception of red wines. The analyzed wines can be classified into two groups based on their colloidal stability, whereby large, neutral, and highly esterified polysaccharides promoted tannin and pigment protein precipitability and impaired the age-related formation of pigmented tannins. Small, acidic, and polar pectin fragments show a preventive effect on the precipitation of polyphenols. Sensory analysis revealed that the perception of the astringency sub-qualities "unripe" and "dry" are related to the interactions between pectic polysaccharides and polyphenols. Depending on the polysaccharide composition, the astringency of the wines increased or decreased during aging.

1. Introduction

It is generally accepted that anthocyanins and tannins are two of the major red wine components which determine product quality by providing color and mouthfeel to red wines. Anthocyanins and tannins are extracted from the grape berry to the must during winemaking, but the extractability of these compounds is strongly influenced by various inherent and external factors like the type of polyphenol, winemaking techniques, and grape maturity (Hanlin, Hrmova, Harbertson, & Downey, 2010; Hensen, Hoening, Weilack, Damm, & Weber, 2022; Hernández-Hierro, Quijada-Morín, Rivas-Gonzalo, & Escribano-Bailón, 2012). The latter also determines the composition of cell wall polysaccharides, as the ripening process of grapes is associated with the a balance between biosynthesis and enzymatic degradation of polysaccharides like hemicellulose, cellulose, and in particular pectin (Nunan, Sims, Bacic, Robinson, & Fincher, 1998). This includes the de-esterification of methylated galacturonic acid units followed by the breakdown of unesterified polygalacturonans and (1 → 4)-β-galactans of pectic polysaccharides (Minic & Jouanin, 2006; Nunan et al., 1998). Because the strength of interactions between polysaccharides and polyphenols depends on structural features like branching complexity and degree of esterification, the alterations in polysaccharide composition affect the extractability of tannins and anthocyanins as they constantly adsorb and de-adsorb on grape cell material (Hanlin et al., 2010; Liu, Le Bourvellec, & Renard, 2020; Medina-Plaza et al., 2020). Together with polyphenols, pectin fragments end up in the wine where interactions driven by hydrogen bonding, hydrophobic and electrostatic forces take place (Gao, Fangel, Willats, Vivier, & Moore, 2015; Weber,

Abbreviations: p-PP, precipitable polymeric pigments; np-PP, non-precipitable polymeric pigments.

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https://doi.org/10.1016/j.foodhyd.2024.110402
Received 26 March 2024; Received in revised form 5 July 2024; Accepted 8 July 2024
Available online 9 July 2024

2022).

The interactions with polyphenols impact their protein precipitability, whereby prevention and enhancement of protein precipitation were both reported depending on the type of pectic polysaccharide (Carvalho et al., 2006; de Freitas & Mateus, 2001; Watrelot, Schulz, & Kennedy, 2017). Polysaccharides with the ability to stabilize wine color, prevent tannin (self-) aggregation and precipitation are referred to as protective/stable colloids (Alcalde-Eon, García-Estévez, Puente, Rivas-Gonzalo, & Escribano-Bailón, 2014; Guadalupe, Palacios, & Ayestarán, 2007; Osete-Alcaraz, Bautista-Ortín, & Gómez-Plaza, 2020). The enhanced precipitability of tannins and pigments in the presence of certain polysaccharides is due to the formation of unstable colloids, which consist of pigmented protein precipitable polysaccharide-polyphenol complexes (Alcalde-Eon et al., 2014; Guadalupe et al., 2007; Weilack, Mehren, Schieber, & Weber, 2023). This phenomenon was reported to impair the covalent incorporation of anthocyanins into tannins to form pigmented tannins during red wine aging (Weilack et al., 2023). Earlier research (Vidal, Francis, Noble, et al., 2004; Weber, Greve, Durner, Fischer, & Winterhalter, 2013; Weilack, Schmitz, Harbertson, & Weber, 2021) suggests that the formation of pigmented tannins plays a role in the attenuation and softening of astringency associated with wine aging due to changes in physico-chemical properties; hence, the impaired formation of pigmented tannins could possibly impact red wine astringency perception. This highlights the necessity to examine the relationship between the structural features of pectic polysaccharides and the colloidal stability of wines.

In general, polysaccharides can alter red wine astringency in the finished wines either by changing its texture or interacting with polyphenols and/or salivary proteins (Brandão et al., 2017; Luck et al., 1994; Vidal, Francis, Williams, et al., 2004). According to Gawel, Oberholster, and Francis (2000), red wines induce not only the perception of overall astringency, but also astringency related sensations, which can be classified as astringency sub-qualities. Due to the heterogeneous and complex composition of red wines, the exact triggers of different sub-qualities are difficult to unravel, but have received increased attention in recent years (Ferrero-del-Teso et al., 2024; Sáenz-Navajas, Ferrero-del-Teso, Jeffery, Ferreira, & Fernández-Zurbano, 2020; Wang, Olarte Mantilla, Smith, Stokes, & Smyth, 2020). However, most studies are based on model wines and isolated reactions between polyphenols, polysaccharides, and proteins (González-Muñoz et al., 2022).

The present work is a follow-up of a previous study (Weilack et al., 2023), which investigated the implications of the interactions between pectic polysaccharides and polyphenols on the composition of the latter in young wines. They showed that large and neutral pectin fragments led to an increased protein precipitability of tannins and pigments, which might have resulted in an impaired reaction between anthocyanins and tannins to form polymeric pigments. In contrast, small and polar pectin fragments protected polyphenols from precipitation (Weilack et al., 2023). The objective of the present study was to examine the influence of pectic polysaccharides on the composition of polyphenols in bottle aged red wines and the implications for red wine astringency, particularly considering the age-related attenuation of astringency. This was achieved by subjecting the same six commercially available Cabernet Sauvignon wines to forced aging to trigger age-related changes in the polyphenolic composition like the formation of polymeric pigments. Besides the characterization of pectic polysaccharides, polysaccharide-free reconstituted wines were composed, and polyphenols were determined and compared in all samples. Additionally, all samples were subjected to sensory profiling including taste attributes “sour” and “bitter” and mouthfeel attributes “overall astringency”, “unripe” and “dry” to examine the influence of pectic polysaccharides on the astringency perception and the changes thereof during aging.

2. Materials and methods

2.1. Materials

Maleic acid, ferric chloride, triethanolamine (TEA), and tartaric acid were sourced from Alfa Aesar (Kandel, Germany). Urea, bovine serum albumin fraction V, and (+)-catechin were acquired from Carl Roth (Karlsruhe, Germany). Acetic acid, hydrochloric acid (HCl), ethanol, and potassium bisulfite were sourced from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide and sodium nitrate were obtained from Honeywell Fluka (Offenbach, Germany) and Acros Organics (Geel, Belgium), respectively. Propionic acid, *n*-propanol, and sodium azide were purchased from Merck KGaA (Darmstadt, Germany). Sodium chloride, sulfuric acid, and methanol (HPLC grade) were acquired from Th. Geyer GmbH & Co. KG (Renningen, Deutschland). Food-grade sodium hydroxide, ethanol, and acetic acid were obtained from Emprove Essential (Merck KGaA, Darmstadt, Germany), Brennerei Kessler (Bad Peterstal-Griesbach, Germany), and Macron Fine Chemicals (VWR International GmbH, Darmstadt, Germany), respectively. Food grade adsorbent resin (Resinex AD3300) was provided by Jacobi Carbons Group (Frankfurt am Main, Germany). For the model wines, food-grade sucrose (Südzucker AG, Mannheim, Germany), tartaric acid (Otto Fischar GmbH, Saarbrücken, Germany), potassium bitartrate (Natuurlijk, Ede, Netherlands) and lactic acid (Otto Fischar GmbH, Saarbrücken, Germany) were used.

2.2. Wine samples

This study was carried out on commercially available Cabernet Sauvignon wines, which were subjected to forced aging. This was achieved by storing the wines in a heated incubator at 35 °C for 10 weeks. Originally, the six different Cabernet Sauvignon wines were of the 2018 vintage from three wine-growing regions. At the time of the study, the wines were 3 years old. The wines were made in the following wineries and regions: Weinbiet (14 % v/v ethanol) and Emil Bauer (Bundschuh, 13.5 % v/v ethanol) from the Palatinate region in Germany, Adentu and Las Mulas (each 13.5 % v/v ethanol) from Central Valley, Chile, and Beringer and Canyon Road (each 13 % v/v ethanol) from California, USA. The wines were chosen to reflect a broad variability of geographical origins. The sample coding references the respective winery with the prefixes “fresh reference” wines for the reference wines, which were kept at 10 °C for 10 weeks, hence, did not undergo aging, and “aged” for the aged wines. All samples were analyzed after 10 weeks of storage.

The general composition of the “aged” wines was assessed by Fourier-transform mid-infrared (FT-IR) spectroscopy, including the appropriate calibration method (WineScan FT120 Basic, Foss, Hilleroed, Denmark) (Table 1). Free and total SO_2 contents were determined by titration and are included in Table 1. All bottles were closed with screw caps. The “fresh reference” wines were object of the previous study (Weilack et al., 2023), in which their general, phenolic, and pectic polysaccharide composition have been investigated.

2.3. Removal of wine polysaccharides by using solid phase extraction

Solid phase extraction was performed to obtain polysaccharide-free phenolic extracts of the “aged” red wines using a food-grade adsorbent resin and food-grade chemicals. The applied extraction protocol was published by Weber et al. (2013) with a few modifications as described by Weilack et al. (2023). The extracts of two bottles of each wine were combined, concentrated under vacuum, consecutively lyophilized, and yields were determined gravimetrically.

2.4. Polyphenol composition of the wines and polyphenolic extracts

For polyphenol analyses, the lyophilized extracts were combined and

Table 1
General composition of the "aged" Cabernet Sauvignon samples after storage at 35 °C for 10 weeks determined by Fourier-transform mid-infrared (FT-IR) spectroscopy and titration for total and free SO_2. Due to method robustness, analysis was conducted in single determination.

Wine	Glycerol [g/L]	Residual sugars [g/L]	Titratable acidity [g/L TAE[a]]	Tartaric acid [g/L]	Lactic acid [g/L]	pH	Total SO_2 [mg/L]	Free SO_2 [mg/L]
Adentu (CHL)	8.4	2.4	4.7	1.7	1.4	3.7	56	n.d.[b]
Beringer (USA)	9.5	8.0	4.9	1.2	0.9	3.8	99	5
Bundschuh (GER)	9.3	5.7	5.3	1.3	2.0	3.8	90	4
Canyon Road (USA)	9.1	11.2	4.6	1.8	0.9	3.9	72	7
Las Mulas (CHL)	9.5	1.8	4.5	1.2	1.4	3.8	69	5
Weinbiet (GER)	10.5	2.9	4.4	1.1	1.0	3.9	73	23

[a] Titratable acidity is expressed in g/L tartaric acid equivalents (TAE).
[b] n.d. = not detected.

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). The determined phenolic characteristics of the "fresh reference" and "aged" wines and corresponding extracts included total anthocyanins, non-precipitable polymeric pigments (np-PP), precipitable polymeric pigments (p-PP), and were assessed following the photometric assays described by Harbertson, Picciotto, and Adams (2003, 2009, 2015) using a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany). Anthocyanins are expressed as malvidin-3-glucoside equivalents (Mal-3-glu equiv.) based on an empirical factor and tannins were expressed as catechin equivalents according to an external calibration curve. The "fresh reference" wines and corresponding extracts have been analyzed after 10 weeks of storage at 10 °C and results have been published (Weilack et al., 2023). The analyses were conducted in triplicate.

2.5. Precipitation of total soluble polysaccharides

Total soluble polysaccharides (TSP) were obtained from red wines and polyphenol-rich extracts using ethanolic precipitation following the protocol published by Watrelot et al. (2017) with some adjustments outlined by Weilack et al. (2023). The extraction process was carried out twice and yields were determined through gravimetric measurements.

2.6. Characterization of total soluble polysaccharides

2.6.1. Degree of methylation (DM) and degree of acetylation (DA)

According to Larsen, Buerschaper, Schieber, and Weber (2019), the determination of the degree of methylation (DM) and the degree of acetylation (DA) employed headspace solid-phase dynamic extraction gas chromatography (HS-SPDE-GC) with flame ionization detection (FID) after saponification. The SPDE equipment (Chromtech, Idstein, Germany) was integrated in a CTC-Combi-PAL-Autosampler (Bender and Hobein, Zurich, Switzerland) and connected to a GC FID system (Agilent Technologies model 6890). DM and DA were quantified as mol of methyl/acetyl groups per 100 mol of galacturonic acid (GalAc), as previously outlined (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002), and are expressed in percentage. The analysis was conducted in triplicate.

2.6.2. Determination of monosaccharide composition (galacturonic acid, L-rhamnose, and L-fucose)

The quantification of the monomer composition of the total soluble polysaccharides was conducted using the methodology established by Larsen et al. (2019). Sample hydrolysis was carried out as indicated in the enzyme kits from Megazyme (Wicklow, Ireland) using sulfuric acid (2 M) at 100 °C (6 h) for the determination of GalAc and hydrochloric acid (2.4 M) at 100 °C (1 h) for contents of rhamnose and fucose, respectively. After centrifugation at 10947*g* for 10 min, the specific monosaccharides were measured in the supernatant by absorbance readings at 340 nm. The analysis was conducted in triplicate.

2.6.3. Molecular weight distribution of total soluble polysaccharides

The molecular weight (MW) distribution was assessed through high-performance size exclusion chromatography (HPSEC) equipped with a Smartline HPLC system with a RI detector 2300 (Knauer, Berlin, Germany) and two SEC-Diol columns (300 and 120 Å, 3 μm; YMC, Kyoto, Japan) following the method published by Larsen et al. (2019). Samples were dissolved in water (50 °C) and dialyzed against demineralized water (MWCO 12–14 kDa). Elution of polysaccharides was achieved using water containing 50 mM sodium nitrate and 0.25% sodium azide. MWs were determined using eight pullulan standards ranging from 0.504 to 708 kDa (ReadyCal-Kit Pullulan, PSS- Polymer Standards, Mainz, Germany). Chromatograms were segmented into three representative fractions: high molecular weight fraction (15–708 kDa), medium molecular weight fraction (5.5–15 kDa), and low molecular weight fraction (<5.5 kDa). The proportions of each fraction were calculated relative to the total area. The analysis was conducted in triplicate.

2.6.4. Protein content

The proportion of proteins of the total soluble polysaccharide (TSP) precipitates was quantified using the combustion method on a Euro EA - CHNSO Elemental Analyzer (HEKAtech GmbH, Wegberg, Germany) following the instructions of the manufacturer. Samples (2 mg) were weighed into a sample cup and directly analyzed. Acetanilide was used as external standard. Using 6.25 as nitrogen to protein conversion factor, the percentage of proteins was calculated. The analysis was conducted in duplicate.

2.7. Sensory profiling of samples

To determine the effects of interactions between polysaccharides and polyphenols on red wine astringency, the "fresh reference" and "aged" wines and their polysaccharide-free extracts were evaluated by panel tasting. The protocol of the sensory profiling was oriented on the optimized descriptive profiling (ODP) established by Rita de Cássia dos Santos Navarro da et al., 2012, which was shown to be suitable for the correlation of sensory and instrumental measurements. The wines were subjected to a bench tasting to identify the most important attributes and astringency sub-qualities of the wines. The astringency sub-qualities were selected based on the mouth-feel wheel by Gawel et al. (2000). The attributes that were agreed upon were "sour" and "bitter" as taste attributes and "astringency", "unripe" and "dry" as mouthfeel attributes. Gawel et al. (2000) defined the astringency sub-qualities "unripe" and "dry" as "a negative hedonic grouping consisting of an astringent feel associated with excessive acidity and associated green flavour notes" and "feelings of lack of lubrication or desiccation in the mouth", respectively. These definitions were presented to the panel during the tastings. After the pre-selection of the judges, the sensory panel consisted of 19 judges, of which nine were female and 10 were male with ages ranging from 23 to 60 years (mean 41 years). Panelists were recruited from the professional and private environment of the study leader on a strict voluntary basis. The pre-selection of the panel was

composed of two sessions. During the first session panelists were familiarized with the attributes and their differentiation. Solutions of caffeine (1.5 g/L; Siegfried Pharma Chemikalien, Minden, Germany), tartaric acid (2.5 g/L; Otto Fischar GmbH, Saarbrücken, Germany), aluminum sulfate (2.5 g/L; Euro OTC Pharma GmbH, Bönen, Germany), catechin (3 g/L; Sigma Aldrich Chemie GmbH, Steinheim, Germany) and tannic acid (2 g/L; Omikron GmbH, Neckarwestheim, Germany) were presented in a Pinot noir wine from 2018 used as basic wine to train "bitter", "sour", "astringency", "unripe" and "dry", respectively. This was achieved by advising the panel to assign the attributes to the corresponding solutions, whereby the matching had to be 80% correct. The second session was dedicated to the recognition of various concentrations of these solutions. Solutions of the reference materials were presented containing zero, 33%, 66%, and 100% of the standard concentrations used in the first session, whereby only two of the consecutive samples were allowed to be mixed up. Afterwards, the judges were familiarized with the intensity scale of the final tasting, which was a continuous scale from 0 to 10 for "very low intensity" and "very high intensity", respectively, and reference material. Two differently concentrated reference solutions were presented and their position on the scale was discussed. Trial tastings were held in two sessions, each presenting four wines and one reference solution. For the profiling tasting, the extract samples were dissolved in model wine solutions prepared for each wine sample according to their general composition determined by FT-IR (Table 1; compare Weilack et al. (2023) for composition of "fresh reference" wines) except for the SO_2 addition and their yield after extraction (Table A1). Wine samples were presented in a balanced random order in clear, coded glasses and were tasted in duplicate. One reference solution for each attribute was provided in every session. The panelists were advised to taste 10 mL of the samples, keep them in the mouth for 10 s and rank intensities after spitting. They were advised to neutralize the oral cavity with water and bread and to wait 3 min before tasting the following sample. All sessions were conducted as home use tests using the sensory tool RedJade 2021 (RedJade Sensory Solutions, LLC., Martinez, CA).

The study design was submitted to the Ethics Committee of the Rheinische Friedrich-Wilhelms-Universität Bonn, which approved this study (reference number 460/20). Informed consent was obtained from each panelist as part of the submitted request.

2.8. Statistical analysis

The results were statistically analyzed using XLSTAT (Version 2019.1.1, Addinsoft Technologies, Paris, France). Two-way ANOVA with a selected significance level of $\alpha = 0.05$ followed by pairwise comparison (Tukey (HSD)) was applied to analyze protein concentrations and polyphenol compositions. The phenolic composition of the "fresh reference" wines and corresponding extracts, which were previously published by Weilack et al. (2023), were statistically reanalyzed considering the comparison with the results of the "aged" wines and corresponding extracts. Three-way ANOVA with a selected significance level of $\alpha = 0.05$ followed by pairwise comparison (Tukey (HSD)) was applied to analyze the TSP concentrations. Principal Component Analysis (PCA) was performed on all attributes of the sensory profiling to investigate the effect of pectic polysaccharides and aging of wines on their sensory perception. Pearson's correlation analysis was conducted to examine the relationship between analytical parameters and sensory profiling. An agglomerative hierarchic cluster analysis (AHC), which examines the dissimilarity of the "aged" samples, was performed based on the tannin concentrations and polymeric pigment compositions (Ward method).

3. Results and discussion

3.1. Removal of polysaccharides and precipitation of total soluble polysaccharides (TSP)

Table A1 (supplemental data) presents the yields of the polyphenolic extracts obtained by solid phase extraction, the total soluble polysaccharides (TSP) concentrations of the wines and the extracts, and the protein concentrations of the alcohol insoluble precipitates. The results for the "fresh reference" wines, which were stored at 10 °C for 10 weeks, have been published earlier, but are included for convenience (Weilack et al., 2023). The TSP concentrations of the polyphenolic extracts were determined by referencing to the corresponding extract concentration in the wine. The results (Table A1) show that the polyphenolic extracts contain only negligible amounts of TSP compared to the high yields of the wines; hence, the wine polysaccharides were successfully removed ensuring that the differences seen between wines and polysaccharide-free extracts can be assigned to the presence and absence, respectively, of soluble polysaccharides.

Because the alcoholic precipitation of TSP causes the co-precipitation of proteins (Selvendran, 1975), the protein concentrations in the precipitates were determined to rule out that the differences in polyphenol composition of the Cabernet Sauvignon wines and corresponding extracts are due to interactions between polyphenols and proteins. The protein concentrations ranged from 0.9 ± 0.1 % for the "fresh reference" Adentu wine to 6.3 ± 0.2% for the "fresh reference" Beringer wine (Table A1) indicating that the majority of compounds in the precipitates were wine polysaccharides. Moreover, parts of the protein content derive from the protein residues of polysaccharides like arabinogalactan-proteins and mannoproteins. However, after the aging, the TSP yields of the "aged" wines, except for the Weinbiet and Bundschuh wines, are significantly higher than their "fresh reference" counterparts. The higher TSP yields after the accelerated aging are hardly correlated with an increased co-precipitation of proteins ($r = 0.47$; Table A1), which becomes evident by the fact that the "aged" Canyon Road, Beringer, and Bundschuh wines show no difference or even lower protein concentrations compared to the corresponding "fresh reference" wines. Earlier research reported that the structural features of pectic polysaccharides like degree of methylation (DM), proportions of galacturonic acid, and molecular weight highly impact their alcohol solubility (Guo et al., 2016; Karnik, Jung, Hawking, & Wicker, 2016). Because TSP were obtained through alcoholic precipitation, the data hints that the polysaccharides underwent structural changes during the aging that led to an increased alcohol insolubility and higher TSP yields.

3.2. Polyphenol composition of the wines and the corresponding polysaccharide-free counterparts

Anthocyanins, tannins, and the ratio of protein precipitable polymeric pigments (p-PP) and non-precipitable polymeric pigments (np-PP) of the "fresh reference" and "aged" wines and corresponding polysaccharide-free counterparts were determined (Fig. 1). To display and discuss the compositional changes the wines underwent during aging and the removal of polysaccharides, the results of the "fresh reference" wines and their corresponding extracts, which have been published earlier (Weilack et al., 2023), are included in Fig. 1.

While Table 1 shows that the general composition of the wines was not significantly affected by the accelerated aging (data of the "fresh reference" wines is not shown), the polyphenolic composition of the wines changed due to age-related reactions indicating that the "aged" samples can be described as distinct wines (Fig. 1). During aging, anthocyanin concentrations generally decreased in wine and extract samples, which was expected due to degradation, conversion, and incorporation of anthocyanins into polymeric pigments (McRae et al., 2012). Besides the anthocyanins of the "aged" Weinbiet wine, which declined by 33%, the other "aged" wines show a decline of around 50%

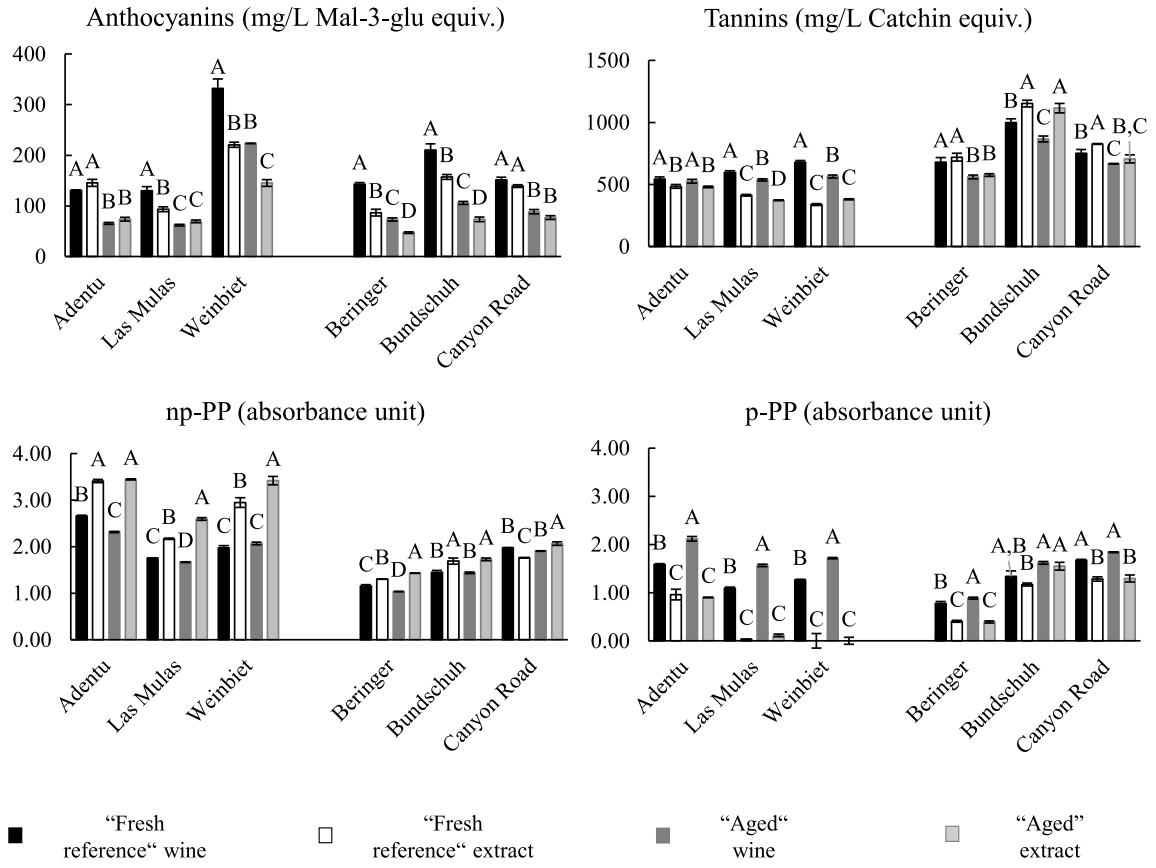

Fig. 1. Phenolic composition of commercially available Cabernet Sauvignon wines, which were subjected to forced aging (35 °C for 10 weeks; "aged"), "fresh reference" wines (10 °C for 10 weeks), and their corresponding polysaccharide-free extracts. Analyses included total anthocyanins, non-precipitable polymeric pigments (np-PP), precipitable polymeric pigments (p-PP), and total tannins and were obtained by photometric assays (Harbertson et al., 2003, 2009, 2015). Weilack et al. (2023) have previously published the results of the "fresh reference" wines and extracts. The gap between the Weinbiet and Beringer samples is implemented to differentiate the two groups according to whether protein precipitation of tannins is enhanced or reduced as mentioned in the text. Means having different letters show a significant difference (α = 0.05) within each wine; n = 3.

in anthocyanins. Comparing the results of the "fresh reference" extracts with the "aged" extracts reveals that the actual decline of Las Mulas and Weinbiet anthocyanins amounts to 26% and 34%, respectively, while the decline in the other extract samples stays at around 50%. This indicates that the anthocyanins found in these two wines were less affected by the age-related changes in polyphenol composition. The "aged" Beringer, Bundschuh and Weinbiet wines show higher anthocyanin concentrations than their corresponding extracts probably due to co-pigmentation-like effects resulting from the interactions between wine polysaccharides and anthocyanins (Padayachee et al., 2012). These effects lead to hyperchromic shifts in the absorbance spectra of anthocyanins (Fernandes et al., 2020) and to seemingly higher anthocyanin concentrations in the wines, as assessed by the photometric assay. However, data shows that these co-pigmentation-like effects became less pronounced during the aging. The co-pigmentation-like effect is based on the adsorption of anthocyanins on pectins, followed by the self-aggregation and parallel stacking of anthocyanins (Padayachee et al., 2012). As anthocyanins generally decreased during the aging, this led to a reduced number of anthocyanins that could take part in the stacking possibly leading the diminished copigmentation.

Tannin concentrations decreased during aging in all wine samples but the Adentu wine, which shows no changes in tannin concentrations. In contrast, the tannin readings of the polysaccharide-free extracts with the exception of Beringer and Canyon Road show negligible changes in tannin concentrations. According to Weilack et al. (2023) the differing tannin readings, which are based on the protein precipitation of tannins, result from the modulation of tannin precipitability by pectic polysaccharides. Thereby, the composition of polysaccharides determines whether tannin precipitability is enhanced or prevented. Small, more polar and acidic polysaccharides, like RG-II and polygalacturonic acids, prevent tannin precipitability, whereas large, more neutral and esterified polysaccharides, like AGP, AG and HG fragments, increase tannin precipitability (Carvalho et al., 2006; de Freitas, Carvalho, & Mateus, 2003; Mateus, Carvalho, Luís, & de Freitas, 2004; Weilack et al., 2023). As seen earlier (Weilack et al., 2023), this allowed the categorization of the "fresh reference" wines into two distinct groups, according to the direction of the effect. Comparing the tannin concentrations of the "aged" wines and corresponding "aged" extracts, it appears that the "aged" samples still divide into the same two groups. Group 1 includes the Adentu, Las Mulas and Weinbiet wines, which show an increased protein precipitability, whereas in group 2 (Beringer, Bundschuh and Canyon Road wines) tannin precipitability decreases in the presence of the respective polysaccharides. This is supported by the cluster analysis of the tannin concentrations and polymeric pigment composition of the "aged" samples, which shows that the wines can be clustered into two main groups, with the Bundschuh and Weinbiet wines forming a separate group based on (pigmented) tannins and np-PP, respectively (Fig. 2).

The aging process led to a slight decline in non-precipitable polymeric pigments (np-PP) in the "aged" Adentu and Beringer wines, while they stagnated in the other wines. On the other hand, precipitable polymeric pigments (p-PP) increased significantly in the "aged" group 1 wines. This is supported by previous publications (Merrell, Larsen, & Harbertson, 2018; Weilack et al., 2021), which postulated that the

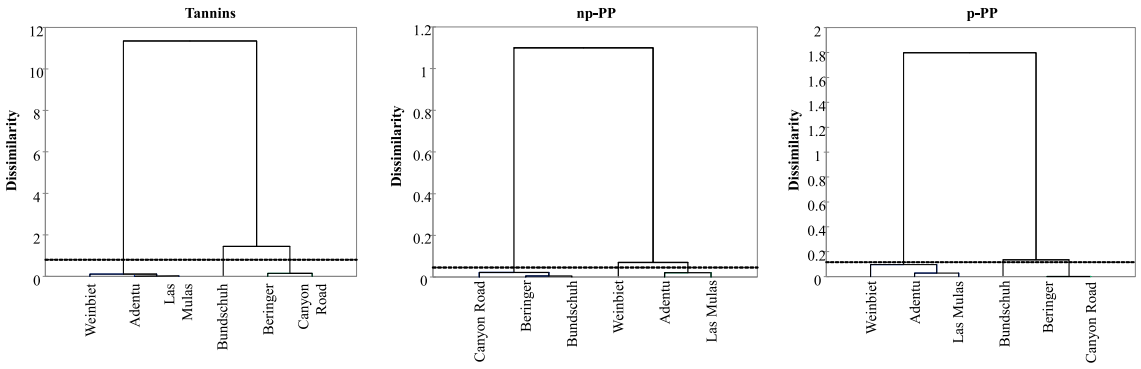

Fig. 2. Results of the agglomerative hierarchic cluster analysis (AHC; Ward method) based on the dissimilarities of the tannin concentrations and polymeric pigment compositions of the "aged" samples. Tannins and polymeric pigments were determined according to Harbertson et al. (2003, 2015) and are presented in Fig. 1.

formation of np-PP would reach a plateau during red wine aging either due to balanced formation and degradation of np-PP or favored formation of p-PP. However, this study shows that np-PP increased in the "aged" polysaccharide-free extracts while p-PP stagnate in all "aged" extracts but the Bundschuh extract, which shows an increase in p-PP. In general, the "aged" wines contain less np-PP and more p-PP than the "aged" polysaccharide-free counterparts, whereas the "aged" polysaccharide-free extracts show higher np-PP proportions and lower p-PP proportions. Thereby, group 1 wines show the bigger differences; hence, in the presence of pectic polysaccharides np-PP are apparently measured as p-PP indicating a higher protein precipitability (Weilack et al., 2023). Ultimately, this leads to the conclusion that certain structural features of pectic polysaccharides result in the formation of protein precipitable pigments, which are measured as p-PP, but are composed of pigmented polysaccharide-polyphenol complexes (Weilack et al., 2023). Altogether, this extends the findings of Graves and Sommer (2021), who showed that polymeric pigment determination using the protein precipitation assay was affected by polysaccharides depending on their concentration they were added to the wines. The authors attributed this effect to the reduced bisulfite bleachability of complexed anthocyanins, which was similarly shown for a decreased discoloration of anthocyanins due to hydration (Fernandes et al., 2021).

The differences in polymeric pigment proportions between wines and extracts become more pronounced with the aging of the wines suggesting the ongoing formation or pigmentation of these protein precipitable pigments. The "fresh reference" Las Mulas and Weinbiet extracts did not contain p-PP, indicating that their specific polysaccharides might have prevented the formation of p-PP (Weilack et al., 2023). The present study shows that the "aged" Las Mulas and Weinbiet extracts contain very few to no p-PP. Together with the fact that anthocyanins were seemingly less affected by age-related reactions, this reinforces the assumption that the aggregation of polysaccharides and pigments could even impair the incorporation of anthocyanins into tannins hindering the formation of conventional, covalently bound p-PP. In contrast, the increase in p-PP of the "aged" Bundschuh polysaccharide-free extract suggests that conventional p-PP were actually formed in this wine during aging. The proceeding incorporation of anthocyanins into tannins renders the tannins more polar, which may lead to an increased interaction with negatively charged RG-II molecules (Dufrechou, Doco, Poncet-Legrand, Sauvage, & Vernhet, 2015). This may result in an enhanced protective effect of RG-II towards the precipitation of tannins in the "aged" Bundschuh wine (Watrelot et al., 2017). It cannot be completely ruled out that storage at 35 °C during the accelerated aging of the wines could have triggered reactions that are not observed during typical aging of red wines at lower temperatures.

Earlier research (Alcalde-Eon et al., 2014; Guadalupe et al., 2007; Osete-Alcaraz et al., 2020; Siebert, Carrasco, & Lynn, 1996) reported that some polysaccharides may act as protective colloids in red wine, stabilizing anthocyanins, anthocyanin-derived pigments, and tannins preventing them from aggregating with proteins and self-aggregation. At the same time, Siebert et al. (1996) and Guadalupe et al. (2007) observed the precipitation of unstable colloidal polyphenol-protein-polysaccharide ternary complexes. Therefore, the altered protein precipitability of tannins and pigments due to polysaccharides can be assigned to the presence and formation of protective/stable or unstable colloids, respectively. Accordingly, the categorization of the wines into two groups can be based on the stability of the colloidal system, being unstable for group 1 (Adentu, Las Mulas and Weinbiet) and stable for group 2 (Beringer, Bundschuh and Canyon Road). In the following, total soluble polysaccharides will be characterized to investigate age-related changes of the structural features of (un-)stable colloids.

3.3. Total soluble polysaccharides (TSP) composition

To characterize the soluble polysaccharides of the "aged" wines, the molecular weight (MW) distribution, the monomeric sugar composition, and the degree of methylation (DM) and acetylation (DA) were determined using size exclusion chromatography (SEC), photometric assays, and GC-FID (Table 2 and Fig. 3). Because all the wines showed an increased precipitation of TSP (Table A1), which may be due to the structural changes of the wine pectic polysaccharides, the composition of TSP may partly be assigned to the composition of the additionally precipitated polysaccharides. The polysaccharide composition of the Cabernet Sauvignon wines that were not subjected to accelerated aging but stored for 10 weeks at 10 °C, following referred to as "fresh reference" wines, was subject of an earlier work and is described by Weilack et al. (2023).

Fig. 1 shows the MW distribution of the polysaccharides of the "aged" wines, which were stored at elevated temperatures for 10 weeks to simulate red wine aging. The polysaccharides show a high polydispersity across all samples and are split into three fractions: the high MW fraction (>15 kDa) comprising substances such as arabinogalactans (AG), arabinogalactan-proteins (AGP), mannans, mannoproteins (MP), as well as small quantities of homo- (HG) and rhamnogalacturonan I (RG-I) (Ayestarán, Guadalupe, & León, 2004; Gao et al., 2015; Guadalupe & Ayestarán, 2007); the medium MW fraction (5.5–15 kDa) including rhamnogalacturonan II (RG-II) dimers (~10 kDa; Pellerin et al., 1996) and medium MW fragments of homogalacturonan (HG), rhamnogalacturonan I (RG-I), AGP, and MP; the low MW fraction (<5.5 kDa) containing rhamnogalacturonan II (RG-II) monomers (~4.6 kDa; Pellerin et al., 1996) and HG, RG-I, AGP, and MP fragments with low molecular weights (Guadalupe & Ayestarán, 2007). With the exception of the "aged" Bundschuh and Beringer wines, the MW distributions of the "aged" wines shift slightly from the high MW to the medium and small MW fractions (Table 2 and Fig. 3). All "aged" wines show a distinct

Table 2
Composition of the pectic polysaccharides of the "aged" Cabernet Sauvignon wine samples after storage at 35 °C for 10 weeks including the proportions of the high and medium molecular weight (MW) fractions, the degree of methylation (DM) and acetylation (DA) and the monosaccharide composition including galacturonic acid (GalAc), rhamnose (Rha), and fucose (Fuc). Means having the same letters are not significantly different at α = 0.05. Means presented with standard deviation; n = 3.

Wine	High MW fraction (15–708 kDa) [%]	Medium MW fraction (5.5–15 kDa) [%]	DM [%]	DA [%]	GalAc [mg/g]	Rha [mg/g]	Fuc [mg/g]
Adentu	55.3 ± 0.6 A	44.7 ± 0.6 D	8.3 ± 0.1 E	0.1 ± 0.1 D	37.0 ± 1.5 A	9.1 ± 0.5 A	1.7 ± 0.1 A
Beringer	47.4 ± 2.4 B	52.6 ± 2.4 C	24.3 ± 1.1 C	8.2 ± 0.1 A	32.9 ± 3.1 A	3.2 ± 0.1 B	1.3 ± 0.1 B
Bundschuh	49.5 ± 0.6 B	50.5 ± 0.6 C	30.7 ± 0.8 B	3.2 ± 0.6 B,C	35.2 ± 0.6 A	2.8 ± 0.1 B,C	1.7 ± 0.1 A
Canyon Raod	34.4 ± 0.6 D	65.6 ± 0.6 A	13.4 ± 0.1 E	3.5 ± 0.2 B,C	33.0 ± 1.8 A	3.3 ± 0.1 B	1.4 ± 0.1 B
Las Mulas	39.7 ± 0.5 C	60.3 ± 0.5 B	19.3 ± 1.0 D	1.9 ± 0.4 C,D	31.1 ± 0.1 A	2.4 ± 0.1 C	1.3 ± 0.1 B
Weinbiet	50.6 ± 1.6 B	49.4 ± 1.6 C	48.5 ± 2.6 A	4.3 ± 0.4 B	34.9 ± 1.0 A	2.3 ± 0.1 C	1.4 ± 0.1 B

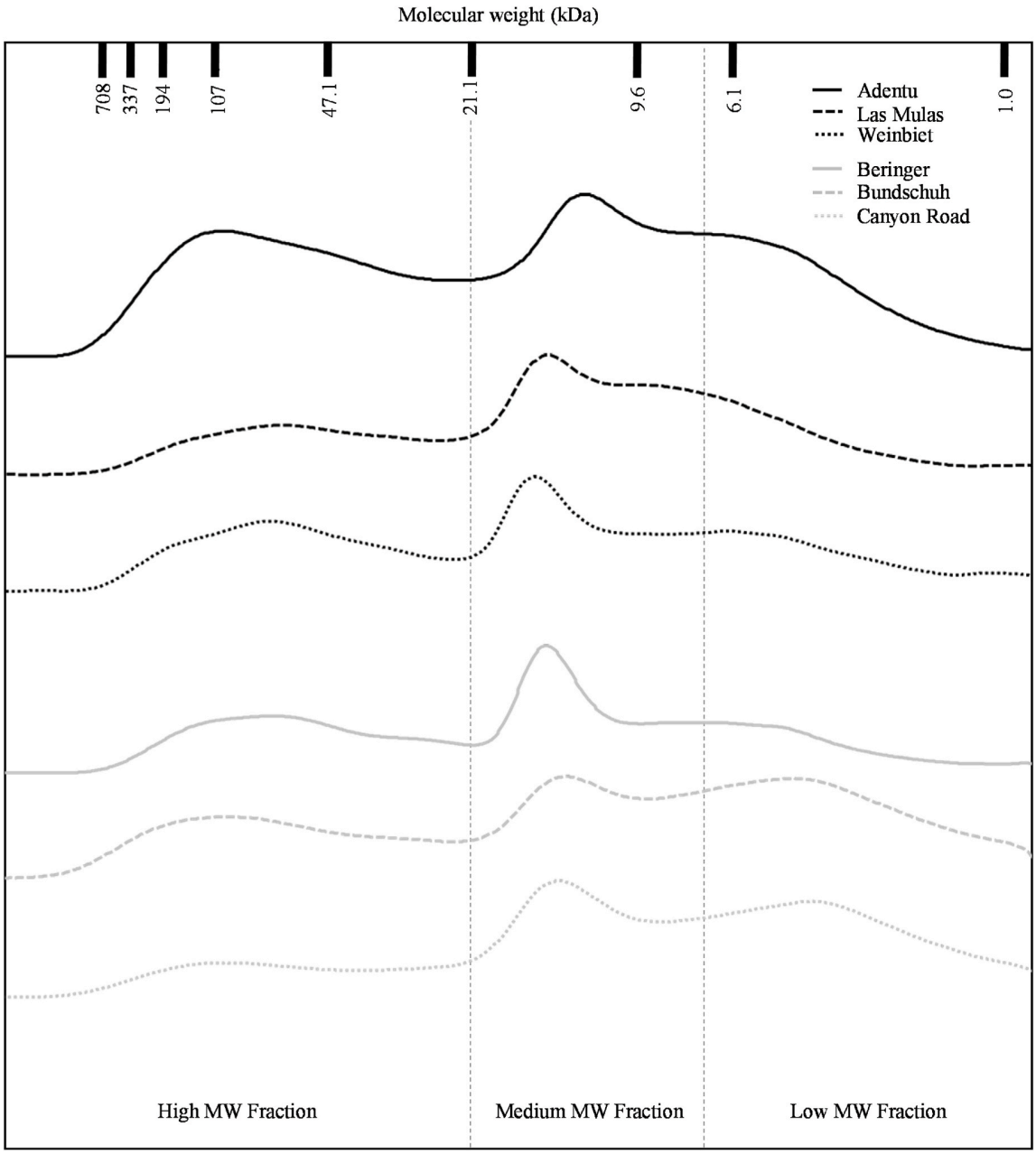

Fig. 3. Size exclusion chromatograms of the pectic polysaccharides of the "aged" Cabernet Sauvignon wine samples after storage at 35 °C for 10 weeks including the high (>15 kDa), medium (15–5.5 kDa) and low (<5.5 kDa) molecular weight (MW) fractions. Chromatograms are overlayed and offset. Chromatograms of the "fresh reference" wine samples were presented earlier (Weilack et al., 2023). MW calculation was calibrated with standards illustrating the peak molecular weight distribution.

peak in the low MW fraction at around 4.6 kDa, suggesting the presence of RG-II monomers and low MW fragments of other pectic polysaccharides, like polygalacturonic acids. While the proportions of fractions are constant for the Bundschuh wines, the "aged" Beringer wine shows a higher proportion of high MW and a lower proportion of low MW polysaccharides after the aging of the wine. Like the "fresh reference" wines (Weilack et al., 2023), the "aged" Adentu and Weinbiet wines show the highest proportions of the high MW fraction (Table 2) suggesting high proportions of AG, AGP, MP, HG and RG-I. In contrast, the "aged" Bundschuh, Beringer, Canyon Road and Las Mulas wines show high proportions of the medium MW fraction, hence, considerable amounts of RG-II molecules and other low MW fragments.

To further characterize the composition of pectic polysaccharides, galacturonic acid (GalAc), rhamnose (Rha) and fucose (Fuc) concentrations were determined (Table 2), which can be correlated with the relative proportions of HG, RG-I and RG-II fragments, respectively, due to their well-defined occurrence in the pectin structure (Larsen et al., 2019). While the "fresh reference" wines showed a wide range of GalAc concentrations (16.0 ± 0.6 mg/g for the Adentu wine to 47.6 ± 0.6 mg/g for the Bundschuh wine; Weilack et al., 2023), the aging of the wines led to the GalAc concentrations approaching a similar level and ranging from 31.1 ± 0.1 to 37.0 ± 1.5 mg/g for the "aged" Las Mulas and Adentu wines, respectively. In contrast to the "aged" Bundschuh and Weinbiet wines, which showed lower GalAc concentrations after aging, the GalAc concentrations of the other wines increased. The shift of the MW distribution of the polysaccharides towards smaller molecules, together with an increased TSP yield (Table A1) indicates the degradation of larger HG chains to smaller oligomers during the simulated aging, which resulted in an enhanced alcohol precipitability as published by Guo et al. (2016) and Karnik et al. (2016).

Data presented in Table 2 and Fig. 3 shows that the polysaccharide composition of the wines became more similar to each other during the accelerated aging, which is particularly evident in the concentrations of the monomeric sugar concentrations. The structural changes observed in this study were similarly reported by Doco, Quellec, Moutounet, and Pellerin (1999), Guadalupe and Ayestarán (2007), and Quijada-Morín, Williams, Rivas-Gonzalo, Doco, and Escribano-Bailón (2014), who showed that various periods of storage of red wines led the polysaccharide composition to shift towards smaller pectin fragments. While these authors stated that the amount of TSP remained unchanged or declined, Guo et al. (2016), and Karnik et al. (2016) showed that lower degrees of esterification, less neutral sugars, higher GalAc concentrations and less RG-I fragments would lead to an increased alcohol insolubility. As the TSP composition of the "aged" wines is characterized by these features, this might explain the increased TSP yields.

The number of free hydroxy groups and in particular the number of free and dissociated carboxylic acid groups, which is represented by the degree of esterification, significantly influences the polarity and hydrophobic nature of pectic polysaccharides. Moreover, the strength of interactions between polysaccharides and polyphenols depend on structural features like the degree of esterification (Liu et al., 2020). During the aging of the wines, the DM and DA decreased in all samples apart from the "aged" Beringer wine, in which the esterification increased. The decrease of DM and DA together with the increase in GalAc concentrations and the shift to lower MW render the resulting polysaccharides more polar, which impacts the interactions with polyphenols and proteins. As the changes of the polysaccharide composition led to smaller proportions of the large, neutral polysaccharides in the "aged" Adentu, Las Mulas and Weinbiet wines and an increase in smaller, less esterified, thus more acidic, pectin fragments, the differences of tannin concentrations between "aged" wines and corresponding polysaccharide-free extracts become less pronounced. This indicates that the structural changes of pectic polysaccharides result in a slightly less enhanced protein precipitation of tannins, thus, less unstable colloids. On the other hand, the "aged" Bundschuh wine shows a higher RG-II concentration than the "fresh reference" wine, which had a Fuc concentration of 1.5 ± 0.1 mg/g (Weilack et al., 2023), thus the Bundschuh polysaccharides show a higher masking effect for tannins.

Overall, as described earlier, the "aged" wines can still be categorized into two groups according to the precipitability of tannins and pigments, thus, the colloidal stability (Fig. 2). Group 1 wines mainly contained large, neutral, and more esterified, HG and RG-I fragments, which formed unstable colloids with polyphenols and proteins, whereas group 2 polysaccharides appear to be protective colloids, which were composed of a larger proportion of small, acidic, more polar, and less esterified pectin fragments including RG-II. This indicates that the polysaccharide composition of the wines at the beginning of the aging process might be decisive for the development of wine polyphenols and particularly for essential compounds like polymeric pigments.

3.4. Sensory analysis

3.4.1. Implications of polyphenol and polysaccharide interactions on the astringency perception of "fresh reference" Cabernet Sauvignon wines

Astringency and bitter perception of red wines are associated with tannin concentrations and their composition, which includes the degree of polymerization, galloylation and the number of trihydroxylated monomers (de Freitas & Mateus, 2001; Noble, 1998; Vidal et al., 2003). To ensure that the results of the sensory analysis can be reasoned by the presence or the lack of polysaccharides rather than tannin composition in the samples, the polysaccharide-free polyphenolic extracts were dissolved in model wines that were composed according to their yields and the general composition of the corresponding wines. This way, differences caused by ethanol content, residual sugar, acidity, and pH value are eliminated.

Fig. 4 shows the principal component analysis (PCA) of the sensory profiling of all Cabernet Sauvignon samples, the wines and the corresponding polysaccharide-free extracts, including the attributes "sour", "bitter", "astringency", "unripe", and "dry". The detailed ratings of the attributes for the wines and extracts are presented in Figure A1. The attribute "sour" correlates negatively with the attributes "bitter", ($r = -0.515$; sign. with $p = 0.010$), "unripe" ($r = -0.414$; sign. with $p = 0.044$), and "dry" ($r = -0.180$) while "astringency" hardly correlates with any of the other attributes ("bitter": $r = 0.075$; "sour": $r = 0.289$; "unripe": $r = 0.049$; "dry": $r = 0.300$). The samples are split into two groups along the first PC showing that the wines tend to be rated more sour than the extracts, whereas the extracts appear to be more bitter, unripe and dry; hence, the samples can be differentiated by whether or not they contain polysaccharides. The sourness of the "fresh reference" wines correlates with the GalAc concentration ($r = 0.792$); thus, the sourness perception may be related to the proportion of HG fragments, which were extracted into the wine. Therefore, the wines taste more sour in the presence of acidic pectic polysaccharides. This is supported by Chong, Cleary, Dokoozlian, Ford, and Fincher (2019), who reported that sourness of Cabernet Sauvignon wines was related to HG concentrations.

While the astringency sub-qualities, "unripe" and "dry", are related to the polysaccharide content of the samples, the overall "astringency" appears to be correlated rather to the aging of the samples, as the "fresh reference" and "aged" samples are spread along the second PC. Therefore, overall astringency and sub-qualities seem to be perceived independently from each other which was also observed by Wang et al. (2020). The PCA shows a higher explained variance (56%) between the wines and their polysaccharide-free extracts than between the "fresh reference" and "aged" samples (23%). According to Sáenz-Navajas et al. (2020), tannin concentrations were correlated to the general dryness and dryness on palate perception of red wines, whereas tannin activity, which is driven by hydrophobic interactions with proteins, was solely related to the dryness on palate. However, the authors could not link other mouthfeel perceptions to the studied chemical parameters highlighting the need of extending the investigations beyond tannin composition (Sáenz-Navajas et al., 2020). Since hydrophobic

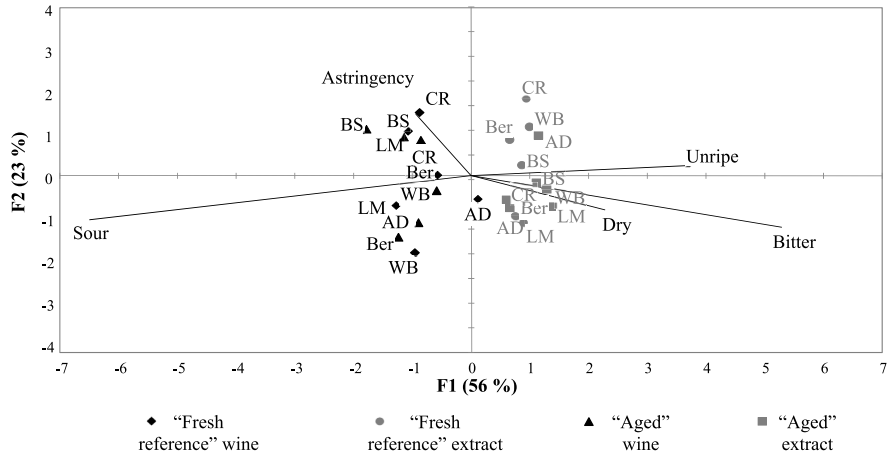

Fig. 4. Principal component analysis (PCA) of the sensory profiling of all Cabernet Sauvignon wines (AD: Adentu, Ber: Beringer, BS: Bundschuh, CR: Canyon Road, LM: Las Mulas, WB: Weinbiet) and corresponding polysaccharide-free extracts, including the attributes "sour", "bitter", "astringency", "unripe", and "dry".

interactions play a role in the interactions of tannins and polysaccharides, mouthfeel perceptions are very likely to be also related to polysaccharide composition.

The "fresh reference" Adentu, Canyon Road and Weinbiet wines are more astringent than their polysaccharide-free extracts, whereas the "fresh reference" Bundschuh wine shows lower "astringency" ratings than the extract (Figure A1). This is more or less in line with the tannin concentrations (Fig. 1), as the Adentu and Weinbiet wines show higher and the Bundschuh wine lower tannin readings than their polysaccharide-free counterparts. However, the higher tannin readings of the Adentu and Weinbiet wines result from an increased tannin precipitability due to interactions between tannins and larger, neutral polysaccharides with a high DM (Weilack et al., 2023). This leads to the assumption, that such tannin-polysaccharide aggregates, which are measured as tannins, contribute to the astringency perception of the wines. Furthermore, not only tannins show a higher precipitability, but also precipitable polymeric pigments are more precipitable. Previous studies (Watrelot et al., 2017; Weber et al., 2013; Weilack et al., 2021) suggested that the formation of pigmented tannins, which are determined as precipitable polymeric pigments, play a role in the attenuation of red wine astringency. The comparison of the "fresh reference" Adentu and Weinbiet wines with their corresponding polysaccharide-free extracts shows that in these wines little to no p-PP were formed (Fig. 1), which may also be adding to the increased astringency perception of the wines.

In contrast, the Bundschuh wine contains a higher proportion of small, acidic polysaccharides and a high concentration of RG-II molecules that lead to the prevention of tannin precipitability (Weilack et al., 2023) and consequently to a lowered astringency perception in the wine when compared to the polysaccharide-free extract (Figure A1). The Canyon Road wine appears to be out of line because the "fresh reference" wine is rated more astringent than the corresponding extract (Figure A1) while having a lower tannin precipitability due to its polysaccharide composition (Weilack et al., 2023). According to Carvalho et al. (2006), RG-II molecules can have different effects on the protein-tannin aggregation depending on the type of protein. In the presence of the globular protein α-amylase, RG-II appeared to prevent tannin precipitability while in the presence of IB8c, a representative of the proline-rich proteins (PRPs), RG-II increased protein-tannin aggregation (Carvalho et al., 2006). Since BSA used for tannin precipitation is a globular protein, the lower tannin readings of the "fresh reference" Bundschuh and Canyon Road wines (Fig. 1) cannot simply be related to the astringency perception that is assigned to the interactions between tannins and PRPs (Charlton et al., 2002). However, Vidal, Francis, Noble, et al. (2004) showed that acidic and neutral polysaccharides alone can decrease the astringency perception of a model wine indicating that they can interact with salivary proteins. Besides the highest RG-II concentration, the "fresh reference" Bundschuh wine contains the highest amount of GalAc and the second highest DM indicating the presence of a high proportion of methylated HG fragments (Table 2). Due to the methylation, these fragments are more hydrophobic and therefore they may take part in hydrophobic interactions with salivary PRPs comparable to the first step of astringency elucidation (Charlton et al., 2002). Consequently, this interaction may lead to a lack of PRPs that could precipitate tannins, thus, to a lower astringency perception of the "fresh reference" Bundschuh wine than of the polysaccharide-free counterpart (Figure A1).

In the "fresh reference" wines and the corresponding extracts, the "unripe" and "dry" perceptions of the wines are significantly correlated with Rha concentrations ("unripe": $r = 0.625$, "dry": $r = 0.634$), GalAc ("unripe": $r = -0.725$) concentrations and the Rha/GalAc ("unripe": $r = 0.817$) ratio indicating a relationship between these mouthfeel attributes and the HG and RG-I proportions of the wine polysaccharides. Wang et al. (2020) hypothesized that polysaccharides could lower saliva precipitation by competing with polyphenols in aggregating saliva proteins indicating that polysaccharides can reduce the amount of saliva proteins that could precipitate polyphenols. Comparable to the maltodextrin used in said study, HG is a linear chain of repeating units, in this case GalAc units, which are also partly methylated leading to a higher hydrophobicity enabling it to interact directly with the saliva proteins (Einhorn-Stoll, Archut, Eichhorn, & Kastner, 2021; Rodrigues et al., 2021). This may lead to a protective effect of HG fragments against an unripe and dry perception of red wine. RG-I, on the other hand, is a branched polysaccharide that may be sterically hindered to align and interact with the salivary proteins. A high number of RG-I fragments in the wines would therefore lead to the loss of the protective effect of HG fragments, resulting in an increased unripe and dry astringency, which was observed in the "fresh reference" Adentu and Beringer wines (Figure A1).

3.4.2. Implications of polyphenol and polysaccharide interactions on the astringency perception of bottle aged Cabernet Sauvignon wines

As mentioned before, the PCA of the sensory analysis (Fig. 4) showed a clear separation of the sample types, wine and polysaccharide-free wine, according to their polysaccharide content. The explained variance between the samples based on aging is not as pronounced. In contrast to the "fresh reference" wines, the "aged" Adentu, Las Mulas and Weinbiet wines (group 1) are less "astringent", "unripe" and "dry" than their polysaccharide-free counterparts (Figure A1). This is accompanied with decreasing tannin concentrations, while the proportions of

p-PP increase during aging (Fig. 1). As described before, these changes in polyphenol composition are not reflected in the results of the corresponding polysaccharide-free extracts, which stagnate at lower concentrations for both parameters; hence, while still having an increased tannin precipitability, tannins of the "aged" group1 wines are less precipitable after aging. Therefore, the prevented tannin precipitability together with the higher proportions of pigmented polysaccharide aggregates formed during aging may have a positive effect on the astringency development of the wines. Considering that these pigmented polysaccharide aggregates are measured as p-PP because of their protein precipitability and ability to absorb light at 520 nm, this supports the hypothesis that the formation of p-PP during red wine aging may be beneficial for astringency perception and quality. Yet, this study shows that this may not only be due to pigmented tannins, but also non-covalently bound polysaccharide-polyphenol-pigments may contribute to the attenuated astringency of aged red wines.

In contrast, the "aged" group 2 wines (Beringer, Bundschuh and Canyon Road) show higher means in the astringency-associated attributes than the "fresh reference" wines, and the "aged" wines are perceived as more astringent than their polysaccharide-free counterparts suggesting an opposing astringency development to the one described before (Figure A1). Contrary to the expectation that astringency would decrease in the presence of pigmented tannins (Vidal, Francis, Noble, et al., 2004; Weber et al., 2013; Weilack et al., 2021), the "aged" Bundschuh wine shows an increased astringency after aging despite the formation of p-PP (Fig. 1). As the "aged" Bundschuh wine contains the highest proportion of RG-II (Table 2) and pigmented tannins (Fig. 1), the increased astringency perception may be assigned to enhanced polar interactions between polysaccharides, pigmented tannins, and proteins. This suggests that the soluble complexes formed between the polymeric pigments, tannins, and the acidic, low molecular weight pectin fragments, including RG-II molecules, of the "aged" group 2 wines may still add to the astringency perception. This is supported by Brossard et al. (2021), who showed that both, soluble and insoluble, protein-tannin aggregates can modulate red wine astringency and sub-qualities.

Based on the polysaccharide composition of the wines, the Bundschuh wine appears to have undergone an enzyme treatment, while the Weinbiet wine is considered not treated (Weilack et al., 2023). Similar to the results presented in this study, Kuhlman, Hansen, Jørgensen, Du Toit, and Moore (2022) showed that Cabernet Sauvignon wines, which were not treated with enzymes, contained higher concentrations of large, neutral pectic polysaccharides and were described as being soft, fine, and velvety, whereas enzyme treated wines were more astringent with hard, chalky, grippy, grainy, and dry sub-qualities. The enzyme treatment was accompanied with higher amounts of polymerized and galloylated polyphenols (Ducasse et al., 2010; Kuhlman et al., 2022), which could lead to a coarser astringency perception (Vidal et al., 2003). Overall, astringency appears to emerge from the combination of the respective polysaccharide and polyphenol compositions, whereby the polysaccharide composition can be modulated by using pectolytic enzymes during the winemaking.

It should be mentioned that the astringency attributes are examined individually in this study, but it is unlikely that the reactions described are triggered independently of each other, since many different molecules and structures come together at the same time during red wine consumption (González-Muñoz et al., 2022).

4. Conclusion

The results of the sensory analysis together with the polyphenolic characterization of the wines and corresponding polysaccharide-free extracts show that the samples can be divided into two groups according to the polysaccharide composition of the "fresh reference" wines and their colloidal stability. On the one hand, the polysaccharides of the "fresh reference" Beringer, Bundschuh and Canyon Road wines consisted of a larger proportion of small, acidic, more polar, and less esterified, thus less hydrophobic pectin fragments, which formed stable colloids with polyphenols and prevented protein precipitation. On the other hand, the "fresh reference" Adentu, Las Mulas, and Weinbiet wines contained a greater portion of large, neutral, and more esterified, thus, more hydrophobic pectin fragments, which form unstable colloids and promote protein precipitation of polyphenols. Moreover, the latter appears to impair the formation of covalent protein precipitable polymeric pigments, which is associated with red wine aging due to the complexation of anthocyanins, tannins, and non-precipitable polymeric pigments and keeping them from further reactions. Instead, pigmented protein precipitable polysaccharide-polyphenol aggregates were formed during aging, which contribute to red wine astringency perception and possibly also to color stability. This suggests that the polysaccharide composition of the wines at the beginning of the aging process may be decisive for the development of the polyphenolic composition and astringency of aging wines. Using enzymes with certain pectolytic activities can modulate the composition of pectic polysaccharides during winemaking. This can influence wine style and its potential for aging, which is why further research on the influence of pectic polysaccharides on the polyphenolic composition and astringency perception of red wine is necessary.

Funding

This IGF project of the FEI (Forschungskreis der Ernährungsindustrie e.V. Bonn) was supported within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economics and Climate Action (BMWK) based on a resolution of the German Parliament. Project AiF 20024N.

CRediT authorship contribution statement

Ingrid Weilack: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Lea Mehren:** Methodology, Investigation, Formal analysis. **Fabian Weber:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank Sandra Feifel from the Wein Campus Neustadt for supporting them with the data on the general composition of the wines.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110402.

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Supplementary Data

Table A.1 Yields of the Cabernet Sauvginon polyphenol-rich extracts that are poor in polysaccharides obtained by solid phase extraction, total soluble polysaccharides (TSP) contents of the wine and extract samples obtained by alcoholic precipitation, and protein concentrations of TSP. Means having the same letters are not significantly different at $\alpha = 0.05$. Means presented with standard deviation; n = 2-3.

Wine		Yield Extraction [g/L]	Wine TSP [g/L]	Extract TSP [g/L]	Protein [%]
Adentu	Fresh	4.32±0.11	3.75±0.23 E,F	0.01±0.01 G	0.93±0.06 H
	Aged	4.45±0.19	4.99±0.31 B,C,D	0.10±0.01 G	3.98±0.07 E
Las Mulas	Fresh	3.76±0.07	3.24±0.14 F	0.03±0.00 G	2.81±0.05G
	Aged	4.09±0.52	4.44±0.08 C,D,E	0.02±0.02 G	3.44±0.02 F
Weinbiet	Fresh	4.63±0.38	4.34±0.27 D,E	0.07±0.02 G	4.55±0.07 D
	Aged	4.61±0.33	5.00±0.11 B,C,D	0.02±0.02 G	4.78±0.11 C,D
Beringer	Fresh	3.99±0.20	4.68±0.12 C,D	0.04±0.01 G	6.32±0.21 A
	Aged	3.90±0.01	6.55±0.45 A	0.04±0.01 G	4.59±0.01 D
Bundschuh	Fresh	5.15±0.07	5.05±0.38 B,C	0.08±0.00 G	5.76±0.16 B
	Aged	5.29±0.29	5.40±0.22 B	0.20±0.32 G	5.04±0.06 C
Canyon Road	Fresh	4.24±0.08	3.18±0.07 F	0.01±0.00 G	4.89±0.06 C,D
	Aged	4.20±0.03	6.12±0.21 A	0.01±0.03 G	4.70±0.03 C,D

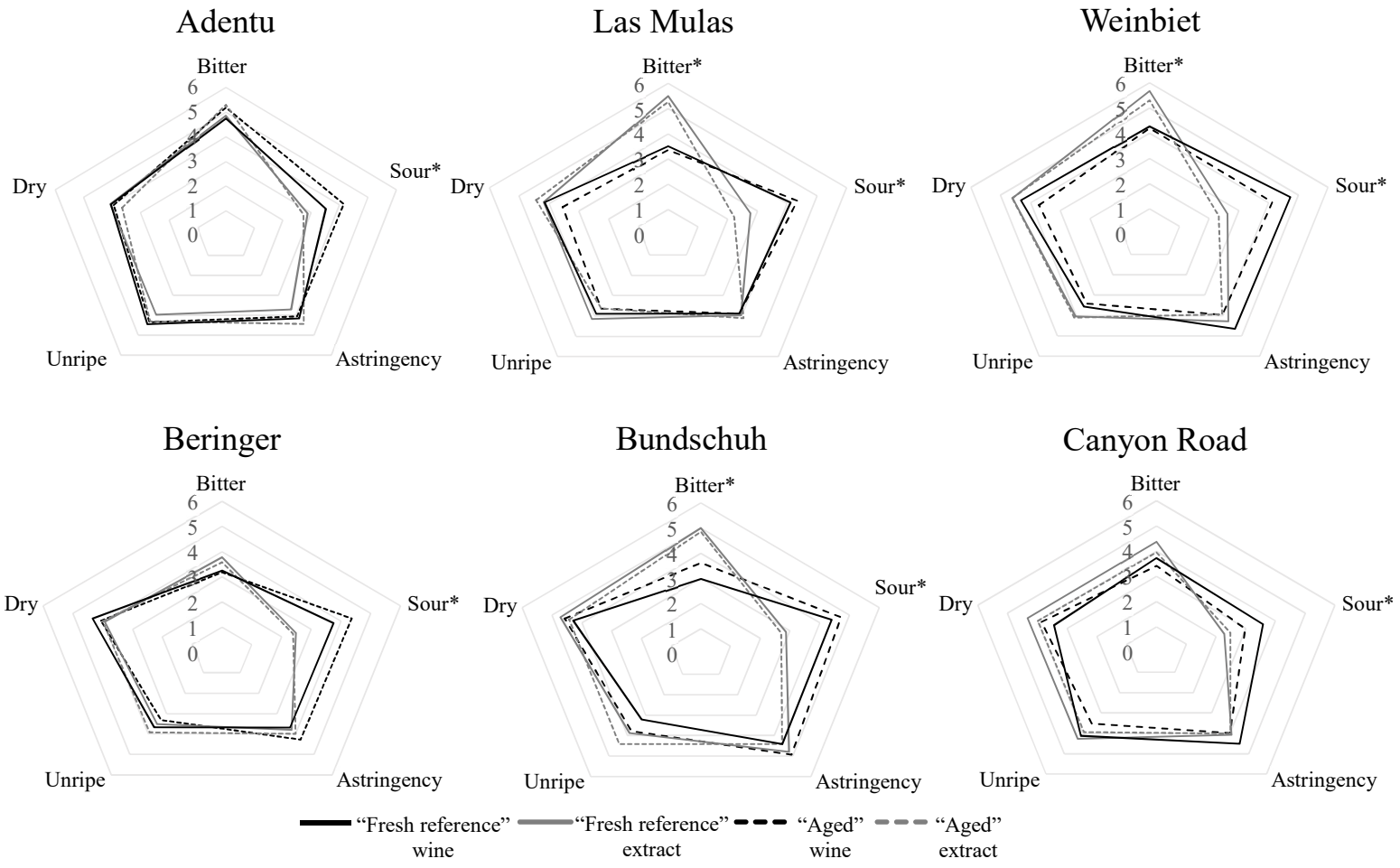

Fig. A.1 Ratings of the sensors profiling of all Cabernet Sauvignon wines and corresponding polysaccharide-free extracts including the attributes "sour", "bitter", "astringency", "unripe", and "dry". Attributes with an asterisk (*) show significant differences ($\alpha = 0.05$) between each wine and corresponding extract.