Institut für Ernährungs- und Lebensmittelwissenschaften
Professur für Molekulare Lebensmitteltechnologie

# Grape phenolic maturity

## Dynamics of polyphenol-polysaccharide interactions and the impact of grape seeds

**Dissertation**

zur Erlangung des Grades

Doktor der Ingenieurwissenschaften (Dr.-Ing.)

der Landwirtschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

**Jan-Peter Hensen**

aus

Bonn-Bad Godesberg

Bonn 2024

Referent: Prof. Dr. Andreas Schieber
Korreferent: Prof. Dr. Fabian Weber

Tag der mündlichen Prüfung: 13.09.2024

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

## Table of contents

# Preliminary remarks

## List of abbreviations

| | |
|---|---|
| ac | acylated |
| ANOVA | analysis of variance |
| BSA | bovine serum albumin |
| Bx | brix |
| CCS | collision cross section |
| CE | catechin equivalent |
| coum | coumaroylated |
| CS | Cabernet Sauvignon |
| Cya | cyanidin |
| Del | delphinidin |
| EU | European Union |
| FLD | fluorescence detector |
| glu | glycosylated |
| ha | hectare |
| HPSEC | high-performance size exclusion chromatography |
| HCl | hydrochloric acid |
| Hl | hectoliter |
| IMS | ion mobility spectrometry |
| kDa | kilo dalton |
| LC | liquid chromatography |
| LOQ | limit of quantification |
| LPP | large polymeric pigments |

| | |
|---|---|
| Mal | malvidin |
| mDP | mean degree of polymerization |
| MS | mass spectrometry |
| *m/z* | mass-to-charge ratio |
| MW | molecular weight |
| MWCO | molecular weight cut-off |
| Nm | Newton meter |
| PCA | principal component analysis |
| PDA | photodiode array detector |
| Peo | peonidin |
| Pet | petunidin |
| pH | potential of hydrogen |
| PN | Pinot noir |
| PP | polymeric pigments |
| QTOF | quadrupole time-of-flight |
| RI | refractive index |
| $R^2$ | coefficient of determination |
| RP | reverse phase |
| rpm | revolutions per minute |
| SEC | size exclusion chromatography |
| SPP | small polymeric pigments |
| TEA | triethylamine |
| TOF | time-of-flight |
| TSS | total soluble solids |
| UV | ultraviolet |
| UHPLC | ultra high performance liquid chromatography |
| UPLC | ultra performance liquid chromatography |
| *Vitis vinifera* L. | Common grapevine, "L." refers to Linnaeus |

## List of publications

Hensen, J. P., Hoening, F., Weilack, I., Damm, S., and Weber, F. 2022. Influence of Grape Cell Wall Polysaccharides on the Extraction of Polyphenols during Fermentation in Microvinifications. *J. Agric. Food. Chem.* **70**. 9117-9131. DOI: 10.1021/acs.jafc.2c02697.

Nemetz, N. J., Winter, A. R., Hensen, J.-P., Schieber, A., and Weber, F. 2023. Toward gentle chokeberry juice production by ultrasound-assisted enzymatic maceration. *Curr. Res. Food Sci.* **6**. 100518. DOI: 10.1016/j.crfs.2023.100518.

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

Hensen, J.-P., Dörner, M., Etzbach, L., Schieber, A., and Weber, F. 2023. Seed Maturation Dynamics in Cabernet Sauvignon and Pinot Noir Grapes During Berry Ripening in Cool Climate Zones. *Food Sci. Technol.* **4**. 161-172. DOI: 10.1021/acsfoodscitech.3c00445.

Hensen, J.-P., Hoening, F., Bogdanovic, T., Schieber, A., and Weber, F. 2024. Pectin forms polymeric pigments by complexing anthocyanins during red winemaking and ageing. *Food Res. Int.* **188**. 114442. DOI: 10.1016/j.foodres.2024.114442.

## Conferences

Hensen, J. P., Weilack, I., Weber, F., Schieber, A. and Harbertson, J. 2019. Comparison of Tannin Analysis by Protein Precipitation and Normal-Phase-HPLC. 11th International Symposion of Oenology, Bordeaux, France, June 25-28, 2019. *Abstracts*, 162. [Oral presentation]

Hensen, J. P., Hoening, F. and Weber, F. 2021. Influence of Polysaccharides on Extraction of Polyphenols during Maceration at Different Grape Maturities. 72nd ASEV National Conference, Virtual, June 21-24, 2021. *Abstracts,* 43-44. [Poster]

Weber, F., Weilack, I., Hensen, J. P., Feifel, S., Wegmann-Herr, P., and Durner, D. 2022. Phenolische Traubenreife – Definition, Bestimmung und Bedeutung. Deutscher Lebensmittelchemikertag, Hamburg, Germany, September 19-21, 2022. *Lebensmittelchemie*. **76**. [Poster]

## Declaration of contribution as co-author

The following co-authors contributed to publications presented in Chapter 2, Chapter 3, and Chapter 4.

**Prof. Dr. Andreas Schieber** supervised this thesis and proofread most manuscripts.

**Prof. Dr. Fabian Weber** assisted in designing experimental work, data interpretation, and publication of results. He was the corresponding author of all publications and proofread all manuscripts.

**Fiona Hoening** was responsible for the winemaking and conducted polyphenol analysis during fermentation of those wines.

**Ingrid Weilack** measured the molecular weight distribution of grape cell wall polysaccharides with SEC.

**Dr. Lara Etzbach** analyzed grape seed flavan-3-ols with UHPLC-IMS TOF MS.

**Sandra Damm** determined ethanol concentrations in wines.

**Maria Dörner** conducted analyses of grape seeds.

**Tamara Bogdanovic** measured the phenolic composition of wines with added pectic polysaccharides.

# Chapter 1

## General introduction

Wine is one of the oldest alcoholic beverages and potentially one of the most important in the western World. EU wine consumption in 2021 was 114 mhl and accounted for nearly half of the world wine consumption. For this production, there are 3.3 mha of vineyards in the EU, of which 103 kha are located in Germany (OIV, 2022). This area only contributes to 0.6 % of Germany's agricultural area but to 2 % of the overall agricultural production value (BMEL, 2022a, 2022b). Wine has a somewhat unique role in agriculture because winegrowers are often also winemakers. This allows farmers to add tremendous value to their crops and explains the high production value of vines. Wines are sold at various prices ranging from under 2 € to thousands. These vary obviously by the winery's reputation and marketing, but quality has a substantial impact. Among all foods and beverages, wine has a unique position because the quality of a wine is, in most cases, reviewed publicly. Countless professional wine critics and consumer information rate the quality of wine on a scale. Often, the rating is even part of the marketing of a wine when the points or medals awarded by a critic are prominently displayed on the label. These ratings are as relevant for wine enthusiasts as they are for the occasional wine buyer because wine production varies a lot and produces a variety of qualities.

Wine is the fermented extract from grape berries, containing acids, sugars, phenolic compounds, volatile compounds from the berry, and volatile compounds and alcohols from fermentation. Polyphenols are essential in red wines. Anthocyanins are responsible for the name-giving color, and tannins contribute vastly to the mouthfeel (Gil et al., 2012; Noble, 1998; Robichaud and Noble, 1990; Somers and Evans, 1974). After fermentation, wines are usually aged for some time, during which reactions produce different volatile and phenolic compounds that impact the sensory characteristics.

However, all begins with the compounds extracted from the berries during winemaking. Therefore, berry maturity is potentially the most critical factor influencing wine quality (Conde et al., 2007; Guidoni and Hunter, 2012; Torchio et al., 2010). Berries are ripe when a specific sugar-to-acid concentration ratio is accumulated in the fruit. At this ratio, which is often specific

to grape varieties and winemakers' preferences, berries are technologically ripe. Because polyphenols substantially impact red wines, their concentration in the berry has become particularly important in producing quality wines. Compared to technological maturity, phenolic maturity is not easily measurable by winegrowers. Moreover, because not only the concentration of polyphenols in berries seems to impact the phenolic composition of wine but also their extractability, phenolic maturity needs to be understood and assessed to produce high-quality red wines.

## 1 Grape phenolic maturity

Berries used for winemaking are predominantly the fruit of *Vitis vinifera* L. They grow in three distinctive phases, beginning after flowering with the growth of green berries. In the first phase, cells grow rapidly and accumulate tartaric and malic acid, and tannins in seeds and skins (Conde et al., 2007; Kennedy et al., 2000a; Kennedy et al., 2000b; Possner and Kliewer, 1985). In the following phase, cell growth speed reduces, and anthocyanin synthesis begins (Fasoli et al., 2016; Robinson and Davies, 2000). The third phase begins with the visual change of the skin color due to anthocyanin accumulation. This characteristic visual change, called veraison, indicates to winegrowers that their crop will soon be ripe. In this last phase, berries grow to their final size and become softer. Cells accumulate glucose and fructose while organic acid concentrations decrease (Coombe, 1960). Pectin methyl esterase and other enzymes depolymerize pectin and other polysaccharides in the cell wall structure (Brummell, 2006; Brummell and Harpster, 2001). Thereby, cell wall polysaccharide composition changes and increases cell wall porosity (Hanlin et al., 2010). Within the third phase, winemakers finally harvest the berries to produce wine.

### *1.1 Quantitative approaches to assessing phenolic maturity in grape berries*

Phenolic maturity is a concept first proposed in Bordeaux, France, which tries to find an optimized harvest date for grape berries. The original idea was that at a specific time during berry growth, skin tannin concentrations were at their maximum while polyphenol concentrations in the seeds were at their minimum (Saint Cricq de Gaulejac et al., 1998). At this specific time, the phenolic composition of the berry would produce high-quality wines. With this concept, winemakers had a guideline to optimize their harvest management. After its first introduction, multiple alterations and additions to this concept have been proposed.

While, winemakers have focused on the phenolic composition of berries ever since, the complete extraction of polyphenols from grape berries into the wine is not always achieved during the winemaking process. It is generally assumed that the degree of ripeness of the grapes and the extractability of polyphenols are correlated. An advanced stage of ripening is associated with softening of the grape berry and with partial degradation and structural change of the polysaccharide-rich cell walls (Hanlin et al., 2010). Interactions between polyphenols and components of the cell wall, which vary during the ripening process of the berry, might cause the variations of the polyphenol extraction during winemaking (Gao et al., 2019; Kennedy et al., 2000b). Therefore, the degree of grape berry ripeness affects the polyphenol content (e.g., anthocyanins) and, thus, the quality of the resulting wine (Deytieux-Belleau et al., 2008). Regarding the strong impact of polyphenol extractability, phenolic maturity has evolved to combine polyphenol concentrations in grape berries and their interactions with all other cell components.

Phenolic maturity can be measured with various methods. The most reliable approaches use chemical extraction of polyphenols from the grape in combination with spectrophotometric measurements (Kontoudakis et al., 2010). These methods mainly try to mimic the extraction of polyphenols during the winemaking process. Other extraction-based methods try to establish a ratio of extractable and bound polyphenols, which defines phenolic maturity by extractability. Alternatively, seed color has been proposed as a tool to determine phenolic maturity, but this has remained controversial, although it is widely used due to its simplicity (Fredes et al., 2017; Ristic and Iland, 2005). More recently, imaging techniques have been used to assess grape phenolic maturity by analyzing the exterior skin color (Agati et al., 2013; Quijada-Morin et al., 2016; Rodriguez-Pulido et al., 2017; Rodriguez-Pulido et al., 2014). All these tools can assist winemakers in deciding when the harvest should begin but cannot explore the cause of a changing phenolic maturity. The complexity of interactions that limit polyphenol extraction during fermentation and their changes during berry ripening need to be revealed first.

### *1.2 Phenolic composition of grape berries*

Wine quality is vastly determined by polyphenols extracted from grape berries during maceration and fermentation. Berries contain various phenolic substances, with high concentrations in the skin and seeds of the berry. Berries contain phenolic acids like hydroxybenzoic and hydroxycinnamic acids and flavonoids like anthocyanins, flavonols, flavanols, and proanthocyanidins.

Except for some varieties, anthocyanins accumulate exclusively in the berry skin tissue (Cerpa-Calderon and Kennedy, 2008). They are responsible for the red, purple, or even tinted blue color of red grapes and wines, depending on the pH of the surrounding matrix. Their color derives from the fully conjugated A-C ring system, which is cross-conjugated into a B-Ring (Waterhouse et al., 2016). Five different aglycones are found in *Vitis vinifera* L. varieties that are predominantly glucosylated: cyanidin, delphinidin, peonidin, petunidin, and malvidin. Aglycones vary in their B-Ring substitution, of which malvidin-3-*O*-glucoside has the highest concentration in most grape varieties.

The sugar moiety of the anthocyanins can be substituted with an acetyl or coumaroyl group (Dimitrovska et al., 2011). Due to their unique UV absorption spectrum among polyphenols, anthocyanins are analyzed with spectrophotometric methods and liquid chromatography (LC). Nowadays, reversed-phase ultra high-performance liquid chromatography (UHPLC) combined with a photodiode array detector (PDA) has become the standard for anthocyanin analysis. Their UV absorption maximum is 520 nm, which is typically used to detect anthocyanins (Mattioli et al., 2020). Identification of anthocyanins is established by their polarity, which results in specific elution profiles. Before analysis, anthocyanins are extracted with polar solvents, typically water with added methanol or ethanol. Anthocyanins are more stable in their flavylium cation form below pH three. Hence, solvents are usually acidified for extraction and analysis. Although monomeric anthocyanins are extremely important for the color of red wines, their concentration plummets in wines after extended periods of aging because of their instability.

Even in young wines, monomeric anthocyanins lose their color due to an increased pH, shifting the anthocyanins toward their colorless hemiketal form. Additionally, sulfite ions in the wine bleach monomeric anthocyanins (Cheynier et al., 2006). In order for a red wine to keep its color, anthocyanins need stabilization by co-pigmentation or interactions with various compounds, predominantly other polyphenols, to form so-called polymeric pigments (Waterhouse et al., 2016). These pigments are more stable than monomeric anthocyanins, adding a different, red-brown tint to aged red wines (Unterkofler et al., 2020).

Polymeric pigments form at all stages of winemaking through oxidative and acid-catalyzed reactions of anthocyanins and various other polyphenols, mainly tannins and flavan-3-ols (Waterhouse et al., 2016). With some exceptions, all polymeric pigments are more stable than monomeric anthocyanins against long-term degradation and bisulfite bleaching. At the same time, they still absorb light at 520 nm, sometimes even more substantially and at a wider pH range than monomeric anthocyanins (Waterhouse et al., 2016). Multiple polymerization

reactions and polymers could be characterized already. Pyranoanthocyanins are formed from anthocyanin-aldehyde reactions, which can polymerize further with vinylphenols to the so-called portisin. Pinotin is the reaction product of anthocyanins and vinylphenols or hydroxycinnamic acid. Flavan-3-ols and tannins can bind anthocyanins directly or ethyl-linked (Cheynier et al., 2006; Unterkofler et al., 2020). However, most polymeric pigments are still uncharacterized due to their size and large variability. Because a large proportion of polymeric pigments derives from anthocyanin-tannin interactions, the variability of molecular structures is endless (Remy et al., 2000). Modern analytical methods cannot separate these structures, resulting in a typical "polymer hump" in measurements that use liquid chromatography (Ma et al., 2018). Therefore, polymeric pigments are measured with spectrophotometric assays, as the one proposed by Harbertson et al. (2003). The assay considers two types of polymeric pigments that combine resistance against bisulfite bleaching and red color as their qualifying characteristics. So-called large polymeric pigments precipitate with bovine serum albumin protein, while small polymeric pigments do not. However, whether precipitability with proteins can be used to measure the size of a polymeric pigment has been questioned (Weilack et al., 2021).

Flavan-3-ols have the highest concentration among all flavonoids in grapes. The most dominant (+)-catechin or its isomer (-)-epicatechin have a 3'.4'-dihydroxy substitution on the B-ring. (-)-Gallocatechin and (-)-epigallocatechin have a 3',4',5'-trihydroxy substitution on the B-ring. Additionally, position three can be substituted with gallic acid, forming (-)-epicatechin gallate (Waterhouse et al., 2016). Flavan-3-ol concentrations are particularly high in grape seeds and skins, with some variability depending on the grape variety (Mattivi et al., 2009; Obreque-Slier et al., 2010). Galloylated flavan-3-ols are predominately accumulated in grape seeds (Souquet et al., 2000).

A substantial proportion of flavan-3-ols polymerizes to proanthocyanidins in grape berries and wine through condensation. Condensation reaction products can range from dimers to polymers, which are referred to as tannins. In wine, flavan-3-ol subunits form interflavan bonds between positions 4 and 8 or 4 and 6 to build procyanidin and prodelphinidin B1 and B5, respectively. Depending on the number of hydroxy substituents at the B-ring, proanthocyanidins are classified as procyanidins (dihydroxylated) or prodelphinidins (trihydroxylated). Seed and skin proanthocyanidins have mean degrees of polymerization (mDP) of roughly 10 and 30, varying among publications (Gagne et al., 2006; Geny et al., 2003; Kennedy et al., 2001; Kennedy et al., 2000a; Obreque-Slier et al., 2010; Souquet et al., 2000).

30 % of seed proanthocyanidins are galloylated; in skins, the proportion is only 5 %, making galloylated proanthocyanidins characteristic for seeds (Souquet et al., 2000).

With an increase in the degree of polymerization the variety of molecular structures formed rises exponentially. While monomeric flavan-3-ols can precisely be analyzed with UHPLC and PDA or fluorescence detectors, proanthocyanidins cannot. Their structural diversity and high mass prevent thorough separation with LC, which led to the development of multiple analytical methods used to quantify proanthocyanidins in grapes and wine. With advancing technologies, oligomers consisting of up to 20 subunits are nowadays analyzed with centrifugal partition chromatography combined with time of flight (TOF) mass spectrometry (MS) (Ma et al., 2018). Other analytical approaches combine two-dimensional LC with ion mobility spectrometry (IMS) TOF MS (Venter et al., 2018). However, these methods are not widely available and are still limited in the range of tannins they can detect. Besides, reference materials for proper identification and quantification of polymers with a degree of polymerization above three are not available. Therefore, tannins are usually quantified as a sum with spectrophotometric methods. Proanthocyanidins have an absorption maximum of 280 nm and get separated from all other polyphenols before analysis. Two different methods separate tannins by either protein precipitation with bovine serum albumin (BSA) (Harbertson et al., 2002; Harbertson et al., 2015) or precipitation of tannins with methyl cellulose (Sarneckis et al., 2006). However, these methods supply no information on the size of proanthocyanidins in a grape or wine sample. Therefore, alternative approaches have been developed. Using acid-catalyzed cleavage of proanthocyanidins in nucleophile (e.g., phloroglucinol) rich solutions forms flavan-3-ol phloroglucinol adducts while keeping the terminal unit unaltered. After UHPLC MS analysis of these compounds, the mDP of tannins is calculated by the ratio of terminal units to flavan-3-ol phloroglucinol adducts (Kennedy and Jones, 2001).

Proanthocyanidins and monomeric flavan-3-ols have arguably the strongest impact on wine sensorial characteristics. Proanthocyanidins add bitterness and astringency to red wines depending on the degree of polymerization and galloylation. The perceived bitterness decreases while astringency increases with an increasing degree of polymerization (Peleg et al., 1999; Vidal et al., 2003a). Astringency unpleasantly increases with a higher degree of galloylation (Vidal et al., 2003a), which is why winemakers try to minimize the extraction of seed tannins. Further, tannin concentration and structure impact the astringent sensation, as described by various publications (Gawel et al., 2000). The astringent sensation results from complex interactions of tannins with salivary proteins, glycosaminoglycans, and epithelial proteins.

Tannins form aggregates with proteins that precipitate, reducing the saliva's viscosity. This results in a highly variable tactile sensation depending on the precipitated aggregate's characteristics. Moreover, because saliva composition, pH, and flow rate vary among individuals, the perceived astringency is a personal experience (McRae and Kennedy, 2011).

In addition to these anthocyanins and flavan-3-ols, grapes and wine contain flavonols, stilbenes, and phenolic acids. However, their analysis was outside the scope of this thesis, due to their likely lower impact on wine quality.

### *1.3 Structure and function of grape berry cell wall polysaccharides*

Cellulose, pectin, and hemicellulose build the primary cell wall of grape berries. Cellulose is the hydrophobic linear chain of β-(1,4)-D-glucose and is most abundant in the grape berry cell wall (Nunan et al., 1998; Vicens et al., 2009). Hemicellulose is a group of diverse polysaccharides of β-(1,4)-linked monosaccharides, e.g., xylose, galactose, glucose, or arabinose. Hemicellulose cross-links cellulose microfibrils through hydrogen bonds (Goulao et al., 2012; Scheller and Ulvskov, 2010). In this network, pectic polysaccharides form a gel, which controls ion transport, hydration, porosity, and more (Goulao et al., 2012). Pectic polysaccharides have a diverse molecular structure, combining smooth regions consisting of unbranched homogalacturonan and branched so-called hairy regions formed by rhamnogalacturonan (Schols and Voragen, 1996). The smooth region consists of 1,4-linked α-D-galacturonic acid, which can be methyl-esterified or acetylated (Vorwerk et al., 2004). The hairy region consists of rhamnogalacturonan-I, rhamnogalacturonan-II, and xylogalacturonan. Alternating 1,4-linked α-D-galacturonic acid and 1,2-linked α-L-rhamnosyl build rhamnogalacturonan-I. The rhamnose can be substituted with different side chains, which impact the physical properties of pectic polysaccharides (Ochoa-Villarreal et al., 2012; Schols and Voragen, 1996). Rhamnogalacturonan-II consists of nine 1,4-linked α-D-galacturonic acid subunits as a backbone, which carries four heteropolymeric side chains. The side chains contain monosaccharides like apiose, 2-*O*-methyl fucose, and 2-*O*-methyl xylose (Hanlin et al., 2010; Vidal et al., 2001).

The polysaccharide composition and structure change during berry ripening, but their overall concentrations seem consistent in grape berry cell walls (Nunan et al., 1998; Ortega-Regules et al., 2008; Vicens et al., 2009). The relative proportion of cellulose decerases in the last stage of berry growth, while the solubility of pectins increases due to an increase in pectinase activities (Brummell and Harpster, 2001; Nunan et al., 1998; Silacci and Morrison,

1990; Vicens et al., 2009). Further the degradation of arabinogalactan-I side chains in pectin (Gross and Sams, 1984) and a reduced xyloglucan content in the hemicellulose fraction (Fasoli et al., 2016) are typical for late stages during ripening.

During winemaking, mostly arabinogalactan-proteins and rhamnogalacturonans I and II are extracted from the berries and remain structurally intact, unlike most other cell wall polysaccharides (Vidal et al., 2003b; Waterhouse et al., 2016). Additionally, wines contain yeast-derived mannoproteins released by autolysis of yeast cells (Vidal et al., 2003b). Although only a fraction of berry cell wall polysaccharides can be found in a wine, they substantially impact the overall composition of wines. Due to interactions with polyphenols during fermentation, wine quality depends on the cell wall composition. Interactions of grape berry polysaccharides with polyphenols are elucidated in #chapter.

As a result of the diverse molecular structures of polyphenols, characterization usually involves fractionation of the alcohol-insoluble cell wall polysaccharides by their solubility.

## *1.4 Grape seeds as indicators of grape maturity and their impact on wine phenolic composition*

Using grape seeds as an indicator of grape maturity has a long tradition among winemakers. Despite only contributing scarcely to the mass of a berry, they store most of the berry's tannins (Souquet et al., 2000). Further, seeds contain polyphenols widely associated with negative sensorial characteristics, like monomeric flavan-3-ols. Although seed polyphenols are important for the color stability of red wines (Bautista-Ortín et al., 2014), they can make wines bitter, harshly astringent, and generally unpleasant (Harrison, 2017; Peleg et al., 1999). Because of these negative attributes, winemakers try to minimize the extraction of grape seed polyphenols.

Grape seeds have a rough surface, with a central raphe on the ventral side and the chalaza on the dorsal side (Ristic and Iland, 2005). Seeds mostly remain intact during winemaking. Therefore, only the outer seed coat is in contact with the must. The coat comprises five layers, the cuticle, epidermis, outer integument, middle integument, and inner integument (Cadot et al., 2006). Below, the endosperm and embryo are located. During berry ripening, the size and mass of seeds increase, the coat solidifies, and the color turns from green to brown (Ristic and Iland, 2005). Most of these changes occur in the last stage of berry ripening, when coat cells sclerify and dehydrate, thereby increasing the solidity. In order to increase the strength even further, cell walls accumulate lignin. With this process, seeds appear more brown and dull on the outside. Both the textural changes and the visual differences between seeds from unripe and

ripe berries have been used to assess the overall berry maturity, as the processes responsible for the physiological changes occur only in the last stage of berry development (Fredes et al., 2017; Fredes et al., 2010; Letaief et al., 2013; Rolle et al., 2012). For winemakers, seeds became an approachable indicator of berry ripeness. Simply, evaluating the visual appearance and texture adds some information aside from the appearance of the berry itself and potentially tasting the berry.

Polyphenols in the seeds are primarily flavan-3-ols, either monomeric or polymerized to proanthocyanidins. The composition and degree of polymerization vary depending on the variety, cultivation, climate, and degree of berry maturity. In the last growing stage, polyphenol concentrations in the seed diminish (Kennedy et al., 2000a). After veraison, expression of genes responsible for polyphenol synthesis stops, which terminates the accumulation of polyphenols (Bogs et al., 2005). The concentration of polyphenols reduces by multiple reactions and interactions that limit the extraction of polyphenols from the cell structure. Tannins can be associated with proteins or cell wall polysaccharides (Downey et al., 2003; Geny et al., 2003). Further oxidation and dehydration limit the extraction of proanthocyanidins (Bautista-Ortín et al., 2012; Kennedy et al., 2000b).

### *1.5 Winemaking*

Winemaking is the process of fermenting grape berries to produce an alcoholic beverage. For red winemaking, grapes are usually destemmed, crushed, and fermented before pressing, fining, and filling (Figure 1.1). White wines are usually pressed before fermentation. Oenologists vary this basic process to produce the wine style they strive for.

In modern wine production, crushed and destemmed grapes ferment with cultured *Saccharomyces cerevisiae* for roughly two weeks until all fermentable sugars are used, or the wine contains a desired ethanol concentration. In the beginning, when ethanol concentrations are still low, anthocyanins are extracted from the skins (Gao et al., 2019). Because the extraction of anthocyanins is crucial for red wine, winemakers sometimes extend this period by lowering the temperature and later inoculating with yeast to halt fermentation. Alternatively, adding cell wall deconstructing enzymes or grinding the berries can increase anthocyanin extraction (Gao et al., 2019). With an increase in ethanol concentration, tannins and other polyphenols are extracted from the skins and seeds of the berry. Apart from polyphenols, various cell wall polysaccharides, mainly arabinogalactan protein and rhamnogalacturonan, are extracted (Guadalupe and Ayestaran, 2007). Because all value-adding compounds in wine are extracted during fermentation, e.g., polyphenols, or produced, e.g., volatile aromas, fermentation is

arguably the most crucial step in winemaking. After alcoholic fermentation finishes, some winemakers use malolactic fermentation to produce lactic acid from malic acid, depending on the wines they want to create. Then, winemakers can separate the young wine from the residue by pressing it and fine it, if desired. Afterwards, wines age in wooden barrels or, as is nowadays, more typical in steel tanks until they are filled into bottles.

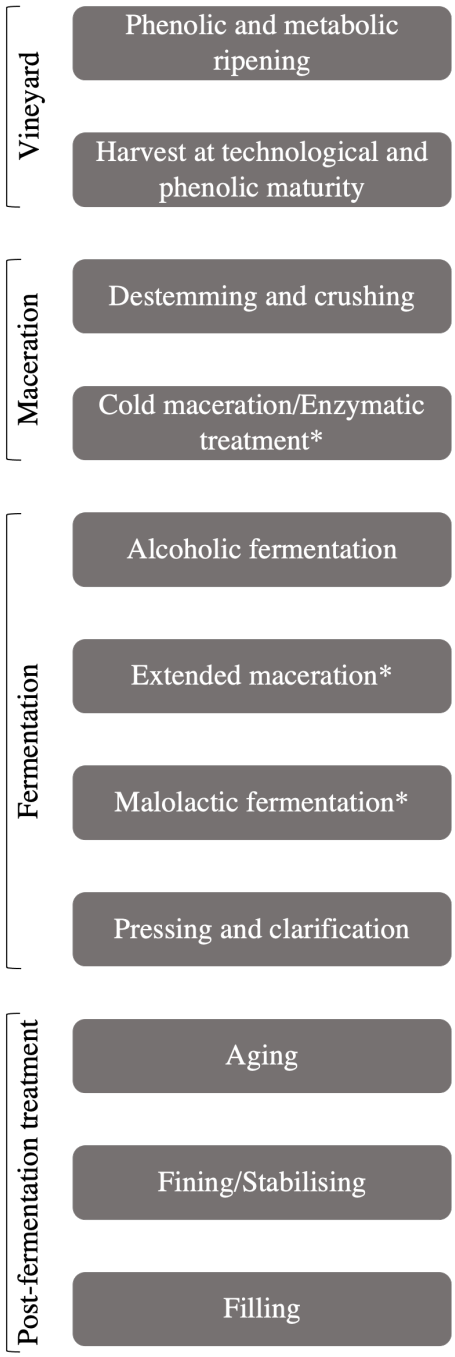



Figure 1.1 Winemaking stages. *Optional processes. Modified according to Unterkofler et al. (2020)

### *1.6 Varietal characteristics of Pinot noir and Cabernet Sauvignon*

Cabernet Sauvignon is the most cultivated grape variety for wine production in the world. Its late ripening nature demands specific climate conditions with moderate temperatures ranging from 16 to 20 °C, considerable sun exposure, and minimal precipitation (Jones, 2018). While Germany's winemaking history with Cabernet Sauvignon is relatively short, the escalating impact of climate change has elevated temperatures in select German wine regions to sufficient levels for quality Cabernet Sauvignon wine production. Traditionally acclaimed Cabernet Sauvignon wines have historical roots in Bordeaux, France, and the past five decades have seen their emergence in California's Napa Valley. Nonetheless, Cabernet Sauvignon vines are planted all over the world. Regardless of their origin, these wines have characteristic attributes. Notably, their grape berries contain high concentrations of anthocyanins and tannins, culminating in wines with an intense coloration and astringency that meet current consumer demands for quality red wines. The characteristic aromas are cassis and green bell pepper in case berries are harvested slightly unripe. With their high tannin concentrations, Cabernet Sauvignon wines are suitable for long-time aging (Robinson et al., 2013). Cabernet Sauvignon berries are relatively firm with thick skins (Letaief et al., 2008a; Letaief et al., 2008b). Berries are small, with a diameter ranging from 11 to 13 mm, and contain on average 1.5 seeds (own unpublished data).

Pinot noir is one of the oldest documented varieties used for winemaking. The first reliably documented Pinot noir vineyards in Germany trace back to 1470, located in Hattenheim, Rheingau (Robinson et al., 2013). Flourishing within the spectrum of moderate temperatures, ranging from 14 to 16 °C, Pinot noir vines are ideally suited for cultivation in cooler climates (Jones, 2018). Viticulture necessitates average temperatures of 12-22 °C in the growing season from April to October in the northern hemisphere. However, in cool climate zones, temperatures in this season are typically between 13 and 16 °C. The majority of German regions, maintaining an average temperature of 14.5 °C or lower, effectively qualify as cool climate zones requiring grape varieties tailored to such conditions, with Pinot noir (locally referred to as Spätburgunder) being a prime choice (Jones and Schultz, 2016). The applications of Pinot noir grapes encompass red winemaking as well as the production of sparkling wines, most notably as one of the trio of grapes contributing to champagne production. Celebrated Pinot noir wines primarily come from Burgundy, France, while Germany's Pinot noir, known as Spätburgunder, boasts an extensive legacy and global repute. Pinot noir wines are typically light in color and astringency, with fruity aromas of strawberries, cherries, blackberries, or cassis. Depending on the berry maturity, the perceived acidity of Pinot noir wines is higher than

in other red wines. Pinot noir wines are considered to reflect the local characteristics of soil, climate, and producers, the terroir, which results in a variability of Pinot noir wine styles around the world (Robinson et al., 2013). Pinot noir berries have delicate skins and a firm texture (Letaief et al., 2008a) with a diameter ranging between 11 and 15 mm and contain 1.2 seeds on average (own unpublished data).

## 2 Interactions between polyphenols and polysaccharides in winemaking

Polyphenols in grape berry cells are stored in vacuoles until the cell structure is compromised through enzymatic, chemical, physical, or mechanical forces. This protective mechanism prevents these secondary metabolites from interacting with other cellular components, serving the plant's defense against environmental threats. However, winemakers go to great lengths to release these polyphenols during maceration and fermentation. With these polyphenols, various other compounds are released, like cell wall polysaccharides and proteins. Within this dynamic matrix, polyphenols have the chance to interact and form complexes with a variety of compounds facilitated by their high reactivity. The mechanisms behind these multifaceted interactions of polyphenols with plant macromolecules have captivated researchers for decades. Initially, the focus was on protein-polyphenol interactions, while more recent years have witnessed a surge of interest in the polysaccharide-polyphenol interplay, particularly in the realm of wine research. Given the pivotal role polyphenols play in wine quality, comprehending these interactions is critical.

Polyphenols can interact with macromolecules in various ways, either forming reversible complexes through hydrogen bonds, hydrophobic interactions, and van der Waals forces, or forming covalent bonds through oxidation or nucleophilic addition (Le Bourvellec and Renard, 2012). Proteins form insoluble complexes with polyphenols via hydrogen bonding and hydrophobic interactions, which creates the astringent mouthfeel characteristic of tannins, or haze in different beverages (Hagerman, 1989). While detailed information on protein-polyphenol interactions is widely available, this thesis primarily delves into the interplay between polysaccharides and polyphenols.

The structural diversity of polysaccharides creates various possibilities for interactions with polyphenols. Mainly, these are ionic interactions, hydrogen bonds, and hydrophobic interactions (Renard et al., 2017). Hydrogen bonds form between hydroxy groups of polyphenols and the homogalacturonan component of pectic polysaccharides. Hydrophobic interactions manifest between hydrophobic sites (e.g., methoxy groups) of polysaccharides and the aromatic ring of polyphenols. Moreover, in some cases, polysaccharides form hydrophobic

pockets that interactions with polyphenols. As these interactions hinge on functional groups, their complex formation is inherently shaped by the structures of both polyphenols and polysaccharides, as well as the surrounding milieu (Weber, 2022).

Polysaccharides released from the cell structure during fermentation are either solved in the matrix or might still be attached to larger cell wall fragments (Bindon et al., 2010b; Gao et al., 2019). The molecular and macroscopic structure of these cell wall polysaccharides determines possible interaction mechanisms and affinity for specific polyphenol structures. Pectic polysaccharides, with their versatile functional groups, exhibit the highest binding capacity for proanthocyanidins, succeeded by xyloglucans, starch, and cellulose (Le Bourvellec et al., 2005; Ruiz-Garcia et al., 2014). The structure of pectin, containing diverse functional groups, creates various interaction sites for all polyphenols. However, the degree of esterification influences the affinity of these interactions. While a higher degree of methylation enhances interactions with procyanidins, interactions with anthocyanins are more robust with pectic polysaccharides exhibiting low methylation (Buchweitz et al., 2013a; Fernandes et al., 2020b; Watrelot et al., 2013). A higher degree of esterification tends to restrict the formation of pectin-anthocyanin complexes, introducing steric hindrances and diminishing potential sites for hydrogen bonding and hydrophobic interactions (Larsen et al., 2019). Because proanthocyanidins form complexes with polysaccharides through hydrophobic interactions, a higher degree of methylation (> 70) increases the possibility for these (Liu et al., 2020). Conversely, anthocyanins form bonds with pectic polysaccharides at a low pH (< 4), mainly through ionic forces. With a higher density of deprotonated carboxyl groups, the ionic forces can potentially create stronger interactions with anthocyanins than highly methylated pectic polysaccharides (Liu et al., 2020).

Generally, the macrostructure of polysaccharides strongly impacts interactions with polyphenols. Neutral sugar side chains within pectic polysaccharides impede the formation of hydrogen bonds and hydrophobic interactions (Watrelot et al., 2014). Similarly, the degree of branching of arabinans negatively impacts the interactions of pectic polysaccharides and polyphenols (Fernandes et al., 2020a). A more linear pectin structure with less neutral sugar side chains seems to interact better with polyphenols. Additionally, the stability of polyphenol-polysaccharide complexes depends on the polysaccharides' molecular weight and structure, as has been shown for pectin-anthocyanin interactions (Larsen et al., 2019).

The divergence in structural attributes of polysaccharides, influenced by grape berry maturity and the specific cell components from which they originate, profoundly shapes their propensity for polyphenol interactions. Multiple publications show that flesh cell wall material

interacts particularly well with proanthocyanidins with a high degree of polymerization. Conversely, skin cell wall polysaccharides exhibit fewer interactions with proanthocyanidins, a tendency that diminishes further as the berry ripens (Bindon et al., 2012; Bindon and Kennedy, 2011; Bindon et al., 2010a; Bindon et al., 2010b). The distinctive composition of skin cell wall material from more mature berries generally favors the formation of complexes with proanthocyanidins (Castro-Lopez et al., 2016). A similar fruit maturity-depending interaction was also reported for pears (Brahem et al., 2019).

The polyphenol structure similarly impacts the binding affinity for polysaccharides. The degree of polymerization positively impacts the affinity for polysaccharide interactions (Bindon et al., 2010b; Fournand et al., 2006; Le Bourvellec et al., 2004; Watrelot et al., 2017). Procyanidins are multidentate ligands with multiple binding sites within the same molecule, increasing the affinity and forming stronger bonds. The configuration of functional groups of polyphenols substantially alters their affinity for interactions. Anthocyanins and prodelphinidins with abundant hydroxy groups on the B-ring demonstrate heightened affinity for polysaccharide interactions compared to their methoxylated counterparts (Buchweitz et al., 2013b; Le Bourvellec et al., 2004). However, it seems that the position of the hydroxy group impacts the affinity (Liu et al., 2020). The degree of galloylation further improves polyphenol-polysaccharide interactions, probably due to the increase in hydroxy groups (Le Bourvellec et al., 2004; Liu et al., 2020).

Furthermore, the composition of the fermenting must significantly influences the dynamics of these interactions. Alterations in temperature and ethanol concentration, for instance, play a pivotal role in modulating the solubility of polyphenols. Elevated temperatures and higher ethanol levels have been found to enhance the solubility of polyphenolic compounds. This, in turn, leads to the liberation of anthocyanins and potentially other polyphenols from intricate complexes that were hitherto reliant on relatively weak intermolecular bonds (Medina-Plaza et al., 2019; Medina-Plaza et al., 2020). Additionally, anthocyanin-pectin interactions are sensitive to specific pH values of the surrounding matrix, as ionic forces can form complexes between both. Although anthocyanins predominantly form complexes with pectic polysaccharides, reports have also indicated interactions with cellulose microfibrils. Notably, anthocyanin-cellulose interactions are primarily characterized by hydrophobic forces, which are considerably less pronounced compared to the interactions between anthocyanins and pectin (Padayachee et al., 2012). Pectic polysaccharides are deprotonated at a pH between 3 and 4, typical for grape must and wines, creating negative charges (Celus et al., 2018). Anthocyanins

predominantly form positively charged flavylium cations at low pH, thereby increasing ionic interactions with deprotonated pectic polysaccharides (Fernandes et al., 2020b; Larsen et al., 2019). As the pH shifts towards higher values, anthocyanins adopt different molecular configurations, such as the quinoidal base or hemiketal forms, both of which lack a net charge. Thereby the interactions between anthocyanins and pectin change.

Polyphenols in must compete for interactions with the available polysaccharides. Significantly high anthocyanin concentrations limit the possibility of tannin-polysaccharide complexation. Because anthocyanins are extracted slightly before tannins during fermentation, due to their better solubility in water, they already occupy polysaccharides, thereby increasing the solubility of tannins (Gao et al., 2019). However, it is important to acknowledge that anthocyanins have the unique capability to self-associate, forming bonds with other attached anthocyanin molecules and resulting in the formation of anthocyanin stacks. This intriguing phenomenon arises from π-π interactions, setting anthocyanins apart from other polyphenols (Padayachee et al., 2012; Weber, 2022).

## 3 Aims of the thesis

Optimal grape harvest timing stands as a critical determinant in the winemaking process. While the assessment of technological maturity through measures such as total soluble solids (TSS) and pH remains prevalent, its relevance is diminishing due to the possibility of manipulating fermentable sugars and pH levels and a shift in consumer demands for quality in red wines. In contemporary winemaking, the emphasis on red wine quality has shifted toward the phenolic composition, relegating acidity and alcohol levels to secondary importance. However, phenolic maturity does not develop like the TSS concentration during berry ripening. Analyzing the phenolic composition of grape berries requires specialized equipment and trained personnel, which is not an option for most winemakers. Therefore, winemakers adapted methods to estimate phenolic maturity by seed or skin characteristics. However, it is crucial to recognize that the extraction of polyphenols during fermentation involves intricate interactions among various grape components. Understanding these interactions is the key to finding suitable tools to evaluate grape phenolic maturity to optimize harvest management and winemaking.

For this thesis Cabernet Sauvignon and Pinot noir have been selected because these two varities hold paramount importance both globally (Cabernet Sauvignon) and within cooler climatic zones (Pinot noir). Winemakers in cool climate zones are challenged by shorter vegetation periods due to the earlier onset of cold and rainy weather. Nonetheless, the climate

changes and gives winemakers in these zones new opportunities to explore. Recent years have granted winemakers the flexibility to deliberate the harvest timing, transcending the previous practice of waiting until grape berries are jeopardized by unfavorable weather shifts. Furthermore, grape varieties such as Cabernet Sauvignon, once considered ill-suited for these climates, are now being cultivated. Facilitating these newfound prospects, the primary objectives of this thesis are delineated as follows:

- Profiling the polysaccharide dynamics in Cabernet Sauvignon and Pinot noir berries throughout the ripening process
- Unraveling the significance of interactions between polyphenols and polysaccharides during fermentation
- Discerning the diverse effects of polysaccharides from distinctly mature grape berries on wine polyphenols
- Investigating the potential formation of polymeric pigments from polysaccharide anthocyanin interactions
- Scrutinizing the transformations in grape seeds during ripening and their ramifications for wine quality
- Evaluating the viability of grape seeds as indicators of phenolic maturity in cool climate zones

## References


Agati, G., D'Onofrio, C., Ducci, E., Cuzzola, A., Remorini, D., Tuccio, L., Lazzini, F., and Mattii, G. 2013. Potential of a multiparametric optical sensor for determining in situ the maturity components of red and white Vitis vinifera wine grapes. *J. Agric. Food. Chem.* **61**. 12211-12218.

Bautista-Ortín, A. B., Busse-Valverde, N., López-Roca, J. M., Gil-Muñoz, R., and Gómez-Plaza, E. 2014. Grape seed removal: effect on phenolics, chromatic and organoleptic characteristics of red wine. *Int. J. Food Sci. Technol.* **49**. 34-41.

Bautista-Ortín, A. B., Rodríguez-Rodríguez, P., Gil-Muñoz, R., Jiménez-Pasqual, E., Busse-Valverde, N., Martínez-Cutillas, A., López-Roca, J. M., and Gómez-Plaza, E. 2012. Influence of berry ripeness on concentration, qualitative composition and extractability of grape seed tannins. *Aust. J. Grape Wine R.* **18**. 123-130.

Bindon, K. A., Bacic, A., and Kennedy, J. A. 2012. Tissue-specific and developmental modifications of grape cell walls influence the adsorption of proanthocyanidins. *J. Agric. Food. Chem.* **60**. 9249-9260.

Bindon, K. A., and Kennedy, J. A. 2011. Ripening-induced changes in grape skin proanthocyanidins modify their interaction with cell walls. *J. Agric. Food. Chem.* **59**. 2696-2707.

Bindon, K. A., Smith, P. A., Holt, H., and Kennedy, J. A. 2010a. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. *J. Agric. Food. Chem.* **58**. 10736-10746.

Bindon, K. A., Smith, P. A., and Kennedy, J. A. 2010b. Interaction between grape-derived proanthocyanidins and cell wall material. 1. Effect on proanthocyanidin composition and molecular mass. *J. Agric. Food. Chem.* **58**. 2520-2528.

BMEL. 2022a. Daten und Fakten. *Bundesministreium für Ernährung und Landwirtschaft*.

BMEL. 2022b. Weinbau in Deutschland. *Bundesministerium für Landwirtschaft und Ernährung*.

Bogs, J., Downey, M. O., Harvey, J. S., Ashton, A. R., Tanner, G. J., and Robinson, S. P. 2005. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol.* **139**. 652-663.

Brahem, M., Renard, C., Bureau, S., Watrelot, A. A., and Le Bourvellec, C. 2019. Pear ripeness and tissue type impact procyanidin-cell wall interactions. *Food Chem.* **275**. 754-762.

Brummell, D. A. 2006. Cell wall disassembly in ripening fruit. *Funct Plant Biol.* **33**. 103-119.

Brummell, D. A., and Harpster, M. H. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol.* **47**. 311-340.

Buchweitz, M., Speth, M., Kammerer, D. R., and Carle, R. 2013a. Impact of pectin type on the storage stability of black currant (Ribes nigrum L.) anthocyanins in pectic model solutions. *Food Chem.* **139**. 1168-1178.

Buchweitz, M., Speth, M., Kammerer, D. R., and Carle, R. 2013b. Stabilisation of strawberry (Fragaria x ananassa Duch.) anthocyanins by different pectins. *Food Chem.* **141**. 2998-3006.

Cadot, Y., Minana-Castello, M. T., and Chevalier, M. 2006. Anatomical, histological, and histochemical changes in grape seeds from Vitis vinifera L. cv Cabernet franc during fruit development. *J. Agric. Food. Chem.* **54**. 9206-9215.

Castro-Lopez, R., Gomez-Plaza, E., Ortega-Regules, A., Lozada, D., and Bautista-Ortin, A. B. 2016. Role of cell wall deconstructing enzymes in the proanthocyanidin-cell wall adsorption-desorption phenomena. *Food Chem.* **196**. 526-532.

Celus, M., Kyomugasho, C., Van Loey, A. M., Grauwet, T., and Hendrickx, M. E. 2018. Influence of pectin structural properties on interactions with divalent cations and its associated functionalities. *Compr. Rev. Food. Sci. Food Saf.* **17**. 1576-1594.

Cerpa-Calderon, F. K., and Kennedy, J. A. 2008. Berry integrity and extraction of skin and seed proanthocyanidins during red wine fermentation. *J. Agric. Food. Chem.* **56**. 9006-9014.

Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J. M., Sarni-Manchado, P., and Fulcrand, H. 2006. Structure and properties of wine pigments and tannins. *Am. J. Enol. Vitic.* **57**. 298-305.

Conde, C., Silva, P., Fontes, N., Dias, A. C. P., Tavares, R. M., Sousa, M. J., Agasse, A., Delrot, S., and Gerós, H. 2007. Biochemical changes throughout grape berry development and fruit and wine quality. *Food.* **1**. 1-22.

Coombe, B. G. 1960. Relationship of Growth and Development to Changes in Sugars, Auxins, and Gibberellins in Fruit of Seeded and Seedless Varieties of Vitis Vinifera. *Plant Physiol.* **35**. 241-250.

Deytieux-Belleau, C., Vallet, A., Doneche, B., and Geny, L. 2008. Pectin methylesterase and polygalacturonase in the developing grape skin. *Plant Physiol. Biochem.* **46**. 638-646.

Dimitrovska, M., Bocevska, M., Dimitrovski, D., and Murkovic, M. 2011. Anthocyanin composition of Vranec, Cabernet Sauvignon, Merlot and Pinot Noir grapes as indicator of their varietal differentiation. *Eur. Food Res. Technol.* **232**. 591-600.

Downey, M. O., Harvey, J. S., and Robinson, S. P. 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine R.* **9**. 15-27.

Fasoli, M., Dell'Anna, R., Dal Santo, S., Balestrini, R., Sanson, A., Pezzotti, M., Monti, F., and Zenoni, S. 2016. Pectins, Hemicelluloses and Celluloses Show Specific Dynamics in the Internal and External Surfaces of Grape Berry Skin During Ripening. *Plant Cell Physiol.* **57**. 1332-1349.

Fernandes, P. A. R., Le Bourvellec, C., Renard, C., Wessel, D. F., Cardoso, S. M., and Coimbra, M. A. 2020a. Interactions of arabinan-rich pectic polysaccharides with polyphenols. *Carbohydr. Polym.* **230**. 115644.

Fernandes, P. A. R., Oliveira, J., Fonseca, F., Ferreira-da-Silva, F., Mateus, N., Vincken, J. P., and Freitas, V. 2020b. Molecular binding between anthocyanins and pectic polysaccharides – Unveiling the role of pectic polysaccharides structure. *Food Hydrocoll.* **102**. 105625.

Fournand, D., Vicens, A., Sidhoum, L., Souquet, J. M., Moutounet, M., and Cheynier, V. 2006. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *J. Agric. Food. Chem.* **54**. 7331-7338.

Fredes, C., Mora, M., and Carrasco-Benavides, M. 2017. An Analysis of Seed Colour During Ripening of Cabernet Sauvignon Grapes. *S. Afr. J. Enol. Vitic.* **38**. 38-45.

Fredes, C., Von Bennewitz, E., Holzapfel, E., and Saavedra, F. 2010. Relation between Seed Appearance and Phenolic Maturity: A Case Study Using Grapes cv. Carménère. *Chil. J. Agric. Res.* **70**. 381-389.

Gagne, S., Saucier, C., and Geny, L. 2006. Composition and cellular localization of tannins in Cabernet Sauvignon skins during growth. *J. Agric. Food. Chem.* **54**. 9465-9471.

Gao, Y., Zietsman, A. J. J., Vivier, M. A., and Moore, J. P. 2019. Deconstructing wine grape cell walls with enzymes during winemaking: New insights from glycan microarray technology. *Molecules.* **24**.

Gawel, R., Oberholster, A., and Francis, I. L. 2000. A 'Mouth-feel Wheel': terminology for communicating the mouth-feel characteristics of red wine. *Aust. J. Grape Wine R.* **6**. 203-207.

Geny, L., Saucier, C., Bracco, S., Daviaud, F., and Glories, Y. 2003. Composition and cellular localization of tannins in grape seeds during maturation. *J. Agric. Food. Chem.* **51**. 8051-8054.

Gil, M., Kontoudakis, N., Gonzalez, E., Esteruelas, M., Fort, F., Canals, J. M., and Zamora, F. 2012. Influence of grape maturity and maceration length on color, polyphenolic composition, and polysaccharide content of Cabernet Sauvignon and Tempranillo wines. *J. Agric. Food. Chem.* **60**. 7988-8001.

Goulao, L. F., Fernandes, J. C., Lopes, P., and Amâncio, S. 2012. Tackling the cell wall of the grape berry. *In* The biochemistry of the grape berry. H. Gerós, M. Manuela Chaves and S. Delrot (eds.), pp. 172-193. Bentham Science Publishers.

Gross, K. C., and Sams, C. E. 1984. Changes in cell wall neutral sugar composition during fruit ripening: a species survey. *Phytochemistry*. **23**. 2457-2461.

Guadalupe, Z., and Ayestaran, B. 2007. Polysaccharide profile and content during the vinification and aging of Tempranillo red wines. *J. Agric. Food. Chem.* **55**. 10720-10728.

Guidoni, S., and Hunter, J. J. 2012. Anthocyanin profile in berry skins and fermenting must/wine, as affected by grape ripeness level of Vitis vinifera cv. Shiraz/R99. *Eur. Food Res. Technol.* **235**. 397-408.

Hagerman, A. E. 1989. Chemistry of tannin-protein complexation. *In* Chemistry and significance of condensed tannins. R.W. Hemingway, Karchesy, J.J., Branham, S.J. (ed.), pp. 323-333. Springer, Boston, MA.

Hanlin, R. L., Hrmova, M., Harbertson, J. F., and Downey, M. O. 2010. Review: Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust. J. Grape Wine R.* **16**. 173-188.

Harbertson, J. F., Kennedy, J. A., and Adams, D. O. 2002. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *Am. J. Enol. Vitic.* **53**. 54-59.

Harbertson, J. F., Mireles, M., and Yu, Y. 2015. Improvement of BSA Tannin Precipitation Assay by Reformulation of Resuspension Buffer. *Am. J. Enol. Vitic.* **66**. 95-99.

Harbertson, J. F., Picciotto, E. A., and Adams, D. O. 2003. Measurement of polymeric pigments in grape berry extracts and wines using a protein precipitation assay combined with bisulfite bleaching. *Am. J. Enol. Vitic.* **54**. 301-306.

Harrison, R. 2017. Practical interventions that influence the sensory attributes of red wines related to the phenolic composition of grapes: a review. *Int. J. Food Sci. Technol.* **53**. 3-18.

Jones, G. V. 2018. The Climate Component of Terroir. *Elements*. **14**. 167-172.

Jones, G. V., and Schultz, H. 2016. Climate change and emerging cool climate wine regions.

Kennedy, J. A., Hayasaka, Y., Vidal, S., Waters, E. J., and Jones, G. P. 2001. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food. Chem.* **49**. 5348-5355.

Kennedy, J. A., and Jones, G. P. 2001. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food. Chem.* **49**. 1740-1746.

Kennedy, J. A., Matthews, M. A., and Waterhouse, A. L. 2000a. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry*. **55**. 77-85.

Kennedy, J. A., Troup, G. J., Pilbrow, J. R., Hutton, D. R., Hewitt, D., Hunter, C. R., Ristic, R., Iland, P. G., and Jones, G. P. 2000b. Development of seed polyphenols in berries from Vitis vinifera L. cv. Shiraz. *Aust. J. Grape Wine R.* **6**. 244-254.

Kontoudakis, N., Esteruelas, M., Fort, F., Canals, J. M., and Zamora, F. 2010. Comparison of methods for estimating phenolic maturity in grapes: correlation between predicted and obtained parameters. *Anal. Chim. Acta*. **660**. 127-133.

Larsen, L. R., Buerschaper, J., Schieber, A., and Weber, F. 2019. Interactions of anthocyanins with pectin and pectin fragments in model solutions. *J. Agric. Food. Chem.* **67**. 9344-9353.

Le Bourvellec, C., Bouchet, B., and Renard, C. M. G. C. 2005. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta, Gen. Subj.* **1725**. 10-18.

Le Bourvellec, C., Guyot, S., and Renard, C. M. 2004. Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta, Gen. Subj.* **1672**. 192-202.

Le Bourvellec, C., and Renard, C. M. 2012. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.* **52**. 213-248.

Letaief, H., Maury, C., Symoneaux, R., and Siret, R. 2013. Sensory and instrumental texture measurements for assessing grape seed parameters during fruit development. *J. Sci. Food Agric.* **93**. 2531-2540.

Letaief, H., Rolle, L., and Gerbi, V. 2008a. Mechanical behavior of winegrapes under compression tests. *Am. J. Enol. Vitic.* **59**. 323-329.

Letaief, H., Rolle, L., Zeppa, G., and Gerbi, V. 2008b. Assessment of grape skin hardness by a puncture test. *J. Sci. Food Agric.* **88**. 1567-1575.

Liu, X., Le Bourvellec, C., and Renard, C. 2020. Interactions between cell wall polysaccharides and polyphenols: Effect of molecular internal structure. *Compr. Rev. Food. Sci. Food Saf.* **19**. 3574-3617.

Ma, W., Waffo-Teguo, P., Alessandra Paissoni, M., Jourdes, M., and Teissedre, P. L. 2018. New insight into the unresolved HPLC broad peak of Cabernet Sauvignon grape seed polymeric tannins by combining CPC and Q-ToF approaches. *Food Chem.* **249**. 168-175.

Mattioli, R., Francioso, A., Mosca, L., and Silva, P. 2020. Anthocyanins: A Comprehensive Review of Their Chemical Properties and Health Effects on Cardiovascular and Neurodegenerative Diseases. *Molecules*. **25**.

Mattivi, F., Vrhovsek, U., Masuero, D., and Trainotti, D. 2009. Differences in the amount and structure of extractable skin and seed tannins amongst red grape varieties. *Aust. J. Grape Wine R.* **15**. 27-35.

McRae, J. M., and Kennedy, J. A. 2011. Wine and grape tannin interactions with salivary proteins and their impact on astringency: a review of current research. *Molecules*. **16**. 2348-2364.

Medina-Plaza, C., Beaver, J. W., Lerno, L., Dokoozlian, N., Ponangi, R., Blair, T., Block, D. E., and Oberholster, A. 2019. Impact of temperature, ethanol and cell wall material composition on cell wall-anthocyanin interactions. *Molecules*. **24**.

Medina-Plaza, C., Beaver, J. W., Miller, K. V., Lerno, L., Dokoozlian, N., Ponangi, R., Blair, T., Block, D. E., and Oberholster, A. 2020. Cell wall–anthocyanin interactions during red wine fermentation-like conditions. *Am. J. Enol. Vitic.* **71**. 149-156.

Noble, A. C. 1998. Why do wines taste bitter and feel astringent? *In* Chemistry of wine flavor. A.L. Waterhouse and S.E. Ebeler (eds.), pp. 156-165. American Chemical Society, Washington, DC.

Nunan, K. J., Sims, I. M., Bacic, A., Robinson, S. P., and Fincher, G. B. 1998. Changes in cell wall composition during ripening of grape berries. *Plant Physiol.* **118**. 783-792.

Obreque-Slier, E., Pena-Neira, A., Lopez-Solis, R., Zamora-Marin, F., Ricardo-da Silva, J. M., and Laureano, O. 2010. Comparative study of the phenolic composition of seeds and skins from Carmenere and Cabernet Sauvignon grape varieties (Vitis vinifera L.) during ripening. *J. Agric. Food. Chem.* **58**. 3591-3599.

Ochoa-Villarreal, M., Aispuro-Hernndez, E., Vargas-Arispuro, I., and Martínez-Téllez, M. 2012. Plant Cell Wall Polymers: Function, Structure and Biological Activity of Their Derivatives. *In* Polymerization. G. Ailton De Souza (ed.), pp. Ch. 4. IntechOpen, Rijeka.

OIV. 2022. State of the world vine and world wine sector 2021. *The International Organisation of Vine and Wine*.

Ortega-Regules, A., Ros-Garcia, J. M., Bautista-Ortin, A. B., Lopez-Roca, J. M., and Gomez-Plaza, E. 2008. Changes in skin cell wall composition during the maturation of four premium wine grape varieties. *J. Sci. Food Agric.* **88**. 420-428.

Padayachee, A., Netzel, G., Netzel, M., Day, L., Zabaras, D., Mikkelsen, D., and Gidley, M. J. 2012. Binding of polyphenols to plant cell wall analogues – Part 1: Anthocyanins. *Food Chem.* **134**. 155-161.

Peleg, H., Gacon, K., Schlich, P., and Noble, A. C. 1999. Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. *J. Sci. Food Agric.* **79**. 1123-1128.

Possner, D. R. E., and Kliewer, W. M. 1985. The localisation of acids, sugars, potassium and calcium in developing grape berries. *Vitis*. **24**. 229-240.

Quijada-Morin, N., Garcia-Estevez, I., Nogales-Bueno, J., Rodriguez-Pulido, F. J., Heredia, F. J., Rivas-Gonzalo, J. C., Escribano-Bailon, M. T., and Hernandez-Hierro, J. M. 2016. Trying to set up the flavanolic phases during grape seed ripening: A spectral and chemical approach. *Talanta*. **160**. 556-561.

Remy, S., Fulcrand, H., Labarbe, B., Cheynier, V., and Moutounet, M. 2000. First confirmation in red wine of products resulting from direct anthocyanin–tannin reactions. *J. Sci. Food Agric.* **80**. 745-751.

Renard, C. M. G. C., Watrelot, A. A., and Le Bourvellec, C. 2017. Interactions between polyphenols and polysaccharides: Mechanisms and consequences in food processing and digestion. *Trends Food Sci. Tech.* **60**. 43-51.

Ristic, R., and Iland, P. G. 2005. Relationships between seed and berry development of Vitis Vinifera L. cv Shiraz: Developmental changes in seed morphology and phenolic composition. *Aust. J. Grape Wine R.* **11**. 43-58.

Robichaud, J. L., and Noble, A. C. 1990. Astringency and bitterness of selected phenolics in wine. *J. Sci. Food Agric.* **53**. 343-353.

Robinson, J., Harding, J., and Vouillamoz, J. 2013. *Wine grapes: a complete guide to 1,368 vine varieties, including their origins and flavours*. Penguin UK.

Robinson, S., and Davies, C. 2000. Molecular biology of grape berry ripening. *Aust. J. Grape Wine R.* **6**. 175-188.

Rodriguez-Pulido, F. J., Gonzalez-Miret, M. L., and Heredia, F. J. 2017. Application of imaging techniques for the evaluation of phenolic maturity of grape seeds. *Opt. Pura Apl.* **50**. 1-11.

Rodriguez-Pulido, F. J., Hernandez-Hierro, J. M., Nogales-Bueno, J., Gordillo, B., Gonzalez-Miret, M. L., and Heredia, F. J. 2014. A novel method for evaluating flavanols in grape seeds by near infrared hyperspectral imaging. *Talanta*. **122**. 145-150.

Rolle, L., Siret, R., Río Segade, S., Maury, C., Gerbi, V., and Jourjon, F. 2012. Instrumental Texture Analysis Parameters as Markers of Table-Grape and Winegrape Quality: A Review. *Am. J. Enol. Vitic.* **63**. 11-28.

Ruiz-Garcia, Y., Smith, P. A., and Bindon, K. A. 2014. Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls. *Carbohydr. Polym.* **114**. 102-114.

Saint Cricq de Gaulejac, N., Vivas, N., and Glories, Y. 1998. Phenolic maturity: definition and control. *Rev. Fr. Oenol.* **173**. 22-25.

Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., Herderich, M. J., and Smith, P. A. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. *Aust. J. Grape Wine R.* **12**. 39-49.

Scheller, H. V., and Ulvskov, P. 2010. Hemicelluloses. *Annu. Rev. Plant Biol.* **61**. 263-289.

Schols, H. A., and Voragen, A. G. J. 1996. Complex Pectins: Structure elucidation using enzymes. *In* Pectins and Pectinases, Proceedings of an International Symposium. J. Visser and A.G.J. Voragen (eds.), pp. 3-19. Elsevier.

Silacci, M. W., and Morrison, J. C. 1990. Changes in Pectin Content of Cabernet-Sauvignon Grape Berries during Maturation. *Am. J. Enol. Vitic.* **41**. 111-115.

Somers, T. C., and Evans, M. E. 1974. Wine quality: Correlations with colour density and anthocyanin equilibria in a group of young red wines. *J. Sci. Food Agric.* **25**. 1369-1379.

Souquet, J. M., Labarbe, B., Le Guerneve, C., Cheynier, V., and Moutounet, M. 2000. Phenolic composition of grape stems. *J. Agric. Food. Chem.* **48**. 1076-1080.

Torchio, F., Cagnasso, E., Gerbi, V., and Rolle, L. 2010. Mechanical properties, phenolic composition and extractability indices of Barbera grapes of different soluble solids contents from several growing areas. *Anal. Chim. Acta.* **660**. 183-189.

Unterkofler, J., Muhlack, R. A., and Jeffery, D. W. 2020. Processes and purposes of extraction of grape components during winemaking: current state and perspectives. *Appl. Microbiol. Biotechnol.* **104**. 4737-4755.

Venter, P., Muller, M., Vestner, J., Stander, M. A., Tredoux, A. G. J., Pasch, H., and de Villiers, A. 2018. Comprehensive Three-Dimensional LC x LC x Ion Mobility Spectrometry Separation Combined with High-Resolution MS for the Analysis of Complex Samples. *Anal. Chem.* **90**. 11643-11650.

Vicens, A., Fournand, D., Williams, P., Sidhoum, L., Moutounet, M., and Doco, T. 2009. Changes in polysaccharide and protein composition of cell walls in grape berry skin (cv. Shiraz) during ripening and over-ripening. *J. Agric. Food. Chem.* **57**. 2955-2960.

Vidal, S., Francis, L., Guyot, S., Marnet, N., Kwiatkowski, M., Gawel, R., Cheynier, V. r., and Waters, E. J. 2003a. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. *J. Sci. Food Agric.* **83**. 564-573.

Vidal, S., Williams, P., Doco, T., Moutounet, M., and Pellerin, P. 2003b. The polysaccharides of red wine: total fractionation and characterization. *Carbohydr. Polym.* **54**. 439-447.

Vidal, S., Williams, P., O'Neill, M. A., and Pellerin, P. 2001. Polysaccharides from grape berry cell walls. Part I: tissue distribution and structural characterization of the pectic polysaccharides. *Carbohydr. Polym.* **45**. 315-323.

Vorwerk, S., Somerville, S., and Somerville, C. 2004. The role of plant cell wall polysaccharide composition in disease resistance. *Trends Plant Sci.* **9**. 203-209.

Waterhouse, A. L., Jeffery, D. W., and Sacks, G. L. 2016. *Understanding wine chemistry*. Wiley. Chichester.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., and Renard, C. M. 2013. Interactions between pectic compounds and procyanidins are influenced by methylation degree and chain length. *Biomacromolecules.* **14**. 709-718.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., and Renard, C. M. 2014. Neutral sugar side chains of pectins limit interactions with procyanidins. *Carbohydr. Polym.* **99**. 527-536.

Watrelot, A. A., Schulz, D. L., and Kennedy, J. A. 2017. Wine polysaccharides influence tannin-protein interactions. *Food Hydrocoll.* **63**. 571-579.

Weber, F. 2022. Noncovalent Polyphenol-Macromolecule Interactions and Their Effects on the Sensory Properties of Foods. *J. Agric. Food. Chem.* **70**. 72-78.

Weilack, I., Schmitz, C., Harbertson, J. F., and Weber, F. 2021. Effect of Structural Transformations on Precipitability and Polarity of Red Wine Phenolic Polymers. *Am. J. Enol. Vitic.*

# Chapter 2

## Influence of Grape Cell Wall Polysaccharides on the Extraction of Polyphenols During Fermentation in Microvinifications

Grape cell wall polysaccharides influence the extraction of phenolic compounds during winemaking and consequently polyphenol concentrations in the final wine. During ripening, both compound groups undergo pronounced structural and compositional changes, resulting in a dynamic change of extractability. Grape cell wall polysaccharides from differently ripe grapes were added to fermentations of Cabernet Sauvignon and Pinot noir grapes. Polyphenol-polysaccharide interactions affected the concentrations of tannins and monomeric flavanols in the wines depending on the maturity of the added polysaccharides. With a higher polysaccharide maturity, the effects became more pronounced. Polysaccharides protected monomeric flavanols and tannin in Pinot noir, thereby increasing the concentrations, but they precipitated or masked these compounds in Cabernet Sauvignon. The added polysaccharides affected concentrations in anthocyanins and polymeric pigments much less compared to the ripening status of the grapes. It was concluded that, structural changes of polysaccharides during ripening affect the extraction of tannins and monomeric flavanols most.

**Keywords**: Pinot noir, Cabernet Sauvignon, tannin, anthocyanin, polymeric pigments, extractability, grape cell wall polysaccharides, winemaking

---

Reprinted with permission from:

Hensen, J. P., Hoening, F., Weilack, I., Damm, S., and Weber, F. 2022. Influence of Grape Cell Wall Polysaccharides on the Extraction of Polyphenols during Fermentation in Microvinifications. *J. Agric. Food. Chem.* **70**. 9117-9131. DOI: 10.1021/acs.jafc.2c02697.

## 1 Introduction

The choice of the exact timing of grape harvest is crucial for the quality of the resulting wine. Throughout the ripening period, the winemaker has the difficult task to determine the right harvest time where all grape associated parameters like sugar and acid content, aroma profile, and polyphenol extractability are optimal for producing the desired wine style. Since mouthfeel and color properties are important quality determining attributes of a red wine, the extraction of the corresponding compounds during maceration is decisive for the overall quality of the product. The extraction of tannins and anthocyanins has been shown to be tremendously influenced by interactions with polysaccharides.(Hanlin et al., 2010; Hernandez-Hierro et al., 2014; Medina-Plaza et al., 2020) During grape ripening, not only the concentrations of individual polyphenols evolve, but their interactions with polysaccharides and other cell material is altered as well.(Bindon et al., 2014b; Bindon et al., 2010a) Polysaccharides can interact with polyphenols either integrated in the cell wall, dissolved, (Gao et al., 2019) or insoluble in the must. (Bindon et al., 2010b) The interactions are influenced by the morphological and molecular structure of the polysaccharide as well as other molecules like proteins present in the must. (Springer et al., 2016) During berry ripening, the total polysaccharide amount does not change, (Vicens et al., 2009) but the structure and specific ratio of the main polysaccharides cellulose, hemicellulose, and pectin evolves continuously. (Nunan et al., 1998; Ruiz-Garcia et al., 2014) Since pectin remnants with linear short side chains and a low molecular weight interact more likely with polyphenols, (Larsen et al., 2019; Watrelot et al., 2014) and the degree of pectin esterification further influences polyphenol interactions, (Liu et al., 2020) the maturity of cell wall polysaccharides may impact the affinity for polyphenol interactions. Macroscopic changes of the cell wall structure during ripening further increase the interactions with polyphenols. (Bindon et al., 2012; Bindon et al., 2014b) The connection between polysaccharide composition and polyphenol concentrations in a wine is however highly complex. A multitude of different molecular structures that are released during maceration can interact in countless ways, compete for the same binding sites, and get altered by enzymes at the same time. Some general differences in the extraction of the different groups of polyphenols have been established, e.g. anthocyanins are extracted slightly earlier than tannins due to their water solubility. (Gao et al., 2019) Their competition with tannins for the same polysaccharides could therefore lead to an increased tannin concentration in the wine. (Gao et al., 2019) On the contrary, tannin cell wall interactions can prohibit anthocyanin extraction as well. (Bindon et al., 2014b) The molecular structure of tannins affects the binding affinity for polysaccharides further. A positive correlation of the mean degree of polymerization

(mDP) of tannins with the affinity for polysaccharide interactions has been shown.(Bindon et al., 2010a; Fournand et al., 2006) Galloylation and a higher number of hydroxy groups further increase the binding affinity.(Hanlin et al., 2010; Le Bourvellec et al., 2004; Liu et al., 2020) Besides, the changing conditions in the must during fermentation like an increase in ethanol concentration and high temperatures enhance polyphenol solubility and reduce polyphenol polysaccharide interactions.(Medina-Plaza et al., 2019; Medina-Plaza et al., 2020) Since most influencing factors evolve during berry ripening, (Bindon et al., 2014a) it is necessary to evaluate the impact of berry ripeness on polyphenol polysaccharide interactions. Most of the existing studies are based on model experiments and highlight the factors determining polyphenol polysaccharide interactions and the possible effects on the wine during fermentation. The complexity of the combination of all these factors complicates the transfer of these results to actual winemaking processes. The present study assesses the influence of grape polysaccharides on the extraction of polyphenols during winemaking. As polyphenol extractability changes during berry ripening, a special focus of the present study is the influence of berry ripeness and polysaccharide maturity on polysaccharide polyphenol interactions.

## 2 Material and methods

### *2.1 Material*

Sodium chloride was purchased from neoFroxx GmbH (Einhausen, Germany). Acetic acid, ethanol (100%), hydrochloric acid (HCl) (37%), potassium bisulfite (97.4%), and acetonitrile (100%) were purchased from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide was purchased from Honeywell International Inc. (Seelze, Germany). Urea (≥ 99.5%), bovine serum albumin fraction V (≥ 98%), and (+)-catechin ≥98% were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Triethanolamine (≥ 98%), ferric chloride (97%), L-(+)-tartaric acid, and maleic acid (98%) were purchased from Alfa Aesar (Kandel, Germany). Gallic acid (> 98%) was purchased from Fluka Chemie AG (Buchs, Switzerland). Sodium azide (≥ 99%) was purchased from Merck KGaA (Darmstadt, Germany). Formic acid (≥ 98%), ammonium hydroxide, and sulfuric acid (≥ 95%) were purchased from Sigma Aldrich Chemie (Steinheim, Germany). Potassium pyrosulfate (food grade) was purchased from RWA Raiffeisen Ware Austria AG (Vienna, Austria). Potassium bicarbonate (food grade) was purchased from Erbslöh Geisenheim AG (Geisenheim, Germany). Ultrapure water was obtained from a PURELAB flex 2 water purification system (ELGA 90LabWater, Paris, France).

Pinot noir and Cabernet Sauvignon grapes for winemaking and polysaccharide extraction were picked in Neustadt an der Weinstrasse (Palatinate, Germany). The geodesic coordinates of the variety specific vineyards were 49°23'53.3"N 8°11'02.0"E (Cabernet Sauvignon) and 49°22'14.3"N 8°10'56.3"E (Pinot noir). Grapes for polysaccharide extraction were harvested in 2019 with a low, medium, and high concentration of total soluble solids (TSS) and then frozen at -20 °C. Grapes for winemaking were harvested in 2020 again with a low, medium, and high concentration of TSS and chilled instantly at 7 °C for 12 h. Table 2.1 lists the vintage specific concentrations of TSS measured with a digital refractometer (PAL-α ATAGO CO. LTD., Tokyo, Japan).

**Table 2.1** Concentration of total soluble solids (TSS) in grapes used for polysaccharide extraction (vintage 2019) and winemaking (vintage 2020). Classifiction in low, medium and high TSS is vintage specific.

| | Low TSS | Medium TSS | High TSS |
|---|---|---|---|
| Cabernet Sauvignon 2019 | 17.6 °Bx | 21.2 °Bx | 22.7 °Bx |
| Cabernet Sauvignon 2020 | 17.1 °Bx | 20.9 °Bx | 21.8 °Bx |
| Pinot noir 2019 | 17.6 °Bx | 21.6 °Bx | 22.6 °Bx |
| Pinot noir 2020 | 16.7 °Bx | 19.7 °Bx | 21.6 °Bx |

### *2.2 Grape cell wall polysaccharide extraction*

Polysaccharides were extracted from frozen Pinot noir and Cabernet Sauvignon berries harvested in 2019. Whole, destemmed berries were lyophilized (Beta 2-8 LSCBasic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground to 0.25 mm particles (ZM-200, Retsch GmbH, Haan, Germany). For a sufficient lyophilization despite the high sugar concentration in the grapes, grapes were initially frozen at -80 °C and then lyophilized for 8 days. Before grinding, the lyophilized grapes were frozen again at -80 °C. As reported by Fügel et al. (2004) a suspension of one gram berry powder in 25 mL 80% v/v ethanol at 40 °C was prepared and mixed for one hour. After vacuum filtration, the process was repeated with the solid residue 10 times. The final residue was extracted with 25 mL $g^{-1}$ acetone at room temperature for 18 h, filtered, and dried at 40 °C for 24 h. After drying, the cell wall polysaccharides were ground to 0.25 mm particle size. One polysaccharide extract was made for each maturity level from both grape varieties.

### *2.3 Analyses of monomer composition of polysaccharides*

The monomeric sugar composition of the grape cell wall polysaccharides was analyzed after acid hydrolysis with 1 M sulfuric acid at 120 °C for 1.5 h. Samples were first dissolved in 12 M sulfuric acid with ultrasonication. After adjusting the pH to 2.3 with ammonium hydroxide and centrifugation (Heraeus Megafuge 40R centrifuge, Thermo Fisher Scientific, Braunschweig, Germany) at 10 947*g*, 5 min, monosaccharides in the supernatant were analyzed on a Smartline HPLC system with a RI detector 2300 (Knauer, Berlin, Germany) equipped with a Nucleogel ION 300 OA, 7.8 mm (Macherey-Nagel, Düren, Germany). Samples were eluted for 50 min at 0.3 mL $min^{-1}$ with 2.5 mmol sulfuric acid. D(+)-galacturonic acid ≥93% (Fluka Chemie AG, Buchs, Switzerland), D(+)-glucose monohydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), D(+)-xylose ≥99% (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), D(+)-galactose ≥98% (AppliChem GmbH, Darmstadt, Germany), L(+)-rhamnose standard solution (Megazyme Ltd., Wicklow, Ireland) and L(+)-arabinose ≥99% (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were used for identification of the compounds in the analyzed samples. Samples were measured in triplicate and compared with a semi-quantitative approach by comparing peak areas of the different monomers.

### *2.4 Molecular weight distribution of the water-soluble polysaccharide fraction*

Molecular weight (MW) distribution was determined by high-performance size exclusion chromatography (HPSEC) on a Smartline HPLC system with a RI detector 2300 (Knauer, Berlin, Germany) equipped with two different, connected SEC-Diol columns (300 and 120 Å, 3 µm; YMC, Kyoto, Japan) as described by Larsen et al. (2019). After dissolving the samples in water (50 °C), samples were dialyzed against demineralized water (MWCO 3.5 kDa) and filtered through 0.2 µm Chromafil RC-20/15 MS filters (Macherey-Nagel, Düren, Germany). MWs were calculated with eight pullulan standards ranging from 6.1 to 708 kDa (ReadyCal-Kit Pullulan, PSS-Polymer Standards, Mainz, Germany).

### *2.5 Polysaccharide fractionation*

Pectin, hemicellulose, and cellulose fractions were sequentially extracted from the grape cell wall polysaccharides according to the method described by Fügel et al. (2004). The pectin fraction was extracted with 0.05 M NaOH at 4 °C for 3 h and the hemicellulose fraction was extracted with 4 M NaOH at 30 °C for 8 h. After each extraction, the residue was washed five

times with demineralized water and lyophilized subsequently. The weight loss after each extraction was attributed to the corresponding fraction.

### *2.6 Winemaking in microvinifications*

The chilled grapes (vintage 2020) were crushed and destemmed with a grape mill DMCI.18 (GRIFO Macchine Enologiches s.n.c. di Marchetti Giordano & C., Piadena, Italy). Potential alcohol was adjusted to 14.5% v/v by addition of sucrose and pH was adjusted to 3.3 by the addition of potassium bicarbonate to standardize ethanol concentrations and pH between all wines. 20 g Zymaflore RB2 (Laffort, Bordeaux Cedex, France) and 30 g GO-FERM (Lallemand Inc., Montreal, Canada) per hectoliter must were added according to the manufacturers' instructions. The must was fermented in plastic containers in 1.6 kg batches. Grape cell wall polysaccharides were added in two different sets of experiments at a concentration of 0.3% w/w. In the first set, varietal specific polysaccharides extracted from three differently ripe grape materials were added to the grapes with medium concentration in TSS (19.7 °Bx for Pinot noir and 20.9 °Bx for Cabernet Sauvignon). In the second set, a mixture of grape cell wall polysaccharides extracted from Pinot noir and Cabernet Sauvignon berries of different maturity were added to grapes with either a low or high concentration in TSS. The containers were sealed with a lid equipped with a fermentation lock filled with potassium sulphite solution (5 g $L^{-1}$) for microbiological stability. The must was fermented for 12 days at 22 °C ± 2 °C, shaken three times per day and sampled through a septum in the lid. For daily sampling, TSS were measured with a refractometer (PAL-α ATAGO CO. LTD., Tokyo, Japan), then 10 mL sample were centrifuged at 18500 × *g* for 10 min and filtered through a 0.2 µm syringe filter.

### *2.7 Spectrophotometric analysis of color differences between samples*

Using a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany) absorbance spectra of the wine samples were recorded between 300 and 800 nm in a 1 mm path-length glass cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) at the end of fermentation. According to OIV suggestions, luminance L*, chroma C*, and color hue h* were calculated with Spectra Manager Ver. 2.14G (JASCO Deutschland GmbH, Pfungstadt, Germany) after correcting the measurements to a 10 mm path length. (OIV, 2006) The color deviation between the control sample and the corresponding samples with added polysaccharide were calculated as delta E.

### *2.8 UHPLC analyses of polyphenols*

Anthocyanins (delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-3-*O*-acetylglucoside, cyanidin-3-*O*-acetylglucoside, petunidin-3-*O*-acetylglucoside, peonidin-3-*O*-acetylglucoside, malvidin-3-*O*-acetylglucoside, peonidin-3-*O*-coumaroylglucoside, and malvidin-3-*O*-coumaroylglucoside), catechin, and epicatechin were quantified using a Nexera X2 (Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler SIL-30AC chilled to 10 °C, column oven CTO-20AC set to 40 °C, and a DAD SPD-20A as well as a fluorescence detector RF-20A. The method was adapted from Heffels et al. (2015). Anthocyanins were detected at 520 nm. Monomeric flavanols, catechin and epicatechin, were quantified with the FLD at an emission wavelength of 320 nm after excitation at 280 nm in the samples. A Kinetex C18 100 A, 1.7 µm, 150 * 2.1 mm column (Phenomenex Inc., Torrance, CA) was used with water/formic acid (97/3; v/v) and acetonitrile/formic acid (97/3; v/v) as eluent A and B with a flow rate of 0.4 mL $min^{-1}$ and the following gradient: 0 min, 4.0% B; 2 min, 6.5% B; 5 min, 10.0% B; 6 min, 10.5% B; 7 min, 11.0% B; 11 min, 12.5% B; 13 min, 14.0% B; 15 min, 15.0% B; 26 min, 26.0% B; 26.1 min, 100.0% B; 30 min, 100.0% B; 30.1 min, 4.0% B; 33 min, 4.0% B. Injection volume was 5 µL. Malvidin-3-*O*-glucoside chloride ≥95% (Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany) ($R^2$ = 0.999, LOQ = 0.4 mg $L^{-1}$) and (+)-catechin ≥98% (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) ($R^2$ = 0.997, LOQ = 2.3 mg $L^{-1}$) were used for quantification of anthocyanins as malvidin-3-*O*-glucoside equivalent and the quantified flavanols as catechin equivalents by external calibration

### *2.9 Chemical characterization of phenolic polymers*

Tannins and non-precipitable polymeric pigments (renamed from their original classification as small polymeric pigments, SPP) were measured by protein precipitation combined with bisulfite bleaching according to Harbertson et al. (2002); (2003) with a modified resuspension buffer. (Harbertson et al., 2015) Tannins were expressed as catechin equivalents (CE) according to an external calibration curve. The analysis of anthocyanins was carried out according to Harbertson et al. (2009).

### *2.10 Quantification of ethanol*

The ethanol concentration was determined by headspace solid-phase-dynamic extraction gas chromatography with flame ionization detection (6890 Series, Agilent Technologies Inc.,

Santa Clara, CA) according to Zimdars et al. (2019). The Combi-PAL-autosampler (CTC Analytics, Zwingen, Switzerland) and an OPTIMA WAXplus capillary column (30 m × 250 µm × 0.25 µm, Macherey-Nagel, Düren, Germany) were used in the instrumental setup. 1 mL sample was filled in 10 mL headspace vials with added sodium azide to inhibit further yeast activity. Samples were incubated for 5 min at 60 °C before injection of 500 µL. The carrier gas was nitrogen with a constant flow rate of 0.7 mL $min^{-1}$. Temperature of the inlet was set to 200 °C and the initial oven temperature was set to 40 °C. The following oven temperature program was used: 40 °C (1 min); temperature increase, 5 °C $min^{-1}$; final temperature 50 °C (10 min). The detector temperature was set to 250 °C, using $H_2$ (40 mL $min^{-1}$) and air (450 mL $min^{-1}$) as detector gases. Standard solutions of ethanol were used for quantification by external calibration.

### *2.11 Statistical analysis*

R (Version 3.6.0) with R-studio (Version 1.1.383) and packages dplyr (Version 1.0.4), factoextra (Version 1.0.7), ggplot2 (Version 3.3.3), grid (Version 3.6.0), lemon (Version 0.4.5), rstatix (Version 0.7.0), stat (Version 3.6.0) and tidyr (Version 1.0.2) were used for statistical analysis and graphical illustrations. For pairwise comparison, ANOVA and Tukey´s post hoc test with a significance level of $p<0.05$ were used. Effect strength was calculated as Cohen's d.

## 3 Results and discussion

Minor differences between the wines regarding final ethanol and TSS concentrations lack significance and are within natural variances of fermentation. Therefore, it can be assumed that alcoholic fermentation was not interfered by the addition of polysaccharides and all observed effects derive from the interaction between polysaccharides and polyphenols or other must components.

### *3.1 Grape cell wall polysaccharide composition*

The different cell wall polysaccharide extracts from Pinot noir contained 41.5 to 44.6 % pectin, 33.9 to 37.5 % cellulose, and 20.7 to 23.5 % hemicellulose, whereas Cabernet Sauvignon grapes contained 42.0 to 48.6 % pectin, 30.7 to 38.7 % cellulose, and 19.3 to 20.7 % hemicellulose (Table 2.2).

**Table 2.2** Polysaccharide fractions in extracts from Cabernet Sauvignon and Pinot noir grapes at different levels of maturity determined by sequential extraction (means presented with standard deviation; n = 3). For data comparison a one-way ANOVA and Tukey's post-hoc test were performed for pectin, hemicellulose, and cellulose fractions individually. Significant differences ($p \leq 0.05$) were only present within the hemicellulose fraction and are indicated by letters.

| | | Pectin [%] | Hemicellulose [%] | Cellulose [%] |
|---|---|---|---|---|
| Cabernet Sauvignon | Low TSS | 42.6 ± 2.8 | 20.1 ± 1.4[a] | 37.4 ± 4.1 |
| | Medium TSS | 42.0 ± 1.8 | 19.3 ± 0.2[a] | 38.7 ± 1.97 |
| | High TSS | 48.6 ± 5.3 | 20.7 ± 0.4[a] | 30.7 ± 5.5 |
| Pinot noir | Low TSS | 44.6 ± 1.9 | 21.5 ± 1.4[ab] | 33.9 ± 3.3 |
| | Medium TSS | 41.5 ± 1.6 | 23.5 ± 0.8[b] | 35.0 ± 2.5 |
| | High TSS | 41.9 ± 0.4 | 20.7 ± 0.8[a] | 37.5 ± 1.1 |

Significant differences were only observed for changes to the hemicellulose fraction of extracts from Pinot noir and differences of the hemicellulose fraction between both varieties. After acid hydrolysis, galacturonic acid, galactose, glucose, xylose, rhamnose, and arabinose were identified by HPLC-RI via external standards. Between both varieties and during ripening the differences were small (Figure 2.1), similar to findings from Ruiz-Garcia et al. (2014) who compared Shiraz and Cabernet Sauvignon. In polysaccharide extracts from Pinot noir the galacturonic acid, glucose, and galactose proportion were reduced while the proportion of xylose increased. In Cabernet Sauvignon a similar development can be seen in the data, but

differences were not significant. Varietal specific differences at similar levels of ripeness are only present in extracts from grapes harvested with the lowest concentration of TSS. As reported by Nunan et al. (1998) and Vicens et al. (2009), the structure of polysaccharides changes after veraison, but the overall composition and monosaccharide concentrations remain constant. However, polysaccharides are a diverse group of molecules that can largely differ in their molecular structure and size. Although the monosaccharide composition and the proportion of the polysaccharides that build the grapes' cellular structure remained partly constant, cross-links or the individual structure of molecules might have changed within each polysaccharide group. The SEC separation of the water-soluble polysaccharides in the extracts revealed some of these differences. In polysaccharide extracts of both varieties, the fraction of small molecules (4.2-10.2 kDA) was generally reduced during ripening while the proportion of medium (10.2-162.1 kDA) and large (>162.1 kDA) fractions increased (Table 2.3).

**Table 2.3** Relative composition of water-soluble polysaccharides in extracts from Cabernet Sauvignon and Pinot noir grapes at different levels of maturity determined by HPSEC (means presented with standard deviation; n = 3). For data comparison a one-way ANOVA and Tukey's post-hoc test were performed. Significant differences ($p \leq 0.05$) are indicated by letters for each fraction individually.

| | | Small polysaccharide fraction [%] | Medium polysaccharide fraction [%] | Large polysaccharide fraction [%] |
|---|---|---|---|---|
| Cabernet Sauvignon | Low TSS | $36.1 \pm 1.1^{b}$ | $51.1 \pm 0.2^{a}$ | $12.8 \pm 1.2^{bc}$ |
| | Medium TSS | $30.3 \pm 0.6^{c}$ | $52.8 \pm 0.4^{b}$ | $16.9 \pm 0.2^{d}$ |
| | High TSS | $27.1 \pm 0.4^{a}$ | $56.6 \pm 0.4^{c}$ | $16.3 \pm 0.8^{cd}$ |
| Pinot noir | Low TSS | $44.5 \pm 0.1^{d}$ | $49.8 \pm 1.1^{a}$ | $5.7 \pm 2.1^{a}$ |
| | Medium TSS | $32.6 \pm 1.5^{c}$ | $52.9 \pm 0.4^{b}$ | $14.5 \pm 1.9^{bcd}$ |
| | High TSS | $31.8 \pm 0.2^{c}$ | $56.1 \pm 0.3^{c}$ | $4.6 \pm 0.5^{b}$ |

Although the development was similar for both varieties, Pinot noir polysaccharides contained a higher proportion of small molecules while in Cabernet Sauvignon polysaccharide extracts the proportion of larger molecules exceeded those in Pinot noir polysaccharide extracts. At medium berry ripeness, these differences were absent. These measurements were limited to the water-soluble polysaccharides in the extracts, but changes of other polysaccharides in the extracts can be expected. All these changes in the polysaccharide extracts during berry ripening are defined as the maturity of the polysaccharide extracts.

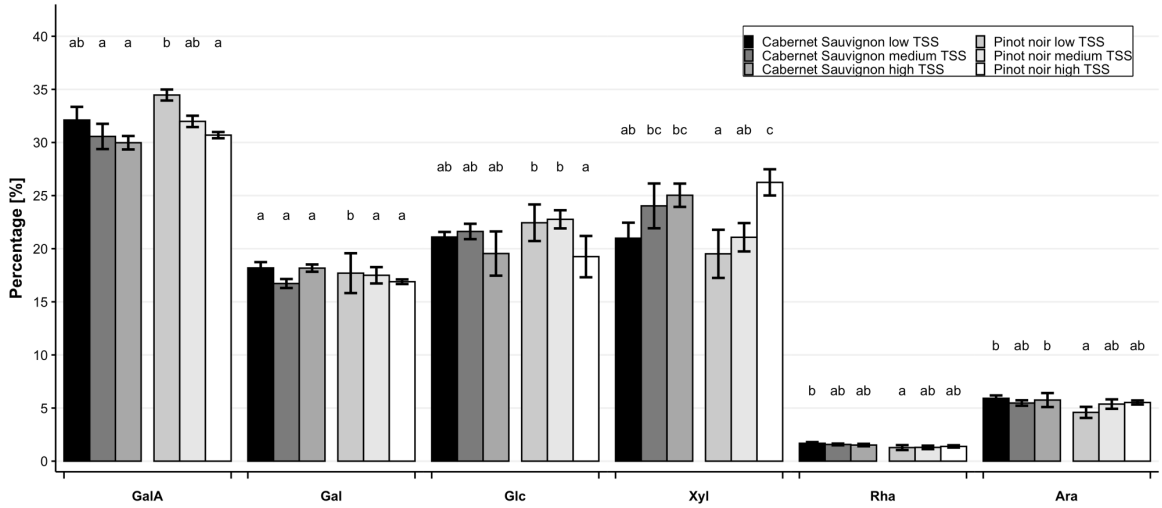



**Figure 2.1** Monosaccharide composition of polysaccharide extracts from Cabernet Sauvignon and Pinot noir grapes harvested with different concentrations of total soluble solids (means presented with standard deviation; n = 3). Galacturonic acid (GalA), xylose (Xyl), glucose (Glc), galactose (Gal), arabinose (Ara), and rhamnose (Rha) were measured with a HPLC after acid hydrolysis. For data comparison a one-way ANOVA and Tukey's post-hoc test were performed. Significant differences ($p \leq 0.05$) are indicated by letters for each analyte individually.

## *3.2 Polyphenol concentrations in reference wines from differently ripe grapes without the addition of polysaccharides*

During grape ripening, changes of polyphenol concentrations in the wines varied between analytes and both varieties (Figure 2.2). In Pinot noir wines the anthocyanins delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, and flavanols catechin and epicatechin were identified by UHPLC-DAD and UHPLC-FLD by their retention order and comparison with commercial standards (malvidin-3-*O*-glucoside chloride and (+)-catechin). In addition to these, the acetylglucoside and coumaroylglucoside derivatives of the mentioned anthocyanins were detected in Cabernet Sauvignon. Among the acylated anthocyanins, only the concentrations of peonidin 3-*O*-*p*-coumaroylglucoside and malvidin 3-*O*-*p*-coumaroylglucoside were sufficient for quantification. The results are presented as sum parameters as there were no changes in the anthocyanin profile. Pinot noir wines from grapes with different concentrations in TSS showed no significant difference of anthocyanin, monomeric flavanol, or tannin concentrations at the end of fermentation. Non-precipitable polymeric pigments (PP) concentration increased slightly in the Pinot noir wines with increasing berry ripeness. Non-precipitable PP, originally named small polymeric pigments, have been defined by the method of Harbertson et al. (2003)

as anthocyanin containing compounds that are not bleached by bisulfite and are not precipitated by bovine serum albumin. In Cabernet Sauvignon wines, anthocyanin and non-precipitable PP concentrations were higher when made from grapes with a high concentration in TSS. This increase in non-precipitable PP concentration during grape ripening has already been described. (Adams et al., 2004) The tannin concentration of Cabernet Sauvignon wines did not change significantly. However, multiple studies suggest that changes of tannin extractability are caused by changes of cell wall porosity and interactions between bound cell wall polysaccharides and tannins during berry ripening. (Bindon et al., 2014a; Bindon et al., 2014b; Hanlin et al., 2010) Catechin and epicatechin concentrations were higher in Cabernet Sauvignon wines from grapes with low levels of TSS compared to wines made from grapes with medium or high levels of TSS.

During fermentation, anthocyanin concentrations reached a maximum after six days of rapid extraction for both varieties and for all concentrations of TSS in the grapes used. Oxidation and polymerization slightly reduce anthocyanin concentrations in the final stage of maceration. (Sommer and Cohen, 2018) Interactions with grape cell wall polysaccharides can further reduce anthocyanin concentrations. (Padayachee et al., 2012) Non-precipitable PP concentrations continuously increase in Cabernet Sauvignon must. In Pinot noir must, non-precipitable PP concentrations reached a maximum after three days of fermentation and leveled at a lower concentration afterwards. Since non-precipitable PPs do not originate from the grapes and are more likely formed in the fermenting must from anthocyanins and other polyphenols, the non-precipitable PP concentration is directly linked to the extraction of these compounds. Tannin extraction starts with the increase in ethanol concentration after two days of fermentation, increasing continuously afterwards.

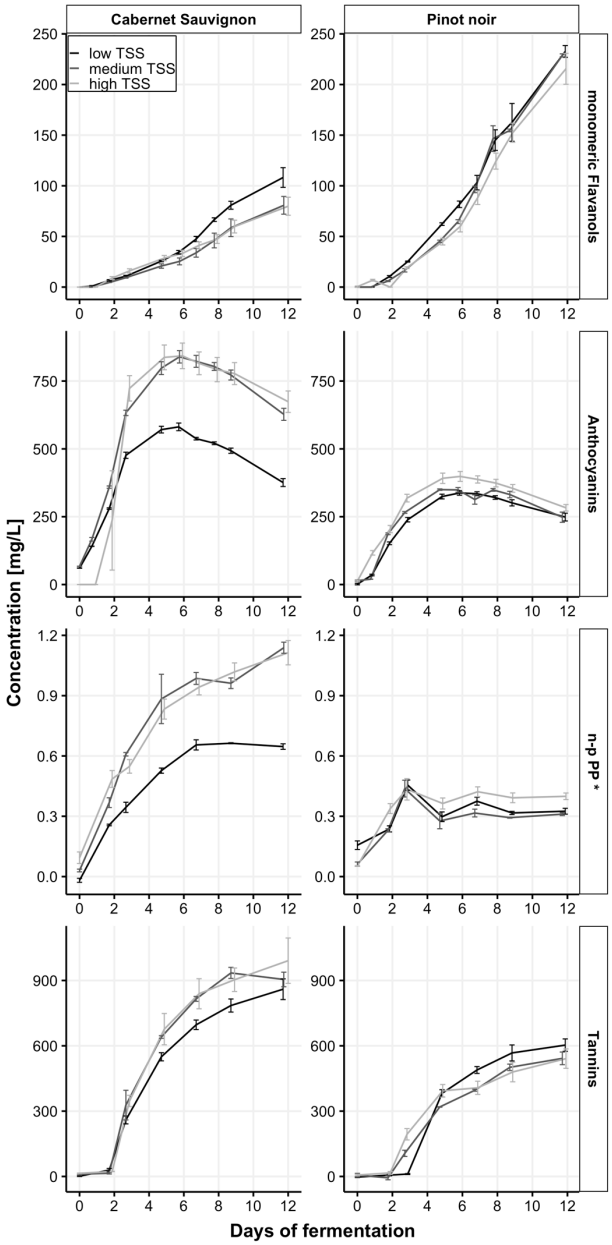



**Figure 1.2** Polyphenol concentration in Cabernet Sauvignon and Pinot noir wines from grapes at different levels of ripeness (means presented with standard deviation; n = 3). The monomer concentrations were measured with UHPLC, polymer concentrations were measured with protein precipitation assay during maceration. (* non-precipitable PP in absorption units) (Pinot noir: low TSS = 16.7 °Bx, medium TSS = 19.7 °Bx, high TSS = 21.6 °Bx, Cabernet Sauvignon: low TSS = 17.1 °Bx, medium TSS = 20.9 °Bx, high TSS = 21.8 °Bx).

### *3.3 Influence of polysaccharide maturity on interactions with polyphenols*

With the addition of cell wall polysaccharides that were extracted from Pinot noir and Cabernet Sauvignon grapes to musts from the same varieties, the influence of cell wall polysaccharide maturity on polyphenol interactions was evaluated. Tannins and monomeric

flavanols were most sensitive to interactions with polysaccharides as their concentration was predominantly influenced and this effect persisted until the end of fermentation (Figure 2.3). While the addition of cell wall polysaccharides increased the concentration of tannins and monomeric flavanols in Pinot noir wines, their concentrations were decreased in Cabernet Sauvignon wines.

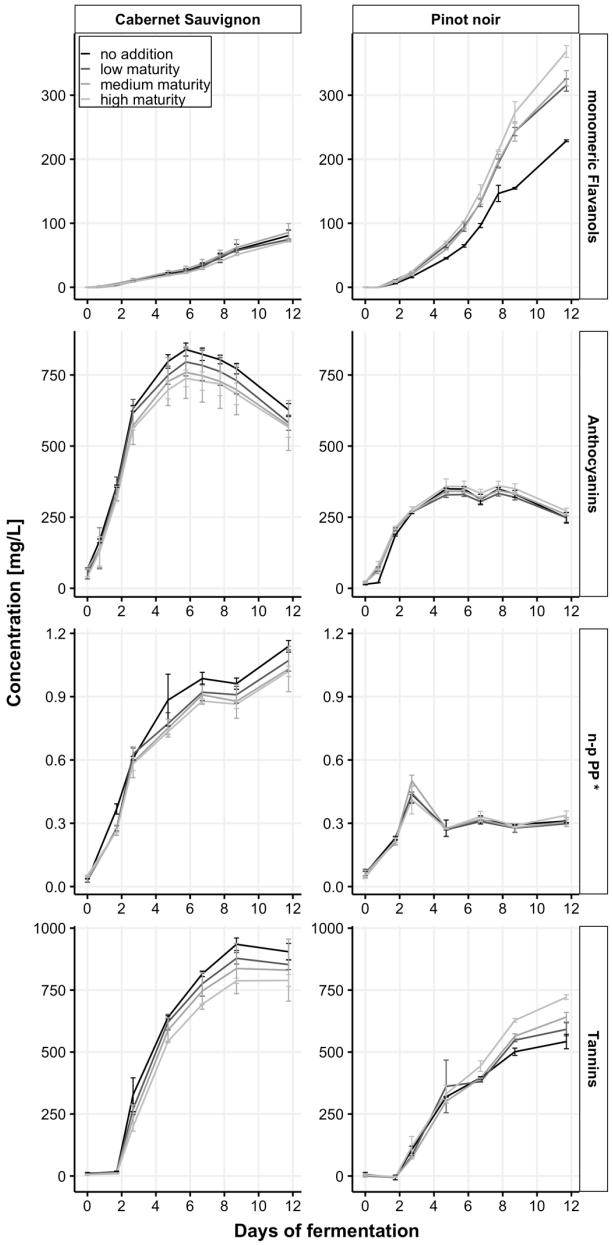



**Figure 2.3** Polyphenol concentrations in Pinot noir and Cabernet Sauvignon wines (means presented with standard deviation; n = 3) after addition of varietal specific grape cell wall polysaccharides with different levels of maturity (indicated by color). The monomer concentrations were measured with UHPLC, polymer concentrations were measured with protein precipitation assay during maceration. (* non-precipitable PP in absorption units)

The anthocyanin concentration was considerably affected only in Cabernet Sauvignon wines (Figure 2.4). On average the concentration was reduced by 6.1% (low maturity polysaccharides added), 9.9% (medium maturity polysaccharides added), and 12.7% (high maturity polysaccharides added) during the observed fermentation period. This reduced concentration was measured with UHPLC and nearly identical with chemical characterization which suggests precipitation of anthocyanins by the added polysaccharides. Because the total amount of anthocyanins in Pinot noir was about one half of the amount in Cabernet Sauvignon but the added amount of polysaccharides was equal in both wines, the effects were expected to be more pronounced in Pinot noir wines due to the excess of polysaccharides. However, cell wall polysaccharides did not change the anthocyanin concentrations in Pinot noir wines. This was not driven by variety specific differences of anthocyanin structures, as the concentration of acetylated anthocyanins, which are only present in Cabernet Sauvignon samples, was equally affected by the addition of polysaccharides as the concentration of non-acetylated anthocyanins. Consequently, the varietal specific polysaccharides and other molecules in the must are preventing or masking the interactions with anthocyanins in Pinot noir wines. Grape cell wall polysaccharides largely contain cellulose, (Hernandez-Hierro et al., 2014; Nunan et al., 1998; Ortega-Regules et al., 2008) that has been contradictorily reported to either bind anthocyanins, (Padayachee et al., 2012) or improve anthocyanin extractability (Hernandez-Hierro et al., 2014) in model experiments. Cellulose can bind anthocyanins by hydrophobic interactions, albeit less compared to pectin or as a complex with pectin. (Padayachee et al., 2012) Hemicellulose has a similarly low affinity toward anthocyanins as cellulose. (Le Bourvellec et al., 2005) Under the acidic conditions in the must (pH 3.3-3.5), anthocyanins exist in an equilibrium between the positively charged flavylium cation, their hemiketal form and quinoidal base, (Fernandes et al., 2020) which limits the formation of hydrophobic interactions with cellulose or hemicellulose but improves interactions with pectin, which was the main polysaccharide in the extracts. Free carboxy groups of pectic polysaccharides with $pk_a$ 3-4 (Celus et al., 2018) are likely deprotonated in this environment, thereby enhancing ionic interactions with the flavylium cation. The coiled structure of pectin molecules further retains anthocyanins in hydrophilic or hydrophobic areas. (Le Bourvellec et al., 2004) These interactions can either lead to the formation of insoluble complexes or stabilize anthocyanins. (Larsen et al., 2019) Since the structure of pectin, in particular after enzymatic modification, influences the interaction with anthocyanins, Cabernet Sauvignon polysaccharides and especially pectin might favor the formation of insoluble anthocyanin complexes, while in Pinot noir wines stabilization and precipitation of anthocyanins is more balanced. Besides, the added polysaccharides were not

necessarily a mixture of individual molecules but might have also contained aggregates of cellulose and pectin fragments still attached as they are within the cell wall. These aggregates might have a different affinity for anthocyanins compared to individual polysaccharides, as results from Padayachee et al. (2012) suggest. With a higher polysaccharide maturity, the effect of the interactions on anthocyanin concentrations increased in Cabernet Sauvignon musts. During berry ripening, cell wall polysaccharides change constantly regarding concentration and composition.(Nunan et al., 1998; Vicens et al., 2009) The increasing activity of degrading enzymes changes the individual molecular structure, modifies side chains, and releases fragments of the polymers.(Deytieux-Belleau et al., 2008; Nunan et al., 2001) One of the effects, a decrease of pectin's degree of methylation, (Barnavon et al., 2001; Ortega-Regules et al., 2008) has been reported to increase the binding capacity for anthocyanins. (Fernandes et al., 2020) Accordingly, the interaction of anthocyanins with cell wall polysaccharides during fermentation increases in the course of berry ripening. In Cabernet Sauvignon wines, this resulted in a progressively reduced anthocyanin concentration when more mature polysaccharides were added. In Pinot noir wines, where the addition of differently mature Pinot noir polysaccharides did not change anthocyanin concentrations, it possibly still affected binding affinity for anthocyanins. However, stabilizing effects and anthocyanin precipitation remained in equilibrium. Since changes to the size distribution of water-soluble polysaccharides increased the proportion of larger polysaccharides during berry ripening, it is likely that these increase in size further affects the binding affinity. The higher proportion of the large polysaccharides in the more mature polysaccharide extracts presumably led to an increased precipitation of the anthocyanins in Cabernet Sauvignon wines. Interestingly, the interactions did not immediately lead to a color deviation measured as L*C*h* and calculated as delta E between the wines with added polysaccharides and those without. Therefore, the interactions might form anthocyanin-polysaccharide complexes that still adsorb at 520 nm in the protein precipitation assay. On the other hand, the visual appearance of Cabernet Sauvignon wines was not altered although anthocyanins were precipitated. These effects might potentially improve long-term color stability of the wines.

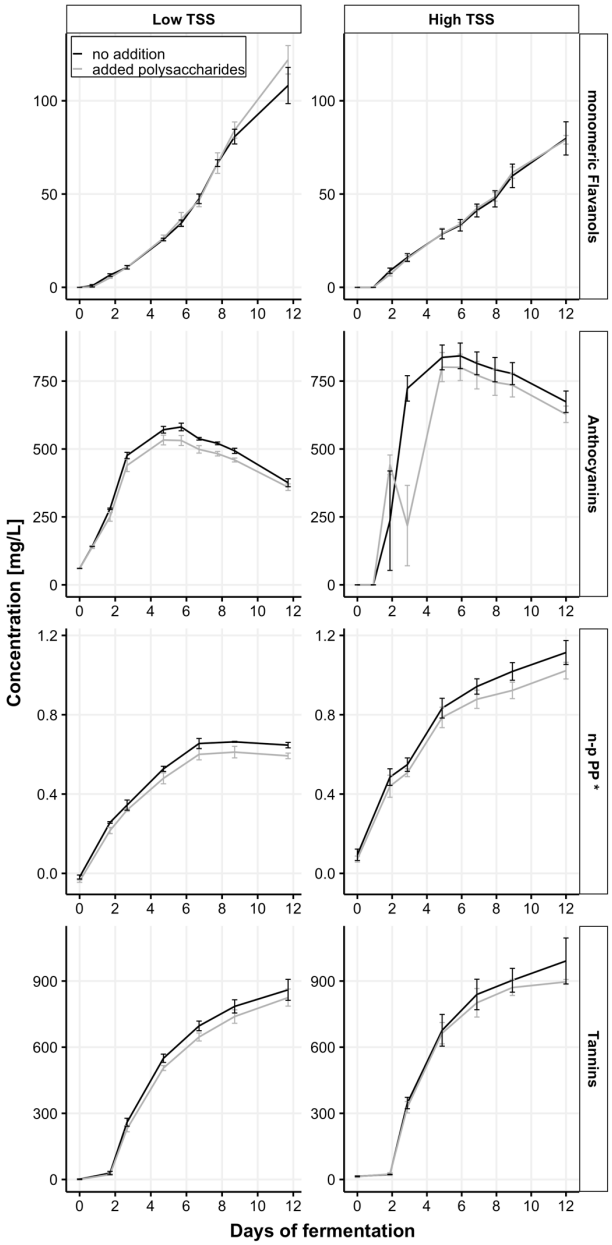



**Figure 2.4** Polyphenol concentrations in Cabernet Sauvignon wines made from grapes with a low and high concentration in TSS with the addition of mixed grape cell wall polysaccharides (means presented with standard deviation; n = 3). Added grape cell wall polysaccharides were a combination of polysaccharide extracts from Pinot noir and Cabernet Sauvignon berries harvested at three different levels of maturity. (* non-precipitable PP in absorption units) (low TSS = 17.1 °Bx, high TSS = 21.8 °Bx)

Tannin concentrations were influenced even stronger by cell wall polysaccharide interactions than anthocyanin concentrations were. In Cabernet Sauvignon wines, the addition of polysaccharides decreased tannin concentrations while in Pinot noir wines, tannin concentrations increased compared to the control wines. The effects were more pronounced when the polysaccharides had a higher maturity, partly due to the already discussed structural

changes of polysaccharides during ripening. Additionally, the decreasing proportion of galacturonic acid and glucose in the polysaccharide extracts in combination with an increasing xylose might enhance the effects of interactions of polysaccharides on the tannin concentration. The opposing effects of the interactions comparing both varieties can derive from the specific polysaccharide composition and structure of the cell wall and/or the tannin structure. Slightly larger polysaccharides in extracts from Cabernet Sauvignon that contain a higher proportion of galacturonic acid, rhamnose and arabinose possibly create structures that are more likely to precipitate tannins. As described, pectin within the added cell wall polysaccharides binds tannins by hydrophobic interactions and hydrogen bonds, (Hanlin et al., 2010; Le Bourvellec et al., 2005; Ruiz-Garcia et al., 2014) whereby structural differences can widely influence selectivity and binding capacity. (Watrelot et al., 2014) Watrelot et al. (2014) have reported that interactions between pectic polysaccharides and tannins with a low mean degree of polymerization (mDP) are driven by hydrophobic interactions. Grape tannin mDP varies depending on vintage, (Chira et al., 2011) ripeness, (Gil et al., 2012) and variety, (Mattivi et al., 2009) but is partly in the range that favors the formation of hydrophobic interactions with pectin. These presumably hydrophobic interactions lead to the precipitation of tannins in Cabernet Sauvignon wines as it has been shown by Bindon et al. (2016) in model experiments. These mechanisms, that have been described specifically for pectin, can also be applied to cell wall material in general, as it has been suggested in multiple publications.(Bautista-Ortin et al., 2016; Castro-Lopez Ldel et al., 2016; Hanlin et al., 2010; Osete-Alcaraz et al., 2019) Cellulose has been reported to have a much lower binding capacity for proanthocyanidins compared to pectic polysaccharides (Ruiz-Garcia et al., 2014) and loosely binds tannins through hydrophobic interaction. (Le Bourvellec and Renard, 2012) However, it is not clear if cellulose contributes to the marked precipitation of tannins during winemaking. As part of the added polysaccharides, Cabernet Sauvignon cellulose might have precipitated tannins in the presented results. Besides tannin precipitation, an additional effect that reduces tannin concentration can be assumed within the analytical determination of tannins. Pectic polysaccharides reportedly hinder the formation of insoluble complexes between procyanidins and bovine serum albumin used in photometric assays. (Mateus et al., 2004) Additionally, polysaccharides increase the solubility of tannins in aqueous solutions by encapsulation, (Watrelot et al., 2017) which concomitantly prohibits interactions with other molecules. (Le Bourvellec and Renard, 2012) Since tannin concentration was measured by protein precipitation, preventing this precipitation by polysaccharide interactions reduces measurable tannin concentration as well. This will

nevertheless change the sensorial characteristics of the wine although tannin is not actually precipitated by the added polysaccharides, as has been shown by Osete-Alcaraz et al. (2019).

The increase in tannin concentration by the polysaccharide addition to Pinot noir wines (Figure 2.3) is clearly not a result of tannin creation but rather of an elevated extraction or increased stabilization of extracted tannins. Similarly to the Cabernet Sauvignon wines, multiple interactions of the added polysaccharides with either polyphenols or other molecules in the must are possible and increase the tannin concentration compared to the control sample. Stabilization of tannins through interactions with grape polysaccharides has been described in the literature, (Osete-Alcaraz et al., 2020) but it can also lead to precipitation as described before. The balance between stabilization and precipitation is partly determined by the tannins' mDP.(Bindon et al., 2016; Osete-Alcaraz et al., 2019) Tannin stabilization results from inhibition of multiple reactions that would otherwise cause tannin degradation or precipitation. Tannin self-aggregation as one reported cause of precipitation (Riou et al., 2002) is inhibited by blocking the necessary hydrogen bonds or hydrophobic interactions by cell wall polysaccharides which occupy the same binding sites to interact with tannins.(Riou et al., 2002; Watrelot et al., 2017) Highly branched pectin molecules with multiple hydrophobic zones however improve the formation of aggregates (Riou et al., 2002) that might precipitate. The specific pectic polysaccharides that were extracted from Pinot noir grapes apparently block tannin aggregation and increase solubility and do not lead to precipitation. The combination of Pinot noir tannins that might favor stabilization and polysaccharides with a structure that improve solubility led to the observed higher tannin concentration in the Pinot noir wines with added polysaccharides compared to the control wine without added polysaccharides. Grape proteins further contribute to tannin precipitation during winemaking.(Bindon et al., 2016; Watrelot et al., 2017) Within the cell wall, proteins have no impact on tannin extractability (Le Bourvellec et al., 2012) but yeast and grape proteins once released from cell structures adsorb and precipitate tannins during maceration.(Mekoue Nguela et al., 2016; Springer et al., 2016) The added polysaccharides might be of further benefit by inhibiting this tannin-protein adsorption. For different proteins, the disruptive effect of polysaccharides on protein-tannin aggregation was reported to be more effective with procyanidins that have a low mDP. (Mateus et al., 2004) The potential structural differences between Pinot noir and Cabernet Sauvignon tannins might have again protected Pinot noir tannins in the presented results. The influence on protein-tannin interactions aside, there is evidence that an increased concentration in soluble polysaccharides in the must increases the release of tannins from the grape cells into the wine. Solubilized polysaccharides compete for the interaction with tannins with polysaccharides that

are still integrated in the cell wall, which reportedly leads to an enhanced release of tannins into the must. (Osete-Alcaraz et al., 2020) This desorption might be driven by the higher affinity of tannins for the solubilized polysaccharides.(Bindon and Kennedy, 2011; Bindon et al., 2010a) Since only Pinot noir tannin concentration increased by the polysaccharide addition, these effects seem specific for the distinct combination of tannin and polysaccharide structures in Pinot noir wines. All the described interactions with proteins, other polysaccharides, and tannin molecules obviously co-exist to increase the measured tannin concentration. Although the general interactions are similar in both varieties, the protective interactions apparently outweigh simultaneous deleterious effects in Pinot noir compared to Cabernet Sauvignon wines. In the beginning of fermentation, the increased release of tannin molecules from the cells is presumably more important, while protection of the extracted tannins stabilizes the achieved tannin concentration in the must afterwards.

Epicatechin and catechin concentrations were affected only by cell wall polysaccharide addition in Pinot noir wines. The added polysaccharides increased monomeric flavanol concentrations significantly whereby the impact grew with an increased polysaccharide maturity. Similar to the observed effects of polysaccharide interactions on tannin concentration, monomeric flavanols are possibly protected from reactions with other molecules in the Pinot noir must. The added polysaccharides might hinder reactions of monomeric flavanols with other polyphenols like anthocyanins, thus preserve the initial monomeric flavanol concentration. Apparently, the specific structure and composition of Pinot noir polysaccharides, which led to enhanced interactions with tannins, is also responsible for monomeric flavanol stabilization. Noteworthy, these protective effects occur despite the much higher monomeric flavanol concentration in Pinot noir. Since an increase in catechin and epicatechin concentrations is not necessarily sensorial desirable, wines might however not directly benefit from this effect.

Non-precipitable PP concentrations were only influenced in Cabernet Sauvignon wines, where their concentrations decreased by the addition of polysaccharides. This might be connected to the decreased anthocyanin concentration, which was also only present in Cabernet Sauvignon wines. Since anthocyanins are the crucial component in polymeric pigments, their reduced concentration might limit the formation of non-precipitable PP during fermentation. This would limit the formation of all polymeric pigments in the wine in a long term, thus affecting color and color stability of the Cabernet Sauvignon wines.

Whenever polysaccharide addition had any measurable influence on the analyzed polyphenols, this effect was always stronger when the polysaccharides had a higher maturity. The differences were not significant in every case, but the results clearly highlight that the effect

strength depends on the different polysaccharides with different maturity levels. It has been reported for Cabernet Sauvignon and Shiraz varieties that the affinity of cell wall material to tannin increases during ripening. (Bindon et al., 2014b) Considering the presented results, the general impact of grape cell wall polysaccharides on the concentrations of multiple polyphenols increases with a higher maturity. This increased effect, which is observed when polysaccharides from more mature grapes were added, could be to some extent the result of the increased size of water-soluble polysaccharides in these extracts. Enzymatic depolymerization of cell wall polysaccharides has already been shown to reduce the binding affinity of proanthocyanidins, which conversely suggests that larger polysaccharides from more mature polysaccharide extracts have a higher binding affinity. (Bindon et al., 2016)

### *3.4 Impact of grape ripeness on the interaction between polyphenols and added polysaccharides*

A mixture of grape cell wall polysaccharides was added to must from Pinot noir and Cabernet Sauvignon grapes that were harvested with low and high concentrations of TSS. This experiment aimed to assess the impact of berry ripeness on polyphenol cell wall polysaccharide interactions. Since grape polyphenols, the cellular structure, and the molecular composition changes during ripening, grape ripeness can influence the interactions between polyphenols and the added cell wall polysaccharides. The addition of the mixed cell wall polysaccharides to differently ripe berries affected polyphenol extraction much lesser compared to the addition of differently matured polysaccharides described above. In this set of experiments, only monomeric flavanol concentration was significantly increased in Pinot noir wines (Figure 2.4). The effect was more pronounced in must from grapes with a high concentration in TSS. This might be the result of protective effects that prevent reactions with other polyphenols as described above. Specifically, the formation of flavanol-anthocyanin oligomers might be inhibited. While the anthocyanin concentrations in the berries increase during ripening, monomeric flavanol concentrations remain constant. In the control sample without polysaccharide addition, this might enhance the formation of flavanol-anthocyanin polymers in wines made from grapes with a high concentration of TSS. Added polysaccharides might inhibit these reactions, thereby preserve the initial monomeric flavanol concentration to some extent. Since the polymerization of monomeric flavanols and anthocyanins is crucial for long-term color stability of red wines, the polysaccharide interactions might reduce the perceived quality of the wine.

The addition of the polysaccharide mixture slightly reduced anthocyanin concentrations in Cabernet Sauvignon wines (Figure 2.5) and Pinot noir wines (Figure 2.4) with a high concentration in TSS but only at certain stages of fermentation. The increasing ethanol concentration in the wine might attenuate the interactions by the end of fermentation, thereby releasing anthocyanins from initially formed complexes as it has been shown by Medina-Plaza et al. (2019). Interestingly, the mixture of polysaccharides affected anthocyanin concentrations of Pinot noir wines, but the addition of distinct Pinot noir polysaccharides did not (see above). This hints at specific structural features of Cabernet Sauvignon polysaccharides in the polysaccharide mixture that lead to anthocyanin precipitation. The slightly higher galacturonic acid, arabinose and rhamnose concentrations in the polysaccharide extracts from Cabernet Sauvignon combined with their larger size, might contribute to the precipitation of anthocyanins. The compositional differences of the monosaccharides hint at potentially more highly branched pectin in Cabernet Sauvignon polysaccharides extracts compared to Pinot noir. This structural difference may benefit the precipitation of anthocyanins. The additional polysaccharides had however no influence on tannin concentrations in both varieties, which again suggests that specific structural features of the polysaccharides are necessary for measurable effects. Although tannins are subjected to changes of their structure, concentration, and composition during ripening (Bautista-Ortín et al., 2012; Harbertson et al., 2002; Kyraleou et al., 2017) that all affect interactions with polysaccharides, measurable changes of the tannin concentration remained absent. Combined with the afore mentioned results of the first experimental setup, differences in polysaccharide structure may have an even greater impact on tannin extractability than changes of the tannin composition during ripening. The overall reduced measurable effects of the added mixed polysaccharides in these experiments compared to the addition of varietal specific, differently mature polysaccharides on polyphenol concentrations suggest a strong impact of a varietal specific polysaccharide composition on the phenolic composition of the wines. Hemicellulose proportions in the polysaccharide extracts differed between varieties, which hints at a possible impact hemicellulose has on the observed interactions with polyphenols. Despite the fact that hemicellulose having a lower affinity for direct interactions with polyphenols (Le Bourvellec et al., 2005), it can be assumed that the hemicellulose in the added extracts, had an impact on the formation of polysaccharide polyphenol complexes in general. Additionally, the different size distributions of the polysaccharides among both varieties might have had an important impact on the specific interactions observed. Polysaccharides in extracts from Pinot noir had a higher proportion in small polysaccharides and a smaller proportion of large polysaccharides compared to extracts

from Cabernet Sauvignon. A mixture of both would level the specific size distribution, possibly limiting the interactions with the varietal specific polyphenol composition.

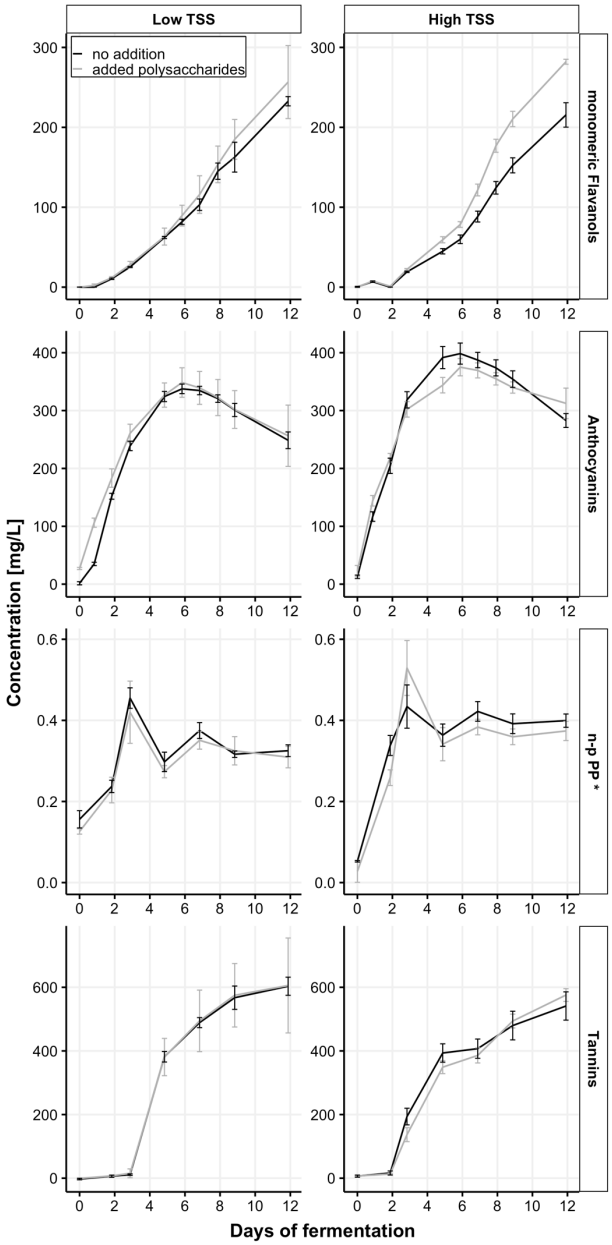



**Figure 2.5** Polyphenol concentrations in Pinot noir wines made from grapes with a low and high concentration in TSS with the addition of grape cell wall polysaccharides (means presented with standard deviation; n = 3). (* non-precipitable PP in absorption units) (low TSS = 16.7 °Bx, high TSS = 21.6 °Bx)

### *3.5 Combined effects of polysaccharide maturity and grape ripeness on the interactions between polyphenols and added polysaccharides*

Principal component analyses were performed with data obtained after fermentation to distinguish the actual effects of grape ripeness and polysaccharide maturity on polyphenol concentrations in the resulting wines (Figure 2.6 and Figure 2.7). Within the varietal specific biplots, samples are grouped by grape ripeness where each group includes the wines with polysaccharide addition and the control wines without polysaccharides. Cabernet Sauvignon wines made from differently ripe grapes can be differentiated along the first principal component. Within these groups, the wines are distributed along the second principal component. Regarding Pinot noir wines, the effects between and within the groups are related similarly but are not aligned with the principal components. Since grape ripeness and polysaccharide maturity effects are orthogonally positioned in both varieties, a clear difference between both factors is revealed. For both varieties, the separation by grape ripeness is positively correlated with the anthocyanin and non-precipitable PP concentration. The distribution within the groups correlates positively with the tannin concentration. Therefore, berry ripeness might impact anthocyanin and non-precipitable PP concentrations in the produced wines while tannin concentration or extractability might depend on polysaccharide interactions. Grape ripeness and extractability are however strongly connected. The impact of the added polysaccharides was determined by either berry ripeness or polysaccharide maturity. Changes of the polysaccharides during grape ripening increased the effects gradually, revealing a strong influence of polysaccharide maturity on measurable tannin concentrations. Polysaccharide maturity might be a crucial factor changing especially tannin extractability during grape ripening, while other developments during ripening, possibly the biosynthesis, impact the anthocyanin and non-precipitable PP concentrations.

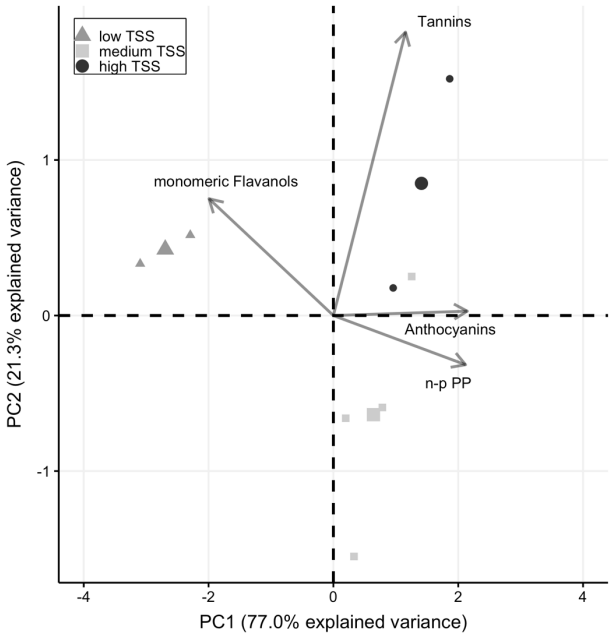



**Figure 2.6** Principal component analysis of the polyphenol concentration in Cabernet Sauvignon wines after 12 days of maceration with and without the addition of grape cell wall polysaccharides. Color and shape indicate berry ripeness at harvest. Enlarged points indicate group means. (low TSS = 17.1 °Bx, medium TSS = 20.9 °Bx, high TSS = 21.8 °Bx)

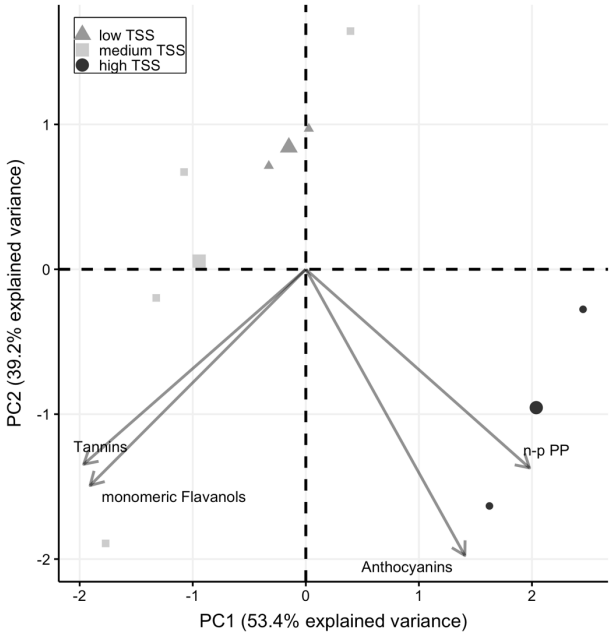



**Figure 2.7** Principal component analysis of the polyphenol concentration in Pinot noir wines after 12 days of maceration with and without the addition of grape cell wall polysaccharides. Color and shape indicate berry ripeness at harvest. Enlarged points indicate group means. (low TSS = 16.7 °Bx, medium TSS = 19.7 °Bx, high TSS = 21.6 °Bx)

Polyphenol extractability during fermentation is a constant balance of adsorption and desorption of polyphenols from cellular structures. Molecular changes of the polysaccharides impact this balance in both directions as demonstrated by the varietal differences. The greater proportion of hemicellulose in the extracts from Pinot noir grapes compared to extracts from Cabernet Sauvignon in combination with a higher proportion of smaller polysaccharides in the water-soluble fraction might improve the polyphenol stabilizing properties of the added polysaccharides. Contrary, larger water-soluble polysaccharides in the Cabernet Sauvignon polysaccharide extracts might have favored the reduced concentrations of tannins. Generally, the pronounced differences between the effects of the added polysaccharides, was not reflected by large differences within the analyzed parameters of the polysaccharides. In addition to these molecular parameters, the ultra-structural properties have been reported to favor aggregate formation by polysaccharides or even bigger cell wall fragments. (Bindon et al., 2016) Synergistic effects and self-association have been reported to further affect cell wall polysaccharide polyphenol interactions.(Le Bourvellec et al., 2012; Ruiz-Garcia et al., 2014)

All the described effects changed the composition of the young wine, but their long-term impact, e.g. on the formation of precipitable PP still needs to be assessed. Even if some of the polyphenol concentrations were not changed within the time frame of the experiment, this might only be the result of balanced interactions that limit future reactions with implications for analytical and sensorial characteristics of the wines.

**Funding**

This research project was financially supported by the German Ministry for Economic Affairs and Energy (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 20024N.

## Supporting information

Significant deviations of polyphenol concentrations between wines made with added polysaccharides and control wines. Representative UHPLC chromatograms of anthocyanin and flavanol measurements in wines with added polysaccharides and in control wines. HPLC-RI chromatograms of monosaccharide composition of cell wall polysaccharide extract from Cabernet Sauvignon and Pinot noir grapes harvested with different levels of maturity.

**Supporting Table 2.1S** Significant deviations of polyphenol concentrations in wines with added varietal polysaccharides extracted from differently ripe Cabernet Sauvignon and Pinot noir grapes in comparison to the control wine without added polysaccharides. Significance was calculated by a one-way ANOVA and Tukey's post-hoc test. (*** $p < 0.001$;** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$)

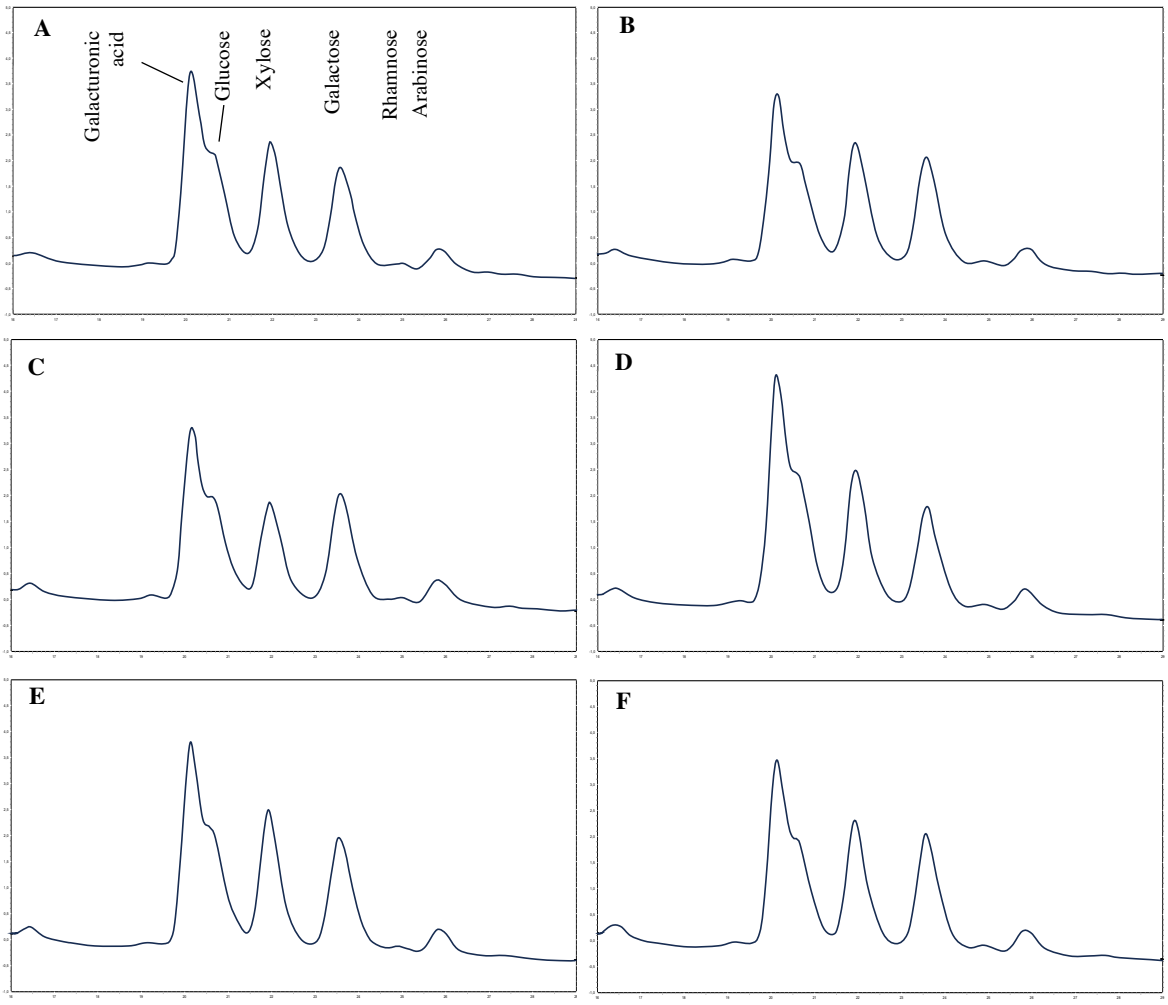
| Variety | Analyte | Day of Fermentation | Maturity level of added polysaccharides | Deviation from control wines [mg/L] |
|---|---|---|---|---|
| Cabernet Sauvignon | tannins | 5 | high | -96.9 ± 4.3* |
| Pinot noir | anthocyanins | 1 | low | 48.1 ± 5.5** |
| | catechin | 2 | low | 2.7 ± 0.4*** |
| | epicatechin | 2 | low | 2.6 ± 0.2*** |
| | catechin | 3 | low | 3.9 ± 0.9** |
| | epicatechin | 3 | low | 3.1 ± 0.5** |
| | catechin | 5 | low | 12.5 ± 1.5*** |
| | epicatechin | 5 | low | 7.6 ± 0.9*** |
| | catechin | 6 | low | 18.4 ± 3.2*** |
| | epicatechin | 6 | low | 11.6 ± 2.0*** |
| | catechin | 7 | low | 22.2 ± 2.9** |
| | epicatechin | 7 | low | 13.7 ± 2.1** |
| | catechin | 8 | low | 29.5 ± 6.0* |
| | epicatechin | 8 | low | 17.9 ± 4.1* |
| | catechin | 9 | low | 55.3 ± 2.8** |
| | epicatechin | 9 | low | 33.3 ± 2.0** |
| | catechin | 12 | low | 55.0 ± 3.9*** |
| | epicatechin | 12 | low | 32.1 ± 2.9** |
| | anthocyanins | 1 | medium | 41.3 ± 6.8** |
| | epicatechin | 2 | medium | 1.2 ± 0.1* |
| | epicatechin | 3 | medium | 1.8 ± 0.7* |
| | catechin | 5 | medium | 8.5 ± 0.7** |

| | | | | |
|---|---|---|---|---|
| | epicatechin | 5 | medium | 5.5 ± 0.3** |
| | catechin | 6 | medium | 16.8 ± 0.9*** |
| | epicatechin | 6 | medium | 10.6 ± 0.6*** |
| | catechin | 7 | medium | 23.1 ± 1.7** |
| | epicatechin | 7 | medium | 14.1 ± 1.8** |
| | catechin | 8 | medium | 31.6 ± 7.1* |
| | epicatechin | 8 | medium | 18.7 ± 4.8* |
| | catechin | 9 | medium | 55.8 ± 7.2** |
| | epicatechin | 9 | medium | 32.8 ± 3.5** |
| | catechin | 12 | medium | 61.0 ± 5.0*** |
| | epicatechin | 12 | medium | 36.6 ± 3.5*** |
| | anthocyanins | 1 | high | 59.9 ± 10.8*** |
| | catechin | 2 | high | 1.8 ± 0.5** |
| | epicatechin | 2 | high | 2.0 ± 0.4** |
| | catechin | 3 | high | 4.3 ± 0.7** |
| | epicatechin | 3 | high | 3.7 ± 0.5*** |
| | catechin | 5 | high | 15.1 ± 0.8*** |
| | epicatechin | 5 | high | 9.7 ± 0.8*** |
| | catechin | 6 | high | 22.7 ± 1.0*** |
| | epicatechin | 6 | high | 15.5 ± 0.6*** |
| | catechin | 7 | high | 32.1 ± 4.0*** |
| | epicatechin | 7 | high | 21.2 ± 3.5*** |
| | catechin | 8 | high | 41.4 ± 5.6** |
| | epicatechin | 8 | high | 25.6 ± 3.5** |
| | catechin | 9 | high | 72.3 ± 6.7** |
| | epicatechin | 9 | high | 46.0 ± 5.4** |
| | tannins | 12 | high | 179.1 ± 21.6** |
| | catechin | 12 | high | 85.8 ± 3.3*** |
| | epicatechin | 12 | high | 53.8± 3.3*** |

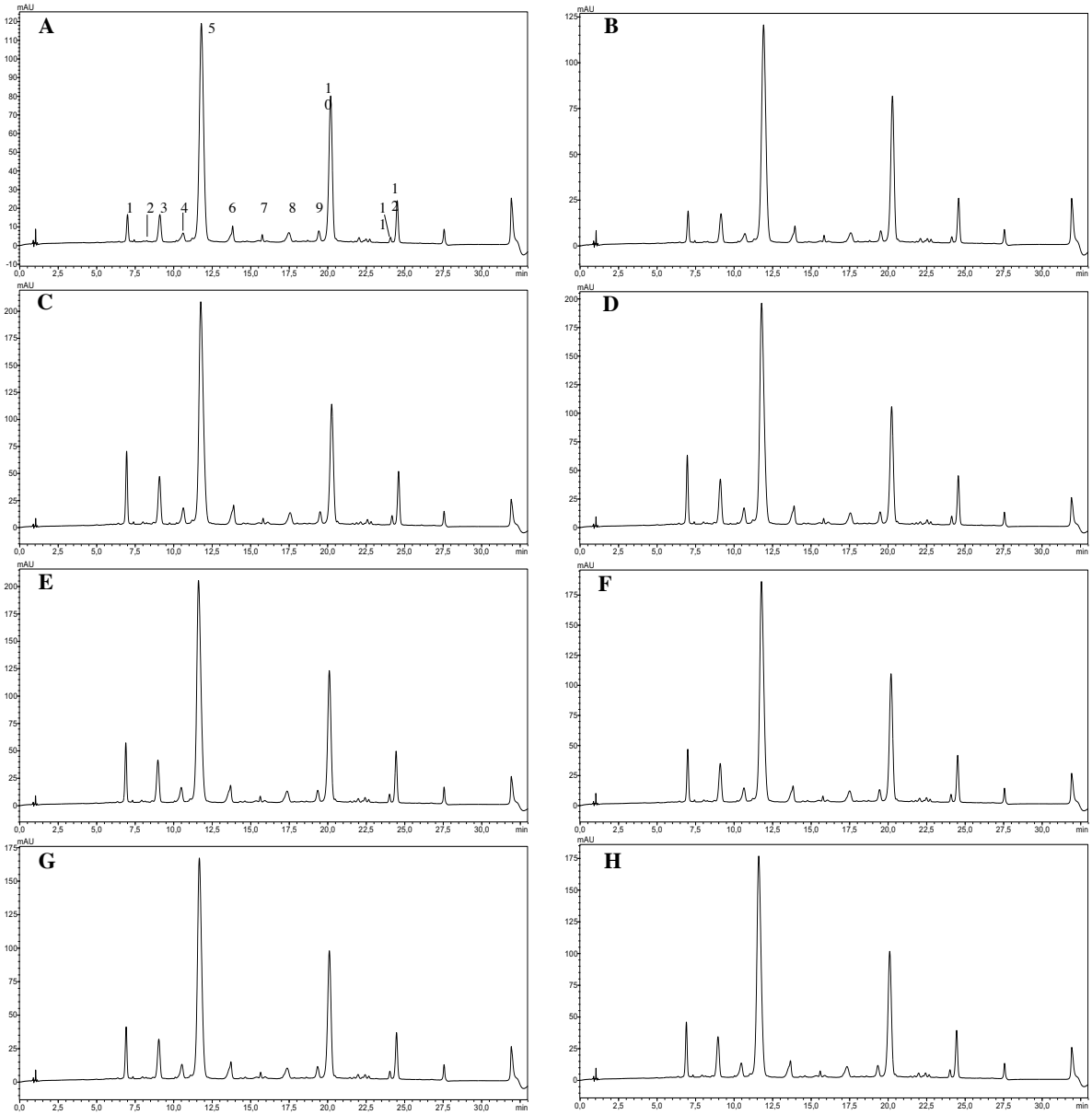
**Supporting Table 2.2S** Significant deviations of polyphenol concentrations in wines made from grapes with different levels of ripeness with the addition of grape polysaccharides in comparison to the control wine without added polysaccharides. Significance was calculated by a one-way ANOVA and Tukey's post-hoc test. (*** $p < 0.001$;** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$)

| Variety | Analyte | Day of Fermentation | TSS concentration in grapes used for winemaking | Deviation from control wines [mg/L] |
|---|---|---|---|---|
| Cabernet Sauvignon | anthocyanins | 6 | low | -49.8 ± 13.4* |
| | anthocyanins | 8 | low | -38.0 ± 5.6* |
| | non-precipitable PP | 9 | low | -0.1 ± 0* |
| | anthocyanins | 3 | high | -505.0 ± 89.6*** |
| Pinot noir | anthocyanins | 0 | low | 25.5 ± 1.9**** |
| | anthocyanins | 1 | low | 71.3 ± 4.8*** |
| | anthocyanins | 1 | high | 27.3 ±7.1** |
| | non-precipitable PP | 2 | high | -0.1 ± 0* |
| | epicatechin | 2 | high | 1.0 ± 0.1* |
| | catechin | 5 | high | 8.4 ± 1.8* |
| | epicatechin | 5 | high | 5.9 ± 1.1* |
| | anthocyanins | 5 | high | -47.7 ± 13.5* |
| | catechin | 6 | high | 11.0 ± 2.3* |
| | epicatechin | 6 | high | 7.5 ± 1.4* |
| | catechin | 7 | high | 20.2 ± 3.5* |
| | epicatechin | 7 | high | 13.0 ± 2.3* |
| | catechin | 8 | high | 33.2 ± 4.4** |
| | epicatechin | 8 | high | 19.4 ± 2.3** |
| | catechin | 9 | high | 36.5 ± 5.0* |
| | epicatechin | 9 | high | 21.5 ± 2.9* |
| | catechin | 12 | high | 42.7 ± 5.4** |
| | epicatechin | 12 | high | 23.9 + 3.8** |

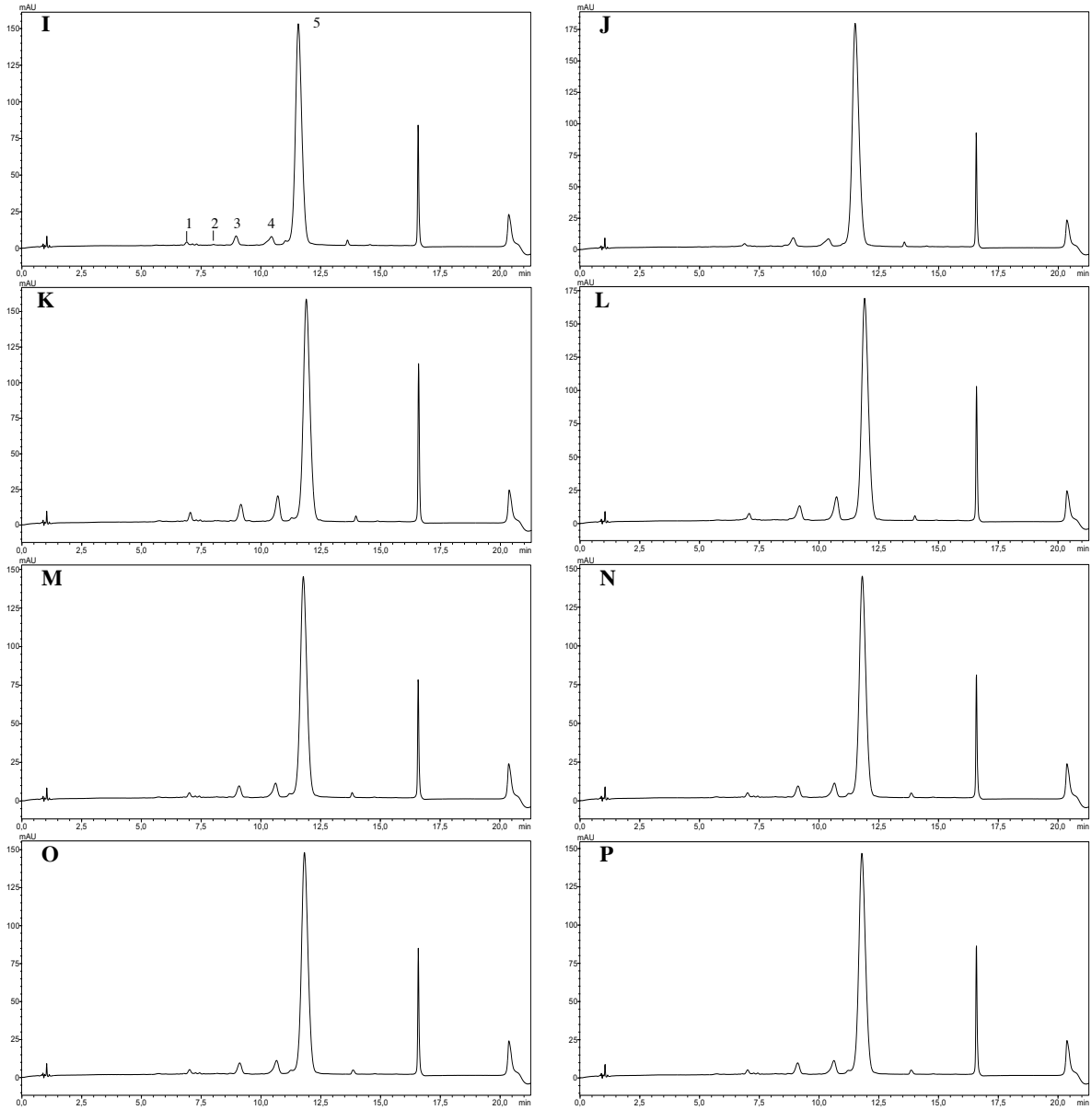
**Supporting Figure 2.1S** Representative chromatograms of monomeric sugars analyzed with HPLC-RI in polysaccharide extracts from Cabernet Sauvignon and Pinot noir grapes harvested with different levels of total soluble solids (TSS). (A: Cabernet Sauvignon TSS 17.6 °Bx, B: Cabernet Sauvignon TSS 21.2 °Bx, C: Cabernet Sauvignon TSS 22.7°Bx, D: Pinot noir TSS 17.6 °Bx, E: Pinot noir TSS 21.6 °Bx, F: Pinot noir TSS 22.6 °Bx)



**Supporting Figure 2.2S** Representative chromatograms of anthocyanins measured with UHPLC-DAD at 520 nm in Cabernet Sauvignon and Pinot noir wines after fermentation for 12 days. Grapes were harvested with different levels of total soluble solids (TSS) and fermented with added grape cell wall polysaccharide extracts from Cabernet Sauvignon and Pinot noir grapes.
(A: Cabernet Sauvignon control TSS 17.1 °Bx, B: Cabernet Sauvignon TSS 17.1 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, C: Cabernet Sauvignon control TSS 21.8 °Bx, D: Cabernet Sauvignon TSS 21.8 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, E: Cabernet Sauvignon control TSS 20.9 °Bx, F: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , G: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , H: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , I: Pinot noir control TSS 16.7 °Bx, J: Pinot noir TSS 16.7 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, K: Pinot noir control TSS 21.6 °Bx, L: Pinot noir TSS 21.6 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, M: Pinot noir control TSS 19.7 °Bx, N: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS , O: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS , P: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS)

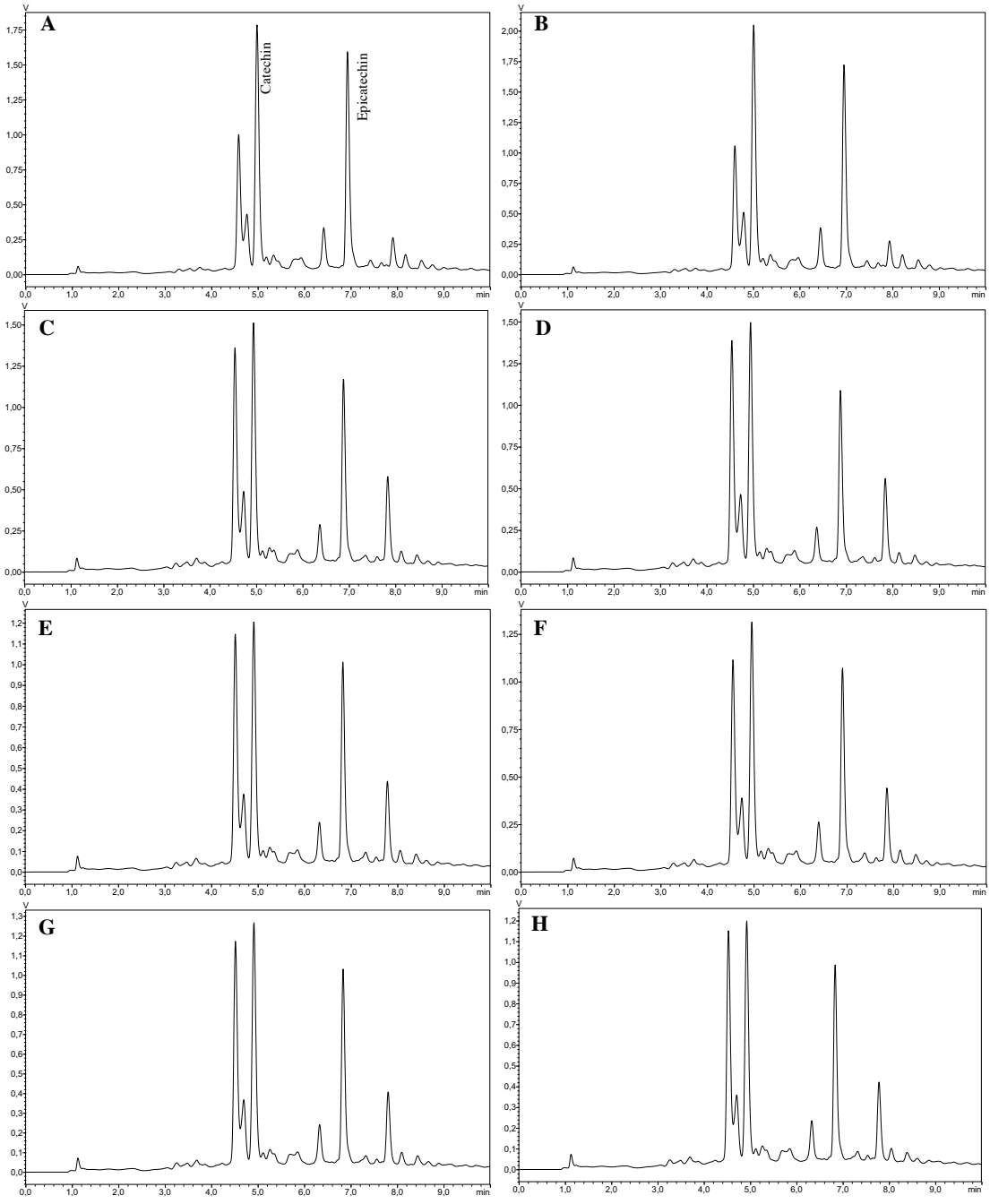


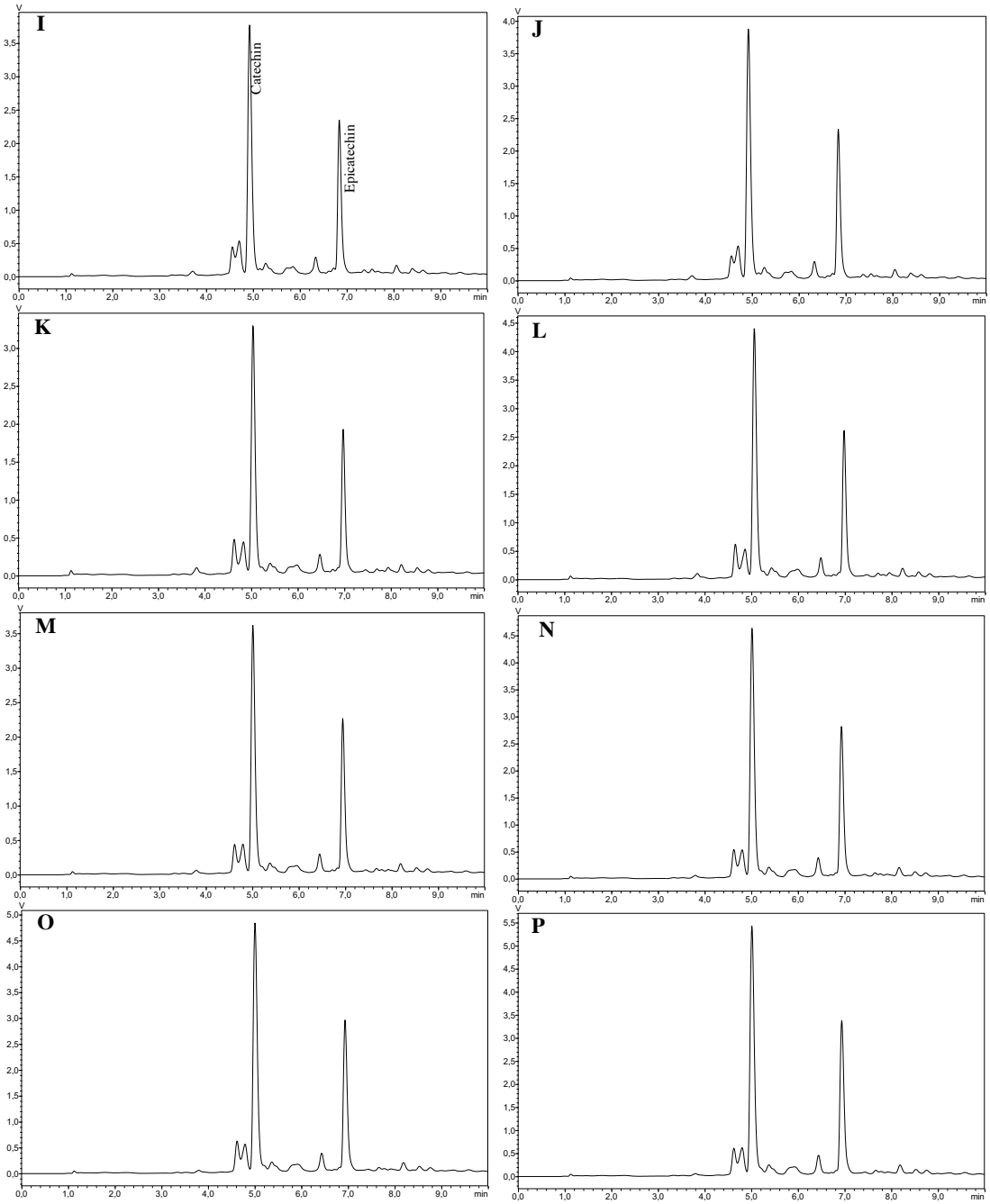

1: delphinidin-3-*O*-glucoside, 2: cyanidin-3-*O*-glucoside, 3: petunidin-3-*O*-glucoside, 4: peonidin-3-*O*-glucoside,
5: malvidin-3-*O*-glucoside, 6: delphinidin-3-*O*-acetylglucoside, 7: cyanidin-3-*O*-acetylglucoside,
8: petunidin-3-*O*-acetylglucoside, 9: peonidin-3-*O*-acetylglucoside, 10: malvidin-3-*O*-acetylglucoside,
11: peonidin-3-*O*-coumaroylglucoside, 12: malvidin-3-*O*-coumaroylglucoside




1: delphinidin-3-*O*-glucoside, 2: cyanidin-3-*O*-glucoside, 3: petunidin-3-*O*-glucoside, 4: peonidin-3-*O*-glucoside, 5: malvidin-3-*O*-glucoside

**Supporting Figure 2.3S** Representative chromatograms of flavanols measured with UHPLC-FLD in Cabernet Sauvignon and Pinot noir wines after fermentation for 12 days. Grapes were harvested with different levels of total soluble solids (TSS) and fermented with added grape cell wall polysaccharide extracts from Cabernet Sauvignon and Pinot noir grapes.(A: Cabernet Sauvignon control TSS 17.1 °Bx, B: Cabernet Sauvignon TSS 17.1 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, C: Cabernet Sauvignon control TSS 21.8 °Bx, D: Cabernet Sauvignon TSS 21.8 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, E: Cabernet Sauvignon control TSS 20.9 °Bx, F: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , G: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , H: Cabernet Sauvignon TSS 20.9 °Bx with added polysaccharides from Cabernet Sauvignon grapes harvested with TSS , I: Pinot noir control TSS 16.7 °Bx, J: Pinot noir TSS 16.7 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, K: Pinot noir control TSS 21.6 °Bx, L: Pinot noir TSS 21.6 °Bx with added mixture of polysaccharides from Cabernet Sauvignon and Pinot noir, M: Pinot noir control TSS 19.7 °Bx, N: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS , O: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS , P: Pinot noir TSS 19.7 °Bx with added polysaccharides from Pinot noir grapes harvested with TSS)




I
Catechin
Epicatechin
J
K
L
M
N
O
P
min

**References**

Adams, D. O., Harbertson, J. F., and Picciotto, E. A. 2004. Fractionation of red wine polymeric pigments by protein precipitation and bisulfite bleaching. *In* Red Wine Color. A.L. Waterhouse and S.E. Ebeler (eds.), pp. 275-288. American Chemical Society, Washington, DC.

Barnavon, L., Doco, T., Terrier, N., Ageorges, A., Romieu, C., and Pellerin, P. 2001. Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. *Phytochemistry*. **58**. 693-701.

Bautista-Ortin, A. B., Ben Abdallah, R., Castro-Lopez Ldel, R., Jimenez-Martinez, M. D., and Gomez-Plaza, E. 2016. Technological implications of modifying the extent of cell wall-proanthocyanidin interactions using enzymes. *Int. J. Mol. Sci.* **17**.

Bautista-Ortín, A. B., Rodríguez-Rodríguez, P., Gil-Muñoz, R., Jiménez-Pasqual, E., Busse-Valverde, N., Martínez-Cutillas, A., López-Roca, J. M., and Gómez-Plaza, E. 2012. Influence of berry ripeness on concentration, qualitative composition and extractability of grape seed tannins. *Aust. J. Grape Wine R.* **18**. 123-130.

Bindon, K. A., Bacic, A., and Kennedy, J. A. 2012. Tissue-specific and developmental modifications of grape cell walls influence the adsorption of proanthocyanidins. *J. Agric. Food. Chem.* **60**. 9249-9260.

Bindon, K. A., Holt, H., Williamson, P. O., Varela, C., Herderich, M., and Francis, I. L. 2014a. Relationships between harvest time and wine composition in Vitis vinifera L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. *Food Chem.* **154**. 90-101.

Bindon, K. A., and Kennedy, J. A. 2011. Ripening-induced changes in grape skin proanthocyanidins modify their interaction with cell walls. *J. Agric. Food. Chem.* **59**. 2696-2707.

Bindon, K. A., Li, S., Kassara, S., and Smith, P. A. 2016. Retention of proanthocyanidin in wine-like solution Is conferred by a dynamic interaction between soluble and insoluble grape cell wall components. *J. Agric. Food. Chem.* **64**. 8406-8419.

Bindon, K. A., Madani, S. H., Pendleton, P., Smith, P. A., and Kennedy, J. A. 2014b. Factors affecting skin tannin extractability in ripening grapes. *J. Agric. Food. Chem.* **62**. 1130-1141.

Bindon, K. A., Smith, P. A., Holt, H., and Kennedy, J. A. 2010a. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. *J. Agric. Food. Chem.* **58**. 10736-10746.

Bindon, K. A., Smith, P. A., and Kennedy, J. A. 2010b. Interaction between grape-derived proanthocyanidins and cell wall material. 1. Effect on proanthocyanidin composition and molecular mass. *J. Agric. Food. Chem.* **58**. 2520-2528.

Castro-Lopez Ldel, R., Gomez-Plaza, E., Ortega-Regules, A., Lozada, D., and Bautista-Ortin, A. B. 2016. Role of cell wall deconstructing enzymes in the proanthocyanidin-cell wall adsorption-desorption phenomena. *Food Chem.* **196**. 526-532.

Celus, M., Kyomugasho, C., Van Loey, A. M., Grauwet, T., and Hendrickx, M. E. 2018. Influence of pectin structural properties on interactions with divalent cations and its associated functionalities. *Compr. Rev. Food. Sci. Food Saf.* **17**. 1576-1594.

Chira, K., Lorrain, B., Ky, I., and Teissedre, P. L. 2011. Tannin composition of cabernet-sauvignon and merlot grapes from the bordeaux area for different vintages (2006 to 2009) and comparison to tannin profile of five 2009 vintage mediterranean grapes varieties. *Molecules*. **16**. 1519-1532.

Deytieux-Belleau, C., Vallet, A., Doneche, B., and Geny, L. 2008. Pectin methylesterase and polygalacturonase in the developing grape skin. *Plant Physiol. Biochem.* **46**. 638-646.

Fernandes, A., Oliveira, J., Fonseca, F., Ferreira-da-Silva, F., Mateus, N., Vincken, J.-P., and Freitas, V. 2020. Molecular binding between anthocyanins and pectic polysaccharides – Unveiling the role of pectic polysaccharides structure. *Food Hydrocoll.* **102**. 105625.

Fournand, D., Vicens, A., Sidhoum, L., Souquet, J. M., Moutounet, M., and Cheynier, V. 2006. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *J. Agric. Food. Chem.* **54**. 7331-7338.

Fügel, R., Carle, R., and Schieber, A. 2004. A novel approach to quality and authenticity control of fruit products using fractionation and characterisation of cell wall polysaccharides. *Food Chem.* **87**. 141-150.

Gao, Y., Zietsman, A. J. J., Vivier, M. A., and Moore, J. P. 2019. Deconstructing wine grape cell walls with enzymes during winemaking: New insights from glycan microarray technology. *Molecules*. **24**.

Gil, M., Kontoudakis, N., Gonzalez, E., Esteruelas, M., Fort, F., Canals, J. M., and Zamora, F. 2012. Influence of grape maturity and maceration length on color, polyphenolic composition, and polysaccharide content of Cabernet Sauvignon and Tempranillo wines. *J. Agric. Food. Chem.* **60**. 7988-8001.

Hanlin, R. L., Hrmova, M., Habertson, J. F., and Downey, M. O. 2010. Review: Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust. J. Grape Wine Res.* **16**. 173-188.

Harbertson, J. F., Kennedy, J. A., and Adams, D. O. 2002. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *Am. J. Enol. Vitic.* **53**. 54-59.

Harbertson, J. F., Mireles, M., and Yu, Y. 2015. Improvement of BSA tannin precipitation assay by reformulation of resuspension buffer. *Am. J. Enol. Vitic.* **66**. 95-99.

Harbertson, J. F., Mireles, M. S., Harwood, E. D., Weller, K. M., and Ross, C. F. 2009. Chemical and sensory effects of saignée, water addition, and extended maceration on high brix must. *Am. J. Enol. Vitic.* **60**. 450-460.

Harbertson, J. F., Picciotto, E. A., and Adams, D. O. 2003. Measurement of polymeric pigments in grape berry extracts and wines using a protein precipitation assay combined with bisulfite bleaching. *Am. J. Enol. Vitic.* **54**. 301-306.

Heffels, P., Weber, F., and Schieber, A. 2015. Influence of Accelerated Solvent Extraction and Ultrasound-Assisted Extraction on the Anthocyanin Profile of Different Vaccinium Species in the Context of Statistical Models for Authentication. *J. Agric. Food. Chem.* **63**. 7532-7538.

Hernandez-Hierro, J. M., Quijada-Morin, N., Martinez-Lapuente, L., Guadalupe, Z., Ayestaran, B., Rivas-Gonzalo, J. C., and Escribano-Bailon, M. T. 2014. Relationship between skin cell wall composition and anthocyanin extractability of *Vitis vinifera* L. cv. Tempranillo at different grape ripeness degree. *Food Chem.* **146**. 41-47.

Kyraleou, M., Kallithraka, S., Theodorou, N., Teissedre, P. L., Kotseridis, Y., and Koundouras, S. 2017. Changes in tannin composition of syrah grape skins and seeds during fruit ripening under contrasting water conditions. *Molecules*. **22**.

Larsen, L. R., Buerschaper, J., Schieber, A., and Weber, F. 2019. Interactions of anthocyanins with pectin and pectin fragments in model solutions. *J. Agric. Food. Chem.* **67**. 9344-9353.

Le Bourvellec, C., Bouchet, B., and Renard, C. M. G. C. 2005. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta, Gen. Subj.* **1725**. 10-18.

Le Bourvellec, C., Guyot, S., and Renard, C. M. 2004. Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta*. **1672**. 192-202.

Le Bourvellec, C., and Renard, C. M. 2012. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.* **52**. 213-248.

Le Bourvellec, C., Watrelot, A. A., Ginies, C., Imberty, A., and Renard, C. M. G. C. 2012. Impact of Processing on the Noncovalent Interactions between Procyanidin and Apple Cell Wall. *J. Agric. Food. Chem.* **60**. 9484-9494.

Liu, X., Le Bourvellec, C., and Renard, C. 2020. Interactions between cell wall polysaccharides and polyphenols: Effect of molecular internal structure. *Compr. Rev. Food Sci. Food Saf.* **19**. 3574-3617.

Mateus, N., Carvalho, E., Luis, C., and de Freitas, V. 2004. Influence of the tannin structure on the disruption effect of carbohydrates on protein-tannin aggregates. *Anal. Chim. Acta.* **513**. 135-140.

Mattivi, F., Vrhovsek, U., Masuero, D., and Trainotti, D. 2009. Differences in the amount and structure of extractable skin and seed tannins amongst red grape varieties. *Aust. J. Grape Wine R.* **15**. 27-35.

Medina-Plaza, C., Beaver, J. W., Lerno, L., Dokoozlian, N., Ponangi, R., Blair, T., Block, D. E., and Oberholster, A. 2019. Impact of temperature, ethanol and cell wall material composition on cell wall-anthocyanin interactions. *Molecules*. **24**.

Medina-Plaza, C., Beaver, J. W., Miller, K. V., Lerno, L., Dokoozlian, N., Ponangi, R., Blair, T., Block, D. E., and Oberholster, A. 2020. Cell wall–anthocyanin interactions during red wine fermentation-like conditions. *Am. J. Enol. Vitic.* **71**. 149-156.

Mekoue Nguela, J., Poncet-Legrand, C., Sieczkowski, N., and Vernhet, A. 2016. Interactions of grape tannins and wine polyphenols with a yeast protein extract, mannoproteins and beta-glucan. *Food Chem.* **210**. 671-682.

Nunan, K. J., Davies, C., Robinson, S. P., and Fincher, G. B. 2001. Expression patterns of cell wall-modifying enzymes during grape berry development. *Planta.* **214**. 257-264.

Nunan, K. J., Sims, I. M., Bacic, A., Robinson, S. P., and Fincher, G. B. 1998. Changes in cell wall composition during ripening of grape berries. *Plant Physiol.* **118**. 783-792.

OIV. 2006. Determination of chromatic characteristics according to CIELab. *Resolution Oeno 1/2006 OIV, Paris, France.*

Ortega-Regules, A., Ros-Garcia, J. M., Bautista-Ortin, A. B., Lopez-Roca, J. M., and Gomez-Plaza, E. 2008. Changes in skin cell wall composition during the maturation of four premium wine grape varieties. *J. Sci. Food Agric.* **88**. 420-428.

Osete-Alcaraz, A., Bautista-Ortin, A. B., and Gomez-Plaza, E. 2019. The role of soluble polysaccharides in tannin-cell wall interactions in model solutions and in wines. *Biomolecules.* **10**. 36.

Osete-Alcaraz, A., Gomez-Plaza, E., Martinez-Perez, P., Weiller, F., Schuckel, J., Willats, W. G. T., Moore, J. P., Ros-Garcia, J. M., and Bautista-Ortin, A. B. 2020. The impact of carbohydrate-active enzymes on mediating cell wall polysaccharide-tannin interactions in a wine-like matrix. *Food R. Int.* **129**.

Padayachee, A., Netzel, G., Netzel, M., Day, L., Zabaras, D., Mikkelsen, D., and Gidley, M. J. 2012. Binding of polyphenols to plant cell wall analogues – Part 1: Anthocyanins. *Food Chem.* **134**. 155-161.

Riou, V., Vernhet, A., Doco, T., and Moutounet, M. 2002. Aggregation of grape seed tannins in model wine - effect of wine polysaccharides. *Food Hydrocoll.* **16**. 17-23.

Ruiz-Garcia, Y., Smith, P. A., and Bindon, K. A. 2014. Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls. *Carbohydr. Polym.* **114**. 102-114.

Sommer, S., and Cohen, S. 2018. Comparison of different extraction methods to predict anthocyanin concentration and color characteristics of red wines. *Fermentation.* **4**. 39.

Springer, L. F., Sherwood, R. W., and Sacks, G. L. 2016. Pathogenesis-related proteins limit the retention of condensed tannin additions to red wines. *J. Agric. Food. Chem.* **64**. 1309-1317.

Vicens, A., Fournand, D., Williams, P., Sidhoum, L., Moutounet, M., and Doco, T. 2009. Changes in polysaccharide and protein composition of cell walls in grape berry skin (cv. Shiraz) during ripening and over-ripening. *J. Agric. Food. Chem.* **57**. 2955-2960.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., and Renard, C. M. 2014. Neutral sugar side chains of pectins limit interactions with procyanidins. *Carbohydr. Polym.* **99**. 527-536.

Watrelot, A. A., Schulz, D. L., and Kennedy, J. A. 2017. Wine polysaccharides influence tannin-protein interactions. *Food Hydrocoll.* **63**. 571-579.

Zimdars, S., Schrage, L., Sommer, S., Schieber, A., and Weber, F. 2019. Influence of glutathione on yeast fermentation efficiency under copper stress. *J. Agric. Food. Chem.* **67**. 10913-10920.

# Chapter 3

## Pectin forms polymeric pigments by complexing anthocyanins during red winemaking and ageing

The long-term stability of red wine color depends on the formation of polymeric pigments from anthocyanins. Although there is still a lot of uncertainty about the specific structure of this diverse group of pigments, there is consensus that they are reaction products of anthocyanins and other polyphenols. Interactions between anthocyanins and pectic polysaccharides have been suggested to stabilize anthocyanins. This study explores the impact of such interactions by adding pectin during red winemaking.

The results demonstrate that these interactions induce the formation of additional polymeric pigments which enhance the pigment stability during fermentation and aging. While initial pigment formation is higher in wines with added pectin, a notable proportion of the complexes degrades in the later stages of fermentation. Presumably, tannins form insoluble complexes with pectin, reducing tannin concentration by more than 300 mg/L. Anthocyanin concentrations decrease by over 400 mg/L, and polymeric pigments double.

Anthocyanins that form polymeric pigments with pectic polysaccharides expand the range of pigments in red wines with possible consequences for the sensory properties of the wine. These findings highlight the complex interactions between pectin, anthocyanins, and tannins, and their influence on pigment formation and wine composition during fermentation and aging.

**Keywords**: pectin interactions, polymeric pigments, anthocyanins, tannins, fermentation, wine aging, wine composition.

---

Reprinted with permission from:

Hensen, J.-P., Hoening, F., Bogdanovic, T., Schieber, A., and Weber, F. 2024. Pectin forms polymeric pigments by complexing anthocyanins during red winemaking and ageing. *Food Res. Int.* **188**. 114442. DOI: 10.1016/j.foodres.2024.114442.

## 1 Introduction

Anthocyanins extracted from grape skins during winemaking are fundamentally responsible for the color of red wine. Beginning immediately after extraction and continuing during maturation of the wine, anthocyanins undergo numerous reactions with other grape constituents or microbial metabolites, which results in color changes and color stabilization (de Freitas et al., 2017; Weber and Schieber, 2023). One major route is the condensation with different other polyphenols like flavanols to form covalent polymeric pigments (Harbertson et al., 2003). Despite their great impact on red wines, there is still considerable uncertainty regarding the structure of these compounds. A second but less investigated pigment-forming route is the interaction of anthocyanins with grape cell wall polysaccharides. They are extracted during maceration and degraded and modified during the whole winemaking process. Although polyphenol-polysaccharide interactions have extensively been investigated, especially in wines, their role in the formation of pigments has not yet been addressed (Bindon et al., 2016; Bindon et al., 2014; Bindon et al., 2010a; Bindon et al., 2010b; Hensen et al., 2022; Le Bourvellec et al., 2005; Le Bourvellec and Renard, 2012; Le Bourvellec et al., 2012; Liu et al., 2020; Mekoue Nguela et al., 2016; Osete-Alcaraz et al., 2020; Renard et al., 2001). Since anthocyanins exist in a pH-dependent equilibrium between the positively charged flavylium cation and their hemiketal form, the pH value of the medium determines the mechanisms and extent of interactions with polysaccharides, particularly with pectin. The latter, as the major polysaccharide involved, carries carboxylic acid groups which can be deprotonated at higher pH. Wines and musts commonly have pH values between 3.0 and 4.0, which leads to various interaction mechanisms based on ionic, hydrophobic, and hydrophilic forces (Celus et al., 2018; Fernandes et al., 2020). Additionally, the coiled structure of pectin molecules can enclose anthocyanins in hydrophilic or hydrophobic pockets (Le Bourvellec et al., 2004).

All polymeric pigments show considerable light absorption around 520 nm and are more or less resistant against bisulfite bleaching and higher pH, which is why they are crucial for the long-term color stability of aging red wines. However, their structural diversity inhibits proper analytical characterization even with mass spectrometry or other advanced analytical methods (Cheynier et al., 2006). Quantification methods based on protein precipitation and bisulfite bleaching have been suggested as feasible alternative methods and are well-established in research and wineries (Harbertson et al., 2002; Harbertson et al., 2003). These methods rely on common physico-chemical characteristics that are typical of (Weilack et al., 2021) but not limited to polymeric pigments consisting only of polyphenols. They differentiate between protein-precipitable and non-precipitable polymeric pigments, so far referred to as large and

small polymeric pigments, respectively. This designation is somewhat misleading, however, as the molecular size does not seem to be the decisive difference (Weilack et al., 2021). Non-precipitable polymeric pigments are usually the only polymeric pigments found in musts and young wines. Only during the subsequent wine ageing, anthocyanins polymerize to form precipitable polymeric pigments. Although the protein precipitation method has been used to quantify wine pigments, its strength lies in detecting compounds with the same phenotype. Without identifying the full molecular structure, the measured pigments share similar characteristics and are grouped together as polymeric pigments.

Complexes formed by interactions between pectin and anthocyanins stabilize or precipitate anthocyanins depending on the various pectin structures (Larsen et al., 2019). Stable anthocyanin-pectin complexes have been reported to have similar characteristics as polymeric pigments formed exclusively from polyphenols, as they absorb at 520 nm and are stable against bisulfite bleaching (Graves and Sommer, 2021). With similar color and stability characteristics, pectin-anthocyanin complexes might be as crucial for long-term color stability in red wines as covalent polymeric pigments.

It can be hypothesized that pectin facilitates the formation of stable pectin-anthocyanin complexes and that this effect can be revealed by comparing the development of pigments in red wines with additional pectin and non-treated wines. The investigation aims to assess the stability of these complexes throughout the aging of the wines, anticipating that their presence will contribute to enhanced color longevity.

## 2 Material and methods

### *2.1 Chemicals*

Acetic acid, ethanol (100%), hydrochloric acid (HCl) (37%), potassium bisulfite (97.4%), and acetonitrile (100%) were purchased from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide was obtained from Honeywell International Inc. (Seelze, Germany). Sodium chloride was acquired from neoFroxx GmbH (Einhausen, Germany). Triethanolamine (≥ 98%), ferric chloride (97%), L-(+)-tartaric acid, and maleic acid (98%) were purchased from Alfa Aesar (Kandel, Germany). Gallic acid (> 98%) was bought from Fluka Chemie AG (Buchs, Switzerland). Urea (≥ 99.5%), bovine serum albumin fraction V (≥ 98%), and (+)-catechin ≥98% were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium azide (≥ 99%) was obtained from Merck KGaA (Darmstadt, Germany).

Formic acid (≥ 98%) was sourced from Sigma Aldrich Chemie (Steinheim, Germany). Potassium pyrosulfate (food grade) was purchased from RWA Raiffeisen Ware Austria AG (Vienna, Austria). Potassium bicarbonate (food grade) was bought from Erbslöh Geisenheim AG (Geisenheim, Germany). Ultrapure water was obtained from a PURELAB flex 2 water purification system (ELGA 90LabWater, Paris, France).

### *2.2 Grape samples*

'Pinot noir' and 'Cabernet Sauvignon' grapes for winemaking were sampled in 2020 with a low, medium, or high concentration of total soluble solids (TSS) measured with a digital refractometer (PAL-α ATAGO CO. LTD., Tokyo, Japan) (Table 3.1). The geodetic coordinates of the variety-specific vineyards in Neustadt an der Weinstrasse (Palatinate, Germany) were 49°23'53.3"N 8°11'02.0"E ('Cabernet Sauvignon') and 49°22'14.3"N 8°10'56.3"E ('Pinot noir'). 'Cabernet Sauvignon' (clone 1Gm on Binova 1Opp rootstock) was planted in 2008 with a vine by row spacing of 2.00 m × 1.20 m and 'Pinot noir' (clone Mariafeld on SO4 rootstock) was planted in1987 with a vine by row spacing of 1.88 m × 1.20 m. After picking, grapes were chilled instantly at 7 °C for 12 h until used for winemaking.

**Table 3.1** Total soluble solids (TSS) and titratable acid (TA) concentrations, and pH of grape samples used for winemaking

| Variety | TSS[°Bx] | TA [g/L] | pH | Harvest Date |
|---|---|---|---|---|
| 'Cabernet Sauvignon' | 17.1 | 10.5 | 3.3 | 09-01-2020 |
| (CS) | 20.9 | 7.3 | 3.6 | 09-15-2020 |
| | 21.8 | 6.6 | 3.7 | 09-28-2020 |
| 'Pinot noir' | 16.7 | 13.3 | 3.0 | 08-18-2020 |
| (PN) | 19.7 | 8.4 | 3.3 | 09-01-2020 |
| | 21.6 | 7.3 | 3.4 | 09-15-2020 |

### *2.3 Winemaking*

A standard winemaking protocol was used to prepare 'Cabernet Sauvignon' and 'Pinot noir' wines according to Hensen et al. (2022). The pH of the must from all grape samples was adjusted to 3.5 through the addition of potassium bicarbonate or tartaric acid. Commercial apple pectin (Classic AU 202, 69% degree of esterification and 75 g/100 g galacturonic acid, kindly provided by Herbstreith & Fox GmbH & Co. KG, Neuenbürg, Germany) was added at a concentration of 0.5% (w/w) to must. A control wine was made accordingly but without the addition of pectin. The potential alcohol of each wine was adjusted by the addition of sucrose. 'Cabernet Sauvignon' and 'Pinot noir' musts were fermented in 5 L cylindric plastic containers in 1.6 kg batches in triplicate. Fermentation containers were sealed with a lid equipped with a fermentation lock filled with potassium sulfate solution. Fermentation lasted for 12 days at 22 °C, during which containers were shaken three times daily. Samples (10 mL) were taken daily through a septum. Total soluble solids were measured with the refractometer PAL-α (ATAGO CO. LTD., Tokyo, Japan). Samples were centrifuged at 18,500*g* for 10 min and filtered through 0.2 µm syringe filters before further analysis.

After fermentation, wines were manually pressed through a mesh, filled in half-liter brown glass bottles with 200 mg/L sulfur dioxide, and stored for 12 months at 17 ±1 °C.

### *2.4 Analysis of anthocyanins by UHPLC-DAD*

Anthocyanins (delphinidin-*3-O*-glucoside, cyanidin-*3-O*-glucoside, peonidin-*3-O*-glucoside, petunidin-*3-O*-glucoside, malvidin-*3-O*-glucoside, delphinidin-*3-O*-acetylglucoside, cyanidin-*3-O*-acetylglucoside, peonidin-*3-O*-acetylglucoside, petunidin-*3-O*-acetylglucoside, malvidin-*3-O*-acetylglucoside, delphinidin-*3-O-p*-coumaroylglucoside, cyanidin-*3-O-p*-coumaroylglucoside, peonidin-*3-O-p*-coumaroylglucoside, petunidin-*3-O-p*-coumaroylglucoside, and malvidin-*3-O-p*-coumaroylglucoside) concentrations were measured according to Hensen et al. (2022) using a Nexera X2 UPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler SIL-30AC thermostatted to 10 °C, column oven CTO-20AC set at 40 °C equipped with a Kinetex C18 100 A, 1.7 µm, 150 × 2.1 mm column (Phenomenex Inc., Torrance, CA), and a DAD SPD-20A. water/formic acid (97/3; v/v) and acetonitrile/formic acid (97/3; v/v) were used as eluents A and B with a flow rate of 0.4 mL $min^{-1}$ and the following gradient: 0 min, 4.0% B; 2 min, 6.5% B; 5 min, 10.0% B; 6 min, 10.5% B; 7 min, 11.0% B; 11 min, 12.5% B; 13 min, 14.0% B; 15 min, 15.0% B; 26 min, 26.0% B;

26.1 min, 100.0% B; 30 min, 100.0% B; 30.1 min, 4.0% B; 33 min, 4.0% B. The injection volume was 5 µL. Anthocyanins were detected at 520 nm. Malvidin-*3*-*O*-glucoside chloride ≥ 95% (PHYTOPLAN Diehm & Neuberger GmbH, Heidelberg, Germany) was used for quantification and identification. Analytes were identified by their elution order.

### *2.5 Chemical characterization of tannin and polymeric pigment concentrations*

The method proposed by Harbertson et al. (2003) was used for tannins and polymeric pigments measurements with a modified resuspension buffer (urea 8.3 M, 5% TEA, pH 7 adjusted with HCl) (Harbertson et al., 2015). Based on an external calibration curve, tannins were expressed as catechin equivalents (CE).

### *2.6 Spectrophotometric analysis of color differences between samples*

The color of the young wines before bottling and after one year of aging was analyzed according to Weilack et al. (2021). Absorbance spectra of the wine samples were recorded between 300 and 800 nm in a 1 mm path-length glass cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) with a Jasco V-730 double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany). $L^*$, $a^*$, and $b^*$ were calculated with Spectra Manager Ver. 2.14G (JASCO Deutschland GmbH, Pfungstadt, Germany) after correcting the measurements to a 10 mm path length according to OIV suggestions (OIV, 2006).

### *2.7 Statistical analysis*

R (Version 4.1.3) with R-studio (Version 2022.02.3) and packages dplyr (Version 2.1.1), ggplot2 (Version 3.3.5), lemon (Version 0.4.5), rstatix (Version 0.7.0), stat (Version 3.6.0), and tidyr (Version 1.0.2) were used for statistical analysis and graphical illustrations. For pairwise comparison, ANOVA and Tukey's post hoc tests with a significance level of $p < 0.05$ were used.

## 3 Results and discussion

As expected, only non-precipitable polymeric pigments were detected during fermentation in all samples. Formation of precipitable polymeric pigments usually begins with wine aging. They are typically not found in grapes and musts (Graves and Sommer, 2021). Concentrations of non-precipitable polymeric pigments differed largely between musts with added pectin and the corresponding control (Figure 3.1). Pectin generally increased the concentration of non-precipitable polymeric pigments. Their highest concentrations were measured between days two and five of fermentation and declined afterwards. In contrast, anthocyanin concentrations were lower compared to the control during the first five days of fermentation due to the added pectin. The observed increase in non-precipitable polymeric pigment concentrations in all samples coincided with lower anthocyanin concentrations. This suggests a reciprocal relationship between the stability and extraction of anthocyanin (Hensen et al., 2022) and the formation of new pigments. It has been shown that anthocyanins and pectin interact and form rather stable and soluble complexes (Larsen et al., 2019), which would be considered as non-precipitable polymeric pigments in wine if the interaction prevented bisulfite bleaching. As a result of this complex formation, the concentration of free anthocyanins decreases. The increase in their concentration due to ongoing extraction compensates for the decline in anthocyanins, leading to a dynamic equilibrium between extraction, complexation, and derivatization. However, after five days of fermentation, pectin-enriched musts had similar concentrations of anthocyanins and non-precipitable polymeric pigments as the control samples. This suggests that most formed complexes were stable only under the initial conditions of fermentation including pH and ethanol concentration. During this stage, anthocyanins are typically extracted (Gao et al., 2019) simultaneously to cell wall polysaccharides and other polyphenols in the control. However, musts with added pectin contained an excess of pectin, which led to a substantial disparity in anthocyanin concentrations between control musts and pectin-enriched musts (Figure 3.2). Furthermore, there was limited competition with other polyphenols, which further facilitated anthocyanin interaction with pectin. After this initial stage, weaker complexes may be susceptible to breaking or precipitation as the ethanol concentration increased. Toward the end of fermentation, ethanol can attenuate the interactions between pectin and anthocyanins, resulting in the release of anthocyanins from the initially formed complexes, as demonstrated by Medina-Plaza et al. (2019). A small but still significant part of pigments formed by pectin interactions were resistant to further modifications or degradation until the end of fermentation, as their concentration persists throughout fermentation.

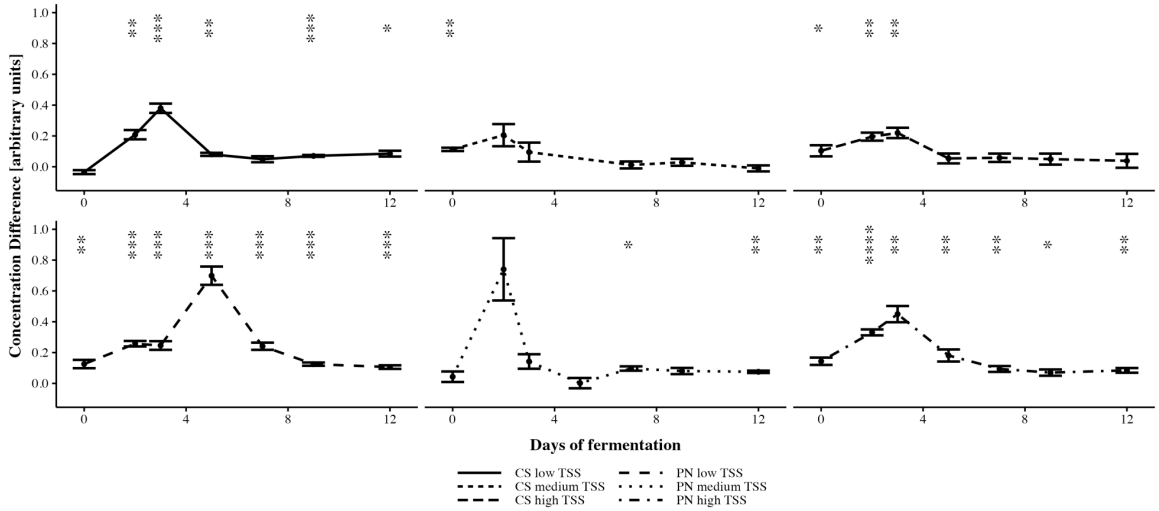



**Figure 3.1** Impact of pectin addition to fermenting musts on the concentration of non-precipitable polymeric pigments at different fermentation stages. A positive concentration difference shows an increase in non-precipitable polymeric pigment concentrations compared to fermenting musts without additional pectin. Means presented with standard error of means (n=3). * indicates significant concentration differences between control and pectin-enriched musts [(*) = $p$<0.05, (**) = $p$<0.01, (***) = $p$<0.001, (****) = $p$<0.0001].

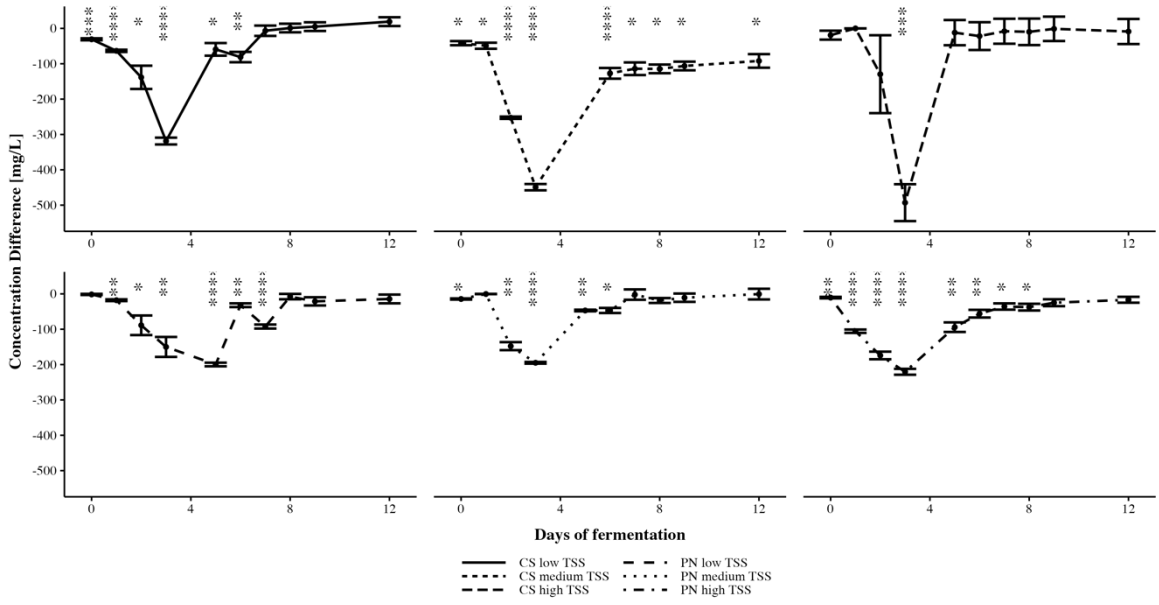



**Figure 3.2** Impact of pectin addition to fermenting musts on the concentration of anthocyanins at different fermentation stages. A negative concentration difference shows a decrease in anthocyanin concentrations compared to fermenting musts without additional pectin. Means presented with standard error of means (n=3). * indicates significant concentration differences between control and pectin-enriched musts [(*) = $p$<0.05, (**) = $p$<0.01, (***) = $p$<0.001, (****) = $p$<0.0001].

As reported by Graves and Sommer (2021), excess pectin in wines can increase the concentration of polymeric pigments. The researchers concluded that anthocyanins interact with pectins by forming stable aggregates resistant to bisulfite bleaching and protein precipitation. On the other hand, pectin can form insoluble complexes with anthocyanins, as shown by Larsen et al. (2019) depending on the pectin structure. The findings revealed that the changes in the concentrations of anthocyanins and non-precipitable polymeric pigments were generally similar, suggesting that the formation of soluble complexes between pectin and anthocyanins was likely more favored than precipitation in this specific experiment. However, the vastly changing concentrations of non-precipitable polymeric pigments during fermentation suggest diverse interactions between anthocyanins and pectin. These pigments include a range of complexes that vary in stability. However, only complexes that are stable can be attributed to polymeric pigments. As the results indicate, pectin forms stable complexes with anthocyanins, that share characteristics of polymeric pigments formed by the polymerization of polyphenols. Combining the present findings with those from recent publications (Graves and Sommer, 2021; Hensen et al., 2022; Weilack et al., 2023), the established concept of polymeric pigments needs to be expanded to include polymeric pigments formed through pectin-anthocyanin interactions. As for now, the quantification of polymeric pigments combines pigments formed through pectin interactions with all other pigments. Whether there is a necessity to apply new methodologies that differentiate the different types of polymeric pigments needs to be assessed.

### *3.1 Interaction effects of pectin on anthocyanin concentrations*

‘Cabernet Sauvignon’ and ‘Pinot noir’ wines contain 3-*O*-glucosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin, whereas ‘Cabernet Sauvignon’ additionally contains the acetyl- and coumaroyl-glucosides. The added pectin significantly changed the concentrations of individual anthocyanins depending on the B-ring substitution ($p<0.05$) (supporting information Figure 3.2S). Due to methoxy groups on the B-ring, anthocyanins interact with the added pectin via hydrophobic interactions (Larsen et al., 2019). Larsen et al. (2019) reported that hydrophobic interactions with pectin stabilize anthocyanins. Although the authors did not measure non-precipitable polymeric pigment concentrations, it is conceivable that stabilization is achieved through the formation of soluble pigments, as seen in the present study. The concentration of delphinidin-*3*-*O*-glucoside, bearing three hydroxy groups on the B-ring, was similarly reduced by pectin interactions. Previous studies indicated that anthocyanins

featuring more hydroxy groups on the B-ring exhibit a stronger affinity for interactions with pectin, which could not be confirmed here (Buchweitz et al., 2013; Fernandes et al., 2014; Larsen et al., 2019). Hydroxy groups located on the B-ring establish hydrogen bonds with pectin, resulting in the formation of stabilized complexes (Larsen et al., 2019). Moreover, the pH value of 3.35 ± 0.15 in the must facilitates the establishment of ionic forces between the positively charged flavylium cation and the negatively charged galacturonic acid. This adds another binding mechanism besides the mentioned hydrophobic and hydrophilic interactions.

Pectin interactions had a significantly lower effect on concentrations of acetylated and coumaroylated anthocyanins. The added pectin reduced coumaroylated anthocyanin concentrations the least. Potential steric hindrances prevented the interaction between the anthocyanin and pectin, while the additional coumaroyl group seemingly does not interact with pectin. Because the coumaroyl group only reduces the interactions between anthocyanins and pectin, the interactions mainly occur with the aglycone. Similarly, Larsen et al. (2019) reported that the B-Ring substitution of the anthocyanin mostly determines the affinity for pectin interactions, rather than the sugar moiety, again showing that interactions might only occur between the aglycone and pectin.

Although pectin reduced individual anthocyanin concentrations differently during fermentation, the observed differences were small toward the end of fermentation. With ethanol concentrations increasing in the must and young wine, polar interactions between anthocyanins and pectin occur much less, reducing the effect of the differently substituted B-ring.

### *3.2 Grape varietal influence on pectin-anthocyanin interactions and pigment formation*

Comparing both varieties, the additional pectin in the must had the same but differently pronounced effects on pigment formation and anthocyanin concentrations. The formation of non-precipitable polymeric pigments from pectin-anthocyanin interactions was slightly higher in ‘Pinot noir’ wines. Anthocyanin concentrations decreased more from pectin interactions in ‘Cabernet Sauvignon’ wines, particularly in wines made from medium mature ‘Cabernet Sauvignon’ grapes. Because this decrease was not accompanied by a comparable increase in non-precipitable polymeric pigment concentration, conditions in ‘Cabernet Sauvignon’ might favor the precipitation of anthocyanins from pectin interactions. Since the must contains all extracted compounds and fragments from the grape, these individual effects obviously reflect varietal differences. Since pH, ethanol, and temperature levels were nearly identical in all wine samples, extracted compounds like polyphenols and grape cell wall polysaccharides apparently

interfered with the interaction of anthocyanins and the added pectin. This accelerated the increased precipitation of anthocyanins in ‘Cabernet Sauvignon’ samples.

Previous research has demonstrated that the interactions between polysaccharides and polyphenols are influenced by the specific composition given by the grape variety (Hensen et al., 2022). Different compounds like cell wall polysaccharides, grape- or yeast-derived proteins, as well as other polyphenols like tannins may affect these interactions and likely cause the different observations made here. The composition of ‘Cabernet Sauvignon’ must enhanced the precipitation of anthocyanin-pectin complexes, whereby the mechanisms causing the varietal-specific effects of anthocyanin-pectin interactions are unknown so far.

### *3.3 Influence of pectin on tannin concentrations and precipitation during fermentation*

Tannins are generally extracted from grape berries with increasing ethanol concentrations during fermentation. After two days of fermentation, a notable increase in tannin concentrations was observed. The addition of pectin to the must reduced tannin concentrations (Figure 3.3). Pectin binds tannins through hydrophobic interactions and hydrogen bonds, depending on the specific molecular structure and the degree of polymerization (Hanlin et al., 2010; Le Bourvellec et al., 2005; Ruiz-Garcia et al., 2014; Watrelot et al., 2014). The formed complexes have been shown to either precipitate or potentially protect tannins (Bindon et al., 2010b; Osete-Alcaraz et al., 2019). Tannins with a higher degree of polymerization have a greater affinity for pectin interactions and tend to precipitate, as shown by Bindon et al. (2016) in model experiments.

The reduced tannin concentration in wines with added pectin suggests that a larger proportion of pectin-tannin complexes precipitated in the presented experiments. After one week of fermentation, a similar increase in tannin concentrations was observed in the control and the pectin-enriched musts. In this final fermentation stage, the tannin concentration gap between control and pectin-enriched musts remained constant. The findings indicate that predominantly those tannins precipitate that are extracted early in the fermentation process. Subsequently, tannin concentrations in all wines changed similarly. During fermentation, skin tannins are typically extracted slightly before seed tannins (González-Manzano et al., 2004). Additionally, skin tannins have the highest degree of polymerization in the berry, facilitating the formation of insoluble complexes with pectin (Downey et al., 2003; Souquet et al., 2000). Both aspects suggest that the gap between tannin concentrations in pectin-enriched wines and control wines is caused by the precipitation of skin tannins through pectin interactions.

Therefore, pectin-enriched wines might contain fewer skin tannins than control wines in the experiments.

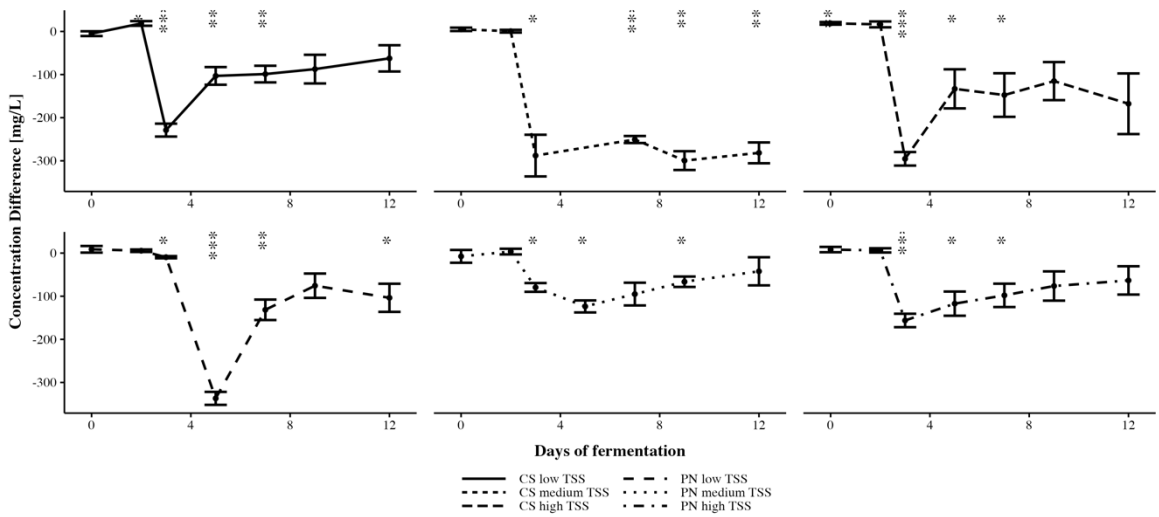



**Figure 3.3** Impact of pectin addition to fermenting musts on the concentration of tannins at different fermentation stages. A negative concentration difference shows a decrease in tannin concentrations compared to fermenting musts without additional pectin. Means presented with standard error of means (n=3). * indicates significant concentration differences between control and pectin-enriched musts [(*) = $p$<0.05, (**) = $p$<0.01, (***) = $p$<0.001, (****) = $p$<0.0001].

Tannins extracted in the final stage of fermentation either formed soluble complexes or could not interact with the added pectin due to its limited availability as it is already occupied by anthocyanins or earlier extracted tannins. As seen for all analytes, differences in the phenolic composition between pectin-enriched and control musts were more pronounced during the first six days of fermentation. Afterward, the impact of pectin interactions was mitigated, likely due to a large proportion of the added pectin being already precipitated or bound in stable soluble complexes. Multiple publications have shown that cell wall polysaccharides can form complexes with tannins, which prevent tannin precipitation during winemaking (Hensen et al., 2022; Osete-Alcaraz et al., 2019; Watrelot et al., 2017). These effects mostly derive from mechanisms preventing polymerization and interactions with proteins that would otherwise precipitate tannins. However, in the present study, pectin causes tannin precipitation rather than preventing it.

### *3.4 Evolution of pectin-polyphenol interactions during wine aging*

During one year of aging, tannin and anthocyanin concentrations decreased, and precipitable and non-precipitable polymeric pigment concentrations increased in most wines. The impact of pectin interactions on polyphenol concentrations was remarkably similar to the effects observed in young wines before filling. Added pectin further reduced the concentrations of tannins and anthocyanins and increased non-precipitable polymeric pigment concentrations (Figure 3.4). There was a high variability of their concentrations among samples that limits the determination of significant differences. The broad range of potential reactions during the aging process is one major source of this variation. Because the composition of the young wine was already altered by pectin interactions during fermentation, reactions during wine aging further increased the differences among samples.

Despite the high variability of samples, the findings outline a similar aging process of wines. Although the added pectin changed the wine composition during fermentation, pectin seems to impact wine composition during subsequent aging only indirectly. Given the lower tannin and anthocyanin concentrations of pectin-enriched wines, the initial composition after filling leaves fewer possibilities for further polymerization reactions that usually occur during wine aging. This seems to be the case in particular for precipitable polymeric pigments arising from the reaction of tannins and anthocyanins. In pectin-enriched wines, the concentrations of precipitable polymeric pigments were lower compared to control wines.

Anthocyanin concentrations were similarly affected in all wines by the added pectin during aging (Figure 3.4 and Figure 3.3S in the supporting information). After one year of aging, anthocyanin concentrations in wines with added pectin were slightly lower compared to control wines, although most differences were not significant. Changes in the anthocyanin concentration caused by pectin interactions during fermentation are preserved throughout aging, but no further impact of pectin can be determined. It can be assumed that anthocyanin concentrations are reduced more by the various degradation and polymerization reactions than non-covalent interactions with pectin (Cheynier et al., 2006). The concentrations of non-precipitable pigments were also still slightly higher in pectin-enriched wines compared to the control. Given this persistent increase in non-precipitable polymeric pigment concentrations in pectin-enriched wines, it can be hypothesized that pectin interactions offer long-term stabilization for anthocyanins through this mechanism.

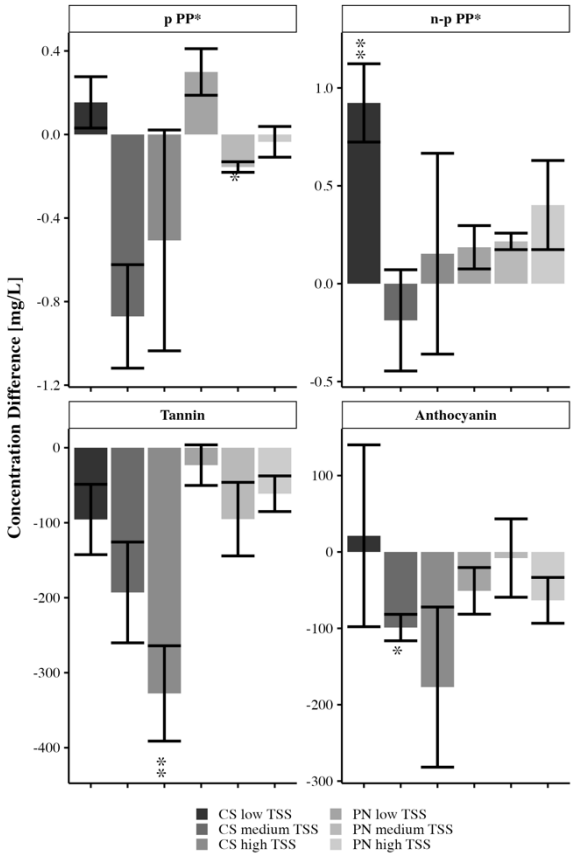



**Figure 3.4** Impact of pectin addition to fermenting musts on the concentration of polyphenols in wines after 12 months aging. A negative concentration difference shows a decrease in polyphenol concentrations compared to wines without additional pectin. Means presented with standard error of means (n=3). * indicates significant concentration differences between control and pectin-enriched wines [(*) = $p$<0.05, (**) = $p$<0.01]. (*Precipitable polymeric pigments (p PP) and non-precipitable polymeric pigments (n-p PP) in absorption units)

Pectin formed a significant amount of insoluble complexes with tannins during fermentation, leading to lower concentrations in the wine after fermentation. During aging, tannin concentrations decreased in all wines caused by oxidation or reactions with other polyphenols and wine compounds (Smith et al., 2015). The control wine of 'Pinot noir' showed a stronger reduction in tannin concentrations than the 'Cabernet Sauvignon', leading to similar tannin concentrations in control and pectin-enriched wines. This implies that the remaining tannins in 'Pinot noir' wines after fermentation are better protected in pectin-enriched wines. It has previously been reported that tannins can be stabilized by polysaccharide interactions (Bindon et al., 2016; Hensen et al., 2022; Osete-Alcaraz et al., 2019). 'Pinot noir' tannins seem to be particularly protected by polysaccharide interactions (Hensen et al., 2022). In 'Cabernet Sauvignon' wines, the added pectin further reduced tannin concentrations during aging. Tannins in high TSS 'Cabernet Sauvignon' wines still formed insoluble complexes with the additional pectin or were influenced by the changed wine composition after fermentation.

Because of the complex nature of reactions and interactions during wine aging, there are plenty of possible pathways by which high pectin concentrations in ‘Cabernet Sauvignon’ must reduce tannin concentrations.

All results combined outline a supposedly positive impact of pectin on red wine composition and properties. With their capability to interact with anthocyanins, pectin increases the concentration of stable non-precipitable polymeric pigments with a potentially positive effect on color stability. Despite this potential, the measured color changes in the samples were found to be insignificant. In young wines the positive impact of an increased pigment formation is obscured by the abundance of anthocyanins, that mainly contribute to the color (Table S1). During aging, different samples developed extremely different. The added pectin had to some extent a stabilizing effect to the natural variability of changes during aging, but overall, this effect was still stronger than any impact by the formation of pigments from pectin.

Although only a small proportion of these non-precipitable polymeric pigments is sufficiently stable to withstand changes during fermentation, they still contribute significantly to the total pigments in wines. In contrast to these stabilizing interactions, pectin precipitated tannins and some anthocyanins, which limited the formation of precipitable polymeric pigments during wine aging. Noteworthy, most of the pectin interactions occurred during fermentation, and effects after filling were generally smaller. The reduced tannin concentration might result in a less astringent mouthfeel, which would need further confirmation. Moreover, polysaccharides play a crucial role in fostering the creation of ternary complexes involving polyphenols and proteins. (Sommer et al., 2019) It is possible that similar ternary complexes might be formed between polysaccharides, anthocyanins, and tannins, although this hypothesis necessitates validation in forthcoming experiments, which should include investigation of the role of ternary complexes in the wine’s sensory properties.

During winemaking, cell wall polysaccharides, in particular pectic polysaccharides, are extracted together with polyphenols from grapes. Only recently, Weilack et al. (2023) demonstrated that grape-derived pectic polysaccharides form pigments with anthocyanins. Together with the present results, it can be assumed that grape pectin forms non-precipitable polymeric pigments in red wines, however to a different extent depending on the grape variety. Especially in wines with high concentrations of polysaccharides, the formation of stable polymeric pigments from pectin and anthocyanins will improve color stability and pigment concentration.

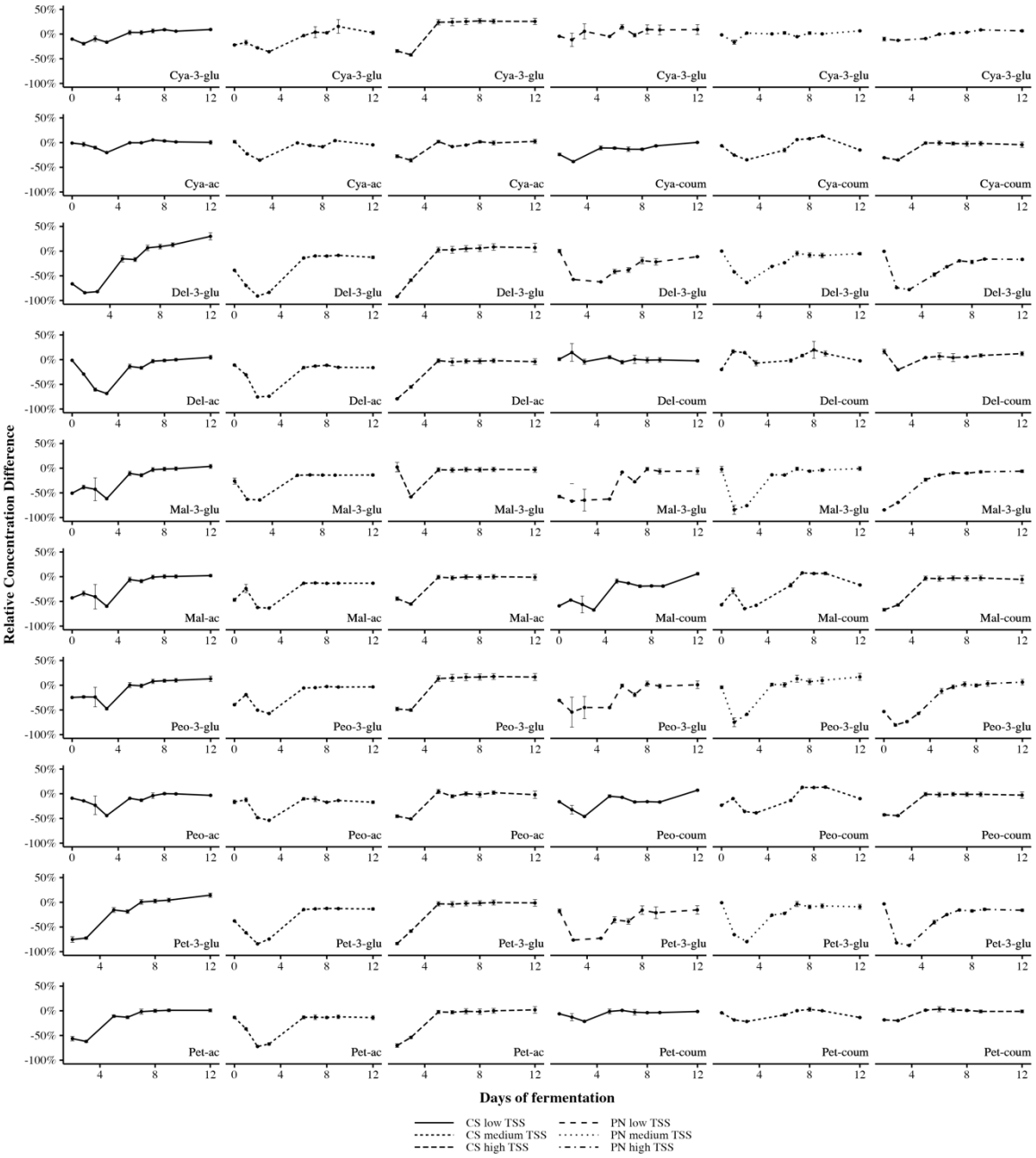
## 4 Conclusions

The presented findings expand the established understanding of structural diversity of polymeric wine pigments. Precipitable and non-precipitable polymeric pigments formed through anthocyanin polymerization with flavanols or tannins must be complemented by pectin-anthocyanin complexes. Regardless of their origin, all compounds comprise similar physico-chemical characteristics that are used for quantification. The current limited diversification of analytes in the assay used for quantification might be overcome by changes in the sample handling. Besides this analytical challenge, the completely different structure of these polymeric pigments might evoke changes in sensory properties, which should be explored further.

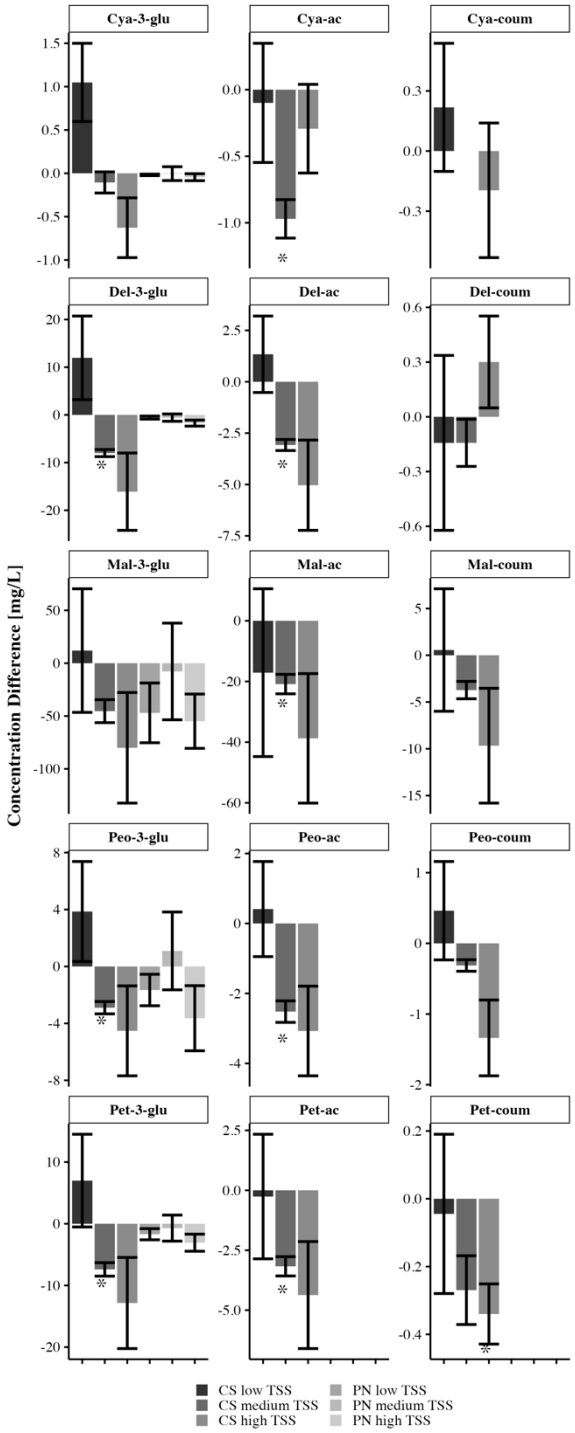
## Funding

This research project was financially supported by the German Ministry for Economic Affairs and Energy (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 20024N.

## Supporting information




**Supporting Figure 3.1S** Effect of pectin interactions on concentrations of delphinidin-*3-O*-glucoside (Del-3-glu), cyanidin-*3-O*-glucoside (Cya-3-glu), peonidin-*3-O*-glucoside (Peo-3-glu), petunidin-*3-O*-glucoside (Pet-3-glu), malvidin-*3-O*-glucoside (Mal-3-glu), delphinidin-*3-O*-acetylglucoside (Del-ac), cyanidin-*3-O*-acetylglucoside (Cya-ac), peonidin-*3-O*-acetylglucoside (Peo-ac), petunidin-*3-O*-acetylglucoside, (Pet-ac) malvidin-*3-O*-acetylglucoside (Mal-ac), delphinidin-*3-O-p*-coumaroylglucoside (Del-coum), cyanidin-*3-O-p*-coumaroylglucoside (Cya-coum), peonidin-*3-O-p*-coumaroylglucoside (Peo-coum), petunidin-*3-O-p*-coumaroylglucoside (Pet-coum), and malvidin-*3-O-p*-coumaroylglucoside (Mal-coum)**.** A negative relative concentration difference shows a decrease in concentrations compared to fermenting musts without additional pectin. Means presented with standard deviation (n=3).




**Supporting Figure 3.2S** Effect of pectin addition to fermenting musts on anthocyanin concentrations in wines after 12 months aging. A negative concentration difference shows a decrease in polyphenol concentrations compared to wines without additional pectin. Means presented with standard error of means (n=3). * indicates significant concentration differences between control and pectin-enriched wines [(*) = $p<0.05$, (**) = $p<0.01$]. For abbreviations see Figure 3.4.

**References**

Bindon, K. A., Li, S., Kassara, S., and Smith, P. A. 2016. Retention of proanthocyanidin in wine-like solution Is conferred by a dynamic interaction between soluble and insoluble grape cell wall components. *J. Agric. Food. Chem.* **64**. 8406-8419.

Bindon, K. A., Madani, S. H., Pendleton, P., Smith, P. A., and Kennedy, J. A. 2014. Factors affecting skin tannin extractability in ripening grapes. *J. Agric. Food. Chem.* **62**. 1130-1141.

Bindon, K. A., Smith, P. A., Holt, H., and Kennedy, J. A. 2010a. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. *J. Agric. Food. Chem.* **58**. 10736-10746.

Bindon, K. A., Smith, P. A., and Kennedy, J. A. 2010b. Interaction between grape-derived proanthocyanidins and cell wall material. 1. Effect on proanthocyanidin composition and molecular mass. *J. Agric. Food. Chem.* **58**. 2520-2528.

Buchweitz, M., Speth, M., Kammerer, D. R., and Carle, R. 2013. Stabilisation of strawberry (Fragaria x ananassa Duch.) anthocyanins by different pectins. *Food Chem.* **141**. 2998-3006.

Celus, M., Kyomugasho, C., Van Loey, A. M., Grauwet, T., and Hendrickx, M. E. 2018. Influence of pectin structural properties on interactions with divalent cations and its associated functionalities. *Compr. Rev. Food. Sci. Food Saf.* **17**. 1576-1594.

Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J. M., Sarni-Manchado, P., and Fulcrand, H. 2006. Structure and properties of wine pigments and tannins. *Am. J. Enol. Vitic.* **57**. 298-305.

de Freitas, V. A. P., Fernandes, A., Oliveira, J., Teixeira, N., and Mateus, N. 2017. A review of the current knowledge of red wine colour. *OENO One*. **51**.

Downey, M. O., Harvey, J. S., and Robinson, S. P. 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine R.* **9**. 15-27.

Fernandes, A., Bras, N. F., Mateus, N., and de Freitas, V. 2014. Understanding the molecular mechanism of anthocyanin binding to pectin. *Langmuir*. **30**. 8516-8527.

Fernandes, P. A. R., Oliveira, J., Fonseca, F., Ferreira-da-Silva, F., Mateus, N., Vincken, J. P., and Freitas, V. 2020. Molecular binding between anthocyanins and pectic polysaccharides – Unveiling the role of pectic polysaccharides structure. *Food Hydrocoll.* **102**. 105625.

Gao, Y., Zietsman, A. J. J., Vivier, M. A., and Moore, J. P. 2019. Deconstructing wine grape cell walls with enzymes during winemaking: New insights from glycan microarray technology. *Molecules*. **24**.

González-Manzano, S., Rivas-Gonzalo, J. C., and Santos-Buelga, C. 2004. Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Anal. Chim. Acta*. **513**. 283-289.

Graves, J., and Sommer, S. 2021. Polysaccharides Influence the Results of Polymeric Pigment Analysis in Red Wines. *Food Sci. Technol.* **1**. 1770-1775.

Hanlin, R. L., Hrmova, M., Habertson, J. F., and Downey, M. O. 2010. Review: Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust. J. Grape Wine Res.* **16**. 173-188.

Harbertson, J. F., Kennedy, J. A., and Adams, D. O. 2002. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *Am. J. Enol. Vitic.* **53**. 54-59.

Harbertson, J. F., Mireles, M., and Yu, Y. 2015. Improvement of BSA Tannin Precipitation Assay by Reformulation of Resuspension Buffer. *Am. J. Enol. Vitic.* **66**. 95-99.

Harbertson, J. F., Picciotto, E. A., and Adams, D. O. 2003. Measurement of polymeric pigments in grape berry extracts and wines using a protein precipitation assay combined with bisulfite bleaching. *Am. J. Enol. Vitic.* **54**. 301-306.

Hensen, J. P., Hoening, F., Weilack, I., Damm, S., and Weber, F. 2022. Influence of Grape Cell Wall Polysaccharides on the Extraction of Polyphenols during Fermentation in Microvinifications. *J. Agric. Food. Chem.* **70**. 9117-9131.

Larsen, L. R., Buerschaper, J., Schieber, A., and Weber, F. 2019. Interactions of anthocyanins with pectin and pectin fragments in model solutions. *J. Agric. Food. Chem.* **67**. 9344-9353.

Le Bourvellec, C., Bouchet, B., and Renard, C. M. G. C. 2005. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta, Gen. Subj.* **1725**. 10-18.

Le Bourvellec, C., Guyot, S., and Renard, C. M. 2004. Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta, Gen. Subj.* **1672**. 192-202.

Le Bourvellec, C., and Renard, C. M. 2012. Interactions between polyphenols and macromolecules: quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.* **52**. 213-248.

Le Bourvellec, C., Watrelot, A. A., Ginies, C., Imberty, A., and Renard, C. M. G. C. 2012. Impact of Processing on the Noncovalent Interactions between Procyanidin and Apple Cell Wall. *J. Agric. Food. Chem.* **60**. 9484-9494.

Liu, X., Le Bourvellec, C., and Renard, C. 2020. Interactions between cell wall polysaccharides and polyphenols: Effect of molecular internal structure. *Compr. Rev. Food. Sci. Food Saf.* **19**. 3574-3617.

Medina-Plaza, C., Beaver, J. W., Lerno, L., Dokoozlian, N., Ponangi, R., Blair, T., Block, D. E., and Oberholster, A. 2019. Impact of temperature, ethanol and cell wall material composition on cell wall-anthocyanin interactions. *Molecules*. **24**.

Mekoue Nguela, J., Poncet-Legrand, C., Sieczkowski, N., and Vernhet, A. 2016. Interactions of grape tannins and wine polyphenols with a yeast protein extract, mannoproteins and beta-glucan. *Food Chem.* **210**. 671-682.

OIV. 2006. Determination of chromatic characteristics according to CIELab. *Resolution Oeno 1/2006 OIV, Paris, France*.

Osete-Alcaraz, A., Bautista-Ortin, A. B., and Gomez-Plaza, E. 2019. The role of soluble polysaccharides in tannin-cell wall interactions in model solutions and in wines. *Biomolecules*. **10**. 36.

Osete-Alcaraz, A., Gomez-Plaza, E., Martinez-Perez, P., Weiller, F., Schuckel, J., Willats, W. G. T., Moore, J. P., Ros-Garcia, J. M., and Bautista-Ortin, A. B. 2020. The impact of carbohydrate-active enzymes on mediating cell wall polysaccharide-tannin interactions in a wine-like matrix. *Food R. Int.* **129**.

Renard, C. M. G. C., Baron, A., Guyot, S., and Drilleau, J. F. 2001. Interactions between apple cell walls and native apple polyphenols: quantification and some consequences. *Int. J. Biol. Macromol.* **29**. 115-125.

Ruiz-Garcia, Y., Smith, P. A., and Bindon, K. A. 2014. Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls. *Carbohydr. Polym.* **114**. 102-114.

Smith, P. A., Mcrae, J. M., and Bindon, K. A. 2015. Impact of winemaking practices on the concentration and composition of tannins in red wine. *Aust. J. Grape Wine R.* **21**. 601-614.

Sommer, S., Weber, F., and Harbertson, J. F. 2019. Polyphenol-Protein-Polysaccharide Interactions in the Presence of Carboxymethyl Cellulose (CMC) in Wine-Like Model Systems. *J. Agric. Food. Chem.* **67**. 7428-7434.

Souquet, J. M., Labarbe, B., Le Guerneve, C., Cheynier, V., and Moutounet, M. 2000. Phenolic composition of grape stems. *J. Agric. Food. Chem.* **48**. 1076-1080.

Watrelot, A. A., Le Bourvellec, C., Imberty, A., and Renard, C. M. 2014. Neutral sugar side chains of pectins limit interactions with procyanidins. *Carbohydr. Polym.* **99**. 527-536.

Watrelot, A. A., Schulz, D. L., and Kennedy, J. A. 2017. Wine polysaccharides influence tannin-protein interactions. *Food Hydrocoll.* **63**. 571-579.

Weber, F., and Schieber, A. 2023. When microbiology meets chemistry: generation, properties and prospects of pyranoanthocyanins as natural food colorants. *Curr. Opin. Food Sci.* **51**. 101019.

Weilack, I., Mehren, L., Schieber, A., and Weber, F. 2023. Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines. *Curr. Res. Food Sci.* **6**. 100506.

Weilack, I., Schmitz, C., Harbertson, J. F., and Weber, F. 2021. Effect of Structural Transformations on Precipitability and Polarity of Red Wine Phenolic Polymers. *Am. J. Enol. Vitic.*

# Chapter 4

## Seed Maturation Dynamics in Cabernet Sauvignon and Pinot noir Grapes during Berry Ripening in Cool Climate Zones

Physical and chemical changes of the polyphenols in grape seeds during berry maturation significantly influence the sensory characteristics of red wine. This study investigates the development of tannins and flavan-3-ols in Cabernet Sauvignon and Pinot noir grape seeds between veraison and full ripeness, alongside concurrent alterations in seed physical characteristics. Our findings demonstrate a peak in tannin and flavan-3-ol concentrations at veraison, followed by a subsequent decline, and varying trends in the mean degree of polymerization of tannins. Moreover, the study reveals that Cabernet Sauvignon seeds exhibit relatively constant hardness and toughness, while Pinot noir seeds experience an increase in these physical properties during ripening. These dynamic changes in seed polyphenols and physical attributes hold considerable implications for red wine quality. Understanding these changes can offer valuable insights for optimizing harvest timing.

**Keywords**: Grape seeds, phenolic maturity, tannins, flavan-3-ols, seed texture

---

Reprinted with permission from:

Hensen, J.-P., Dörner, M., Etzbach, L., Schieber, A., and Weber, F. 2023. Seed Maturation Dynamics in Cabernet Sauvignon and Pinot Noir Grapes During Berry Ripening in Cool Climate Zones. *Food Sci. Technol.* ***4***. 161-172. DOI: 10.1021/acsfoodscitech.3c00445.

## 1 Introduction

Red wine sensory characteristics are known to be highly influenced by grape phenolic maturity.(Harrison, 2017; Peleg et al., 1999) Phenolic maturity summarizes all changes that occur during grape ripening, which impact the phenolic composition and extractability of polyphenols.(Saint Cricq de Gaulejac et al., 1998) In addition to the sugar and acid concentrations, phenolic maturity has emerged as a useful indicator for winemakers to manage their harvest. Among the various components contributing to the overall phenolic profile, grape seeds play a significant role in determining wine tannin and monomeric flavan-3-ol composition.(Kennedy et al., 2000a) Although seeds account for only less than 6% of the berry weight,(Cadot et al., 2006; Hang, 1988) their polyphenols exert a tremendous influence on wine mouthfeel and taste. In particular, seeds from unripe berries can impart bitterness, leading to an astringency often described as green and unpleasant.(Harrison, 2017; Robichaud and Noble, 1990) However, as grape berries ripen, seed polyphenols undergo changes that may positively impact red wine quality.(Bautista-Ortín et al., 2012)

During berry ripening, the concentration and structure of seed polyphenols undergo dynamic shifts. At veraison, the berries reach a pivotal point where tannins and flavan-3-ol monomers reach their maximum concentration, which subsequently decrease by up to 60% and 90%, respectively.(Downey et al., 2003; Kennedy et al., 2000a; Kennedy et al., 2000b) This decline is likely connected to a reduced expression of genes responsible for proanthocyanidin synthesis after veraison.(Bogs et al., 2005) Additionally, enzymatic oxidation(Kennedy et al., 2000b) and a reduced permeability of the integument further reduce the concentration.(Bautista-Ortín et al., 2012; Cadot et al., 2006) Conversely, some studies have reported opposite developments during ripening, with no significant changes or even an increase in tannin concentration.(Bordiga et al., 2011; Harbertson et al., 2002; Saint-Cricq de Gaulejac et al., 2007) Furthermore, the association of proanthocyanidins with cell wall polysaccharides (Downey et al., 2003; Hanlin et al., 2010; Hensen et al., 2022) or proteins (Geny et al., 2003) leads to reduced extractability. Anthocyanins affect these interactions between tannins and cell wall polysaccharides which has the potential to conversely increase the extraction of tannins again. (Bautista-Ortin et al., 2016) The mean degree of polymerization (mDP) of grape seed tannins also exhibits irregular changes during ripening, with some publications reporting an increase and others a decrease.(Bautista-Ortín et al., 2012; Downey et al., 2003; Kennedy et al., 2000a) Such conflicting findings make it challenging to generalize the phenolic ripening process in seeds. The degree of galloylation decreases during berry ripening.(Obreque-Slier et al., 2010)

In this study, seeds from Pinot noir and Cabernet Sauvignon were analyzed. Pinot noir grapes are the dominant red grape variety in cool climate zones due to their good ripening capabilities in these climatic conditions. High-quality wines made from Pinot noir are typically nuanced with delicate flavors. Due to their thin skins, Pinot noir seeds proportionally contribute more polyphenols and, thus, seed ripeness has a strong influence on the wine style in this variety. On the contrary, Cabernet Sauvignon wines often have strong flavors and are rich in skin polyphenols, which attenuates the impact of seed polyphenols on the wine style. Since Cabernet Sauvignon faces challenges reaching full ripeness in cool climate zones, controlling seed ripeness becomes important. In cool climate zones like the one where this study was conducted, grape berries tend to attain a lower degree of ripening, resulting in different sugar concentrations and polyphenol compositions compared to grape in warmer regions. This poses a great challenge on winemakers producing wines of exceptional quality.

There is an apparent discrepancy between technological and phenolic maturity. Understanding and carefully navigating the dynamic relationship between these two aspects are essential for winemakers striving to produce exceptional wines in cool climate zones. The extraction of polyphenols during winemaking is intricately linked to the physical and molecular transformations occurring in the grape berry, emphasizing the need for a nuanced approach in managing grape maturity in these unique viticultural environments.

Influences of variety, climate, and region are seemingly influential factors, highlighting seed analysis as an essential factor in displaying the phenolic berry ripeness. For this reason, grape seeds have been a focal point of scientists and winemakers to assess grape maturity and quality. Without tools to measure polyphenols, winemakers rely on assessing physiological parameters and the taste of seeds to manage their harvest and define berry quality. Moreover, changes in the appearance, color,(Ristic and Iland, 2005) and texture (Letaief et al., 2013) of the seeds may potentially be indicative of broader phenolic changes in the seed or even the entire berry.

This study investigates the molecular and physiological changes that occur in grape seeds during the decisive final weeks of berry ripening. The aim is to establish meaningful connections between these changes and their potential impact on red wine characteristics. The knowledge gained from this research will provide a better understanding of the interplay between grape seed phenolic ripening and the potentially resulting red wine composition and might help winemakers to determine the optimal harvest time.

## 2 Material

Acetone and ethanol (99%) were purchased from Julius Hoesch GmbH & Co. KG (Dueren, Germany). Ammonium oxalate monohydrate (98%), ferric chloride (97%), urea (≥ 98%), triethanol amine (≥ 98%), and tartaric acid (99%) were sourced from Alfa Aesar (Kandel, Germany). Ammonium sulfate (≥ 99%) and sodium hydroxide (≥ 99%) were obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany). Ascorbic acid (100%), acetic acid (glacial), methanol (HPLC-MS grade), and hydrochloric acid (37%) were acquired from VWR International GmbH (Darmstadt, Germany). Bovine serum albumin fraction V (≥ 98%) was sourced from Carl Roth (Karlsruhe, Germany). (+)-Catechin hydrate (≥ 98%), sodium acetate (≥ 99%), and phloroglucinol (≥ 99%) were purchased from Sigma Aldrich (Darmstadt, Germany). (-)-Epicatechin gallate (≥ 97.5%), (-)-epigallocatechin gallate (≥ 98%), and procyanidin C1 (≥ 90%) were obtained from Extrasynthèse (Genay Cedex, France). Methylcellulose (700-1500 mPa s) was acquired from Honeywell Fluka (Offenbach, Germany). Sodium chloride was purchased from neoFroxx (Einhausen, Germany). Acetonitrile (99.99%) and formic acid (99%) were obtained from Biosolve BV (Valkenswaard, The Netherlands). Water (LC-MS grade) was sourced from Fischer Scientific (Loughborough, UK).

### *2.1 Grape samples*

*Vitis vinifera* L. cv. Cabernet Sauvignon and Pinot noir grapes were harvested in Neustadt an der Weinstraße (Palatinate, Germany) between August 27$^{th}$ and October 15$^{th}$ 2019 at three different stages of ripeness after technological maturity. The geodesic coordinates of the variety-specific harvest sites are 49°23'53.3"N 8°11'02.0"E and 49°22'14.3"N 8°10'56.3"E.

At each sampling date, three groups of 100 grapes were selected from 60 grape clusters distributed between a third of the rows within the vineyard. Grape seeds were manually separated from the pulp, washed with deionized water, and patted dry with paper towels. Seeds were counted, weighed, and photographed for visual characterization before textural and chemical analysis. The seed water content was assessed by crushing the seeds, followed by freezing them at -80 °C and then subjecting them to lyophilization (2-8beta LSC-Basic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 0.08 bar for 72 h. The disparity in mass before and after drying accounts for the water content of the seeds.

### *2.2 Texture analysis*

Seed texture was analyzed with a method adapted from Letaief et al. (2013). The break force was measured with a 2 mm cylinder probe on a TA.XT Plus equipped with a 50 kg load cell (Stable Micro Systems Ltd., Godalming, UK). 300 individual seeds collected from all berries were compressed laterally. The compression speed was set at 0.5 mm $s^{-1}$. The force was calibrated after every 100 tests. The software Texture Exponent 32 was used to record the resulting force-time curve and for data analysis.

### *2.3 Seed polyphenol extraction and analysis*

Crushed seeds from 300 berries were frozen at -80 °C and lyophilized (2-8beta LSC-Basic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 0.08 bar for 72 h. Seeds were ground to a fine powder in a mortar and mixed with 25 mL $g^{-1}$ 70% aqueous acetone for 17 h (n = 3). The filtrate (595 Whatmann, GE Healthcare Life Sciences, Solingen, Germany) was evaporated under vacuum and lyophilized at 0.08 bar for 72 h.

The extracts were resuspended in a model wine solution (12% v/v ethanol, 5 g $L^{-1}$ tartaric acid, pH 3.3) at a concentration of 1 mg $mL^{-1}$ for tannin analysis by a methylcellulose precipitation assay and by a protein precipitation assay with a modified resuspension buffer (n = 3).(Harbertson et al., 2002; Harbertson et al., 2015; Mercurio and Smith, 2008; Sarneckis et al., 2006) For the determination of total iron reactive phenolics, a sample aliquot of the suspended extracts was diluted with resuspension buffer to 875 µL and incubated for 10 min as described by Harbertson et al. (2009) (n = 3). The absorbance at 510 nm was measured before and after adding 125 µL ferric chloride. Tannins and total iron reactive phenolics were expressed as catechin equivalents (CE) based on an external calibration curve.

### *2.4 Determination of the tannin mean degree of polymerization (mDP) by phloroglucinolysis*

The mean degree of polymerization was determined after acid catalyzed degradation in the presence of phloroglucinol under the conditions described by Kennedy and Jones (2001). Five milligrams of polyphenolic extract (see 2.3) were dissolved in 1 mL of methanol, sonicated in an ultrasound bath, centrifuged, and 100 µL aliquots of the supernatant were dried under a nitrogen atmosphere until completely dry (n = 3). The dried residue was dissolved in 100 µL reagent (0,1 M HCl in methanol containing 50 g $L^{-1}$ phloroglucinol and 10 g $L^{-1}$ ascorbic acid)

water and treated further according to Kennedy and Jones (2001). Phloroglucinol adducts were analyzed by UHPLC-ESI-MS/MS. The method was adapted from Vallverdu-Queralt et al. (2017) with modifications as stated below.

A UPLC I-Class system (Waters, Milford, MA) consisting of a binary pump, an autosampler cooled at 10 °C, and a column oven set at 40 °C equipped with a Nucleoshell Phenyl-Hexyl column (150 × 2 mm; 2.7 µm particle size) purchased from Macherey-Nagel GmbH & Co. KG (Dueren, Germany) was used for separation. Analytes were eluted with water/formic acid (99.9/0.1% v/v) as eluent A and acetonitrile/formic acid (99.9/0.1% v/v) as eluent B at a flow rate of 0.4 mL $min^{-1}$ with the following gradient: 0 min, 1% B; 3 min, 1% B; 22 min, 20% B; 23 min, 100% B; 25 min, 100% B; 26 min, 1% B; 30 min, 1% B. The injection volume was 2 µL. The UPLC was coupled to an LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Waltham, MA) equipped with an electrospray interface operating in positive ion mode. Mass spectra were recorded in the range of 180–1500 m/z with three consecutive mass scans (MS2, 35% normalized collision energy; MS3, 35% normalized collision energy). The capillary was set at 350 °C with a voltage of 22 V and a source voltage of 4 kV at a current of 100 µA. The tube lens was adjusted to 75 V. The mDP was determined as the sum of terminal and extension subunit amounts divided by the terminal subunit amount.

### *2.5 Time of flight mass spectrometry of monomeric and oligomeric flavanols*

Seed extracts (n = 3) were analyzed using a method adopted from Strassmann et al. (2021) on an Acquity UPLC I-Class system (Waters, Milford, MA) consisting of a binary pump, a sample manager cooled at 10 °C, equipped with an Acquity HSS-T3 RP18 column (150 mm × 2.1 mm; 1.8 µm particle size) combined with a pre-column (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm × 5 mm,1.8 µm), both from Waters (Milford, MA) at 40 °C. Analytes were eluted with water/formic acid (99.9/0.1% v/v) as eluent A and acetonitrile/formic acid (99.9/0.1% v/v) as eluent B at a flow rate of 0.4 mL $min^{-1}$ with the following gradient: 0 min, 0% B; 3 min, 0% B; 14 min, 30% B; 16 min, 100% B; 23 min, 100% B; 24 min, 0% B; 26 min, 0% B.

The UPLC was connected to a Vion IMS QTOF mass spectrometer (Waters, Milford, MA) operating in the negative ion mode. The capillary voltage was 2.0 kV, the source temperature was 120 °C, the cone gas flow was 50 L/h, the desolvation gas temperature was 550 °C, and the desolvation gas flow was 600 L/h. Nitrogen was used as the drift and collision gas. MS mode was set to high definition with a low collision energy of 6 eV and a high collision energy

ramp of 20–60 eV. The measurements were conducted with 100 pg $\mu L^{-1}$ leucine enkephalin as the lock mass with automatic lock correction every 5 min. Data were acquired and processed using UNIFI v1.9.2.045 (Waters, Milford, MA).

Analytes were identified by accurate masses and, when available, by comparison with retention times of reference compounds. Catechin hydrate (≥ 98%) was used for quantification of (epi-)catechin monomers and dimers by external calibration. Procyanidin C1 (≥ 90%) was used for quantification of (epi-)catechin trimers, tetramers, pentamers, and hexamers by external calibration. (-)-Epicatechin gallate (≥ 97.5%) was used for quantification of all galloylated compounds by external calibration. (-)-Epigallocatechin gallate (≥ 98%) was used for quantification of all trihydroxylated monomeric and oligomeric flavanols by external calibration.

### *2.6 Statistical analysis*

R (Version 4.1.3) with R-studio (Version 2022.02.3) and packages dplyr (Version 2.1.1), ggplot2 (Version 3.3.5), lemon (Version 0.4.5), rstatix (Version 0.7.0), stat (Version 3.6.0), and tidyr (Version 1.0.2) were used for statistical analysis and graphical illustrations. For pairwise comparison, ANOVA with Tukey's post hoc or Kruskal-Wallis with Dunn's post hoc tests with a significance level of $p < 0.05$ were used.

## 3 Results

### *3.1 Seed weight dynamics and implications for phenolic maturity during berry ripening*

Throughout the berry ripening process, the total seed weight in Cabernet Sauvignon grapes remained relatively constant, while the seeds in Pinot noir berries exhibited a slight decrease (Table 4.1). Interestingly, in both varieties the seed-to-berry ratio showed a reduction from lowest to medium ripeness levels, which may be attributed to an overall increase in berry weight. According to reports from Ristic and Iland (2005), dehydration of the seeds causes a decreasing seed weight. The water content of Pinot noir seeds was 36.5%, 30.9%, and 29.0%. In Cabernet Sauvignon seeds, the water content was 33.6%, 32.1%, and 30.9%. In the observed period, the dehydration in Pinot noir seeds was more pronounced compared to the relatively slow dehydration in Cabernet Sauvignon seeds. Obreque-Slier et al. (2010) reported a similar stagnation of the seed weight beginning after veraison. Notably, individual seed weight was

found to be higher in Pinot noir grapes, whereas Cabernet Sauvignon berries had higher seeds-per-berry count, resulting in a greater total seed weight per berry compared to Pinot noir berries. It is worth mentioning that the seed weight is influenced not only by grape variety but also by weather and various berry growth-related factors, which may vary from one vintage to another.(Ristic and Iland, 2005) While seed weight showed a slight change during the observed period, the overall berry weight experienced a more significant increase, leading to a reduced seed weight ratio per berry. These different ratios consequently have implications for the general impact of seeds and their polyphenols on the resulting wine. Understanding seed weight dynamics emerges as a crucial aspect in comprehending phenolic maturity.

**Table 4.1** Weight measurements of grape seeds from Cabernet Sauvignon and Pinot noir samples at different levels of grape maturity. Significance was calculated by a one-way ANOVA and Tukey's posthoc test ($p \geq 0.05$).

| Variety | TSS [°Bx] | Seed Weight [g] | Berry Weight [g] | Seed Proportion per Berry [%] |
|---|---|---|---|---|
| Cabernet Sauvignon | 17.6 | 5.74±0.97 ab | 94.83±13.87 b | 6.04±0.16 a |
| | 21.2 | 4.92±0.67 ab | 94.44±8.91 b | 5.19±0.21 b |
| | 22.7 | 5.42±0.21 a | 105.73±0.66 b | 5.12±0.17 b |
| Pinot noir | 17.6 | 5.04±0.75 ab | 98.83±11.10 b | 5.08±0.21 b |
| | 21.6 | 4.89±0.62 ab | 126.81±10.66 a | 3.84±0.20 c |
| | 22.7 | 4.76±0.21 b | 131.68±5.38 a | 3.61±0.04 c |

### *3.2 Visual appearance of Cabernet Sauvignon and Pinot noir seeds during berry ripening*

Visual examination of the seeds revealed distinct varietal differences (Figure 4.1). Pinot noir seeds were larger, tapered, and exhibited significant variations in size. In contrast, Cabernet Sauvignon seeds had a more uniform size and a rounded tip. As berry ripening progressed, the color of seeds of both varieties transitioned from green to light brown and eventually to a darker brown hue, with more pronounced color changes observed on the dorsal side. Cabernet Sauvignon seeds tended to be darker in color and had fewer green portions compared to Pinot noir seeds. The observed color development in seeds during ripening aligns with findings reported in several publications (Fredes et al., 2017; Fredes et al., 2010; Kennedy et al., 2000a; Ristic and Iland, 2005; Rustioni et al., 2018; Van der Weide et al., 2020) and has commonly been utilized as an indicator of overall grape maturity. However, it is worth noting that the final colors reported in these studies were slightly darker than what was observed in the present

study. According to Fredes et al. (2017), the color changes may vary between different parts of the seed, making the chalaza and raphe particularly suitable indicators due to their high contrast with the surrounding area.

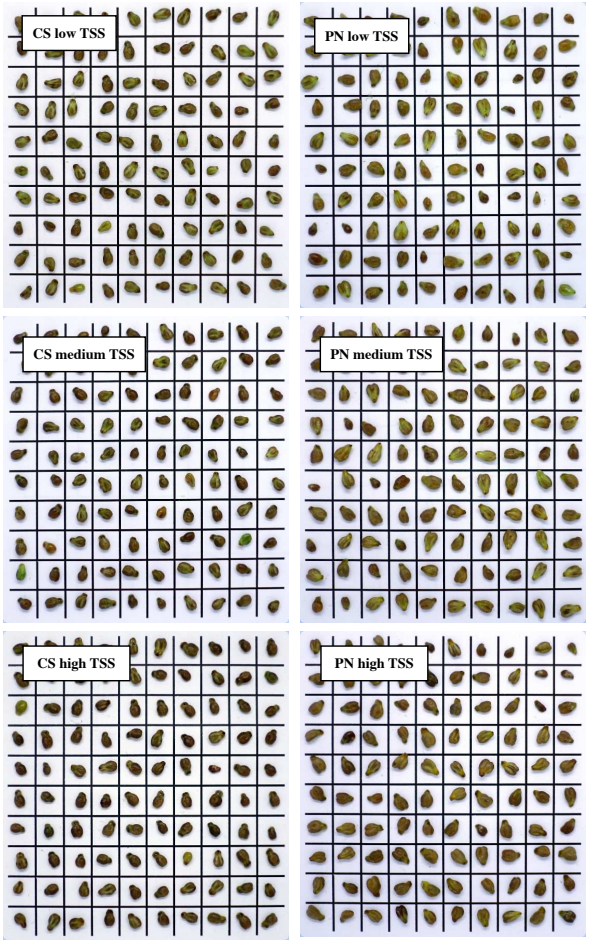



**Figure 4.1** Exemplary representation of seeds from Cabernet Sauvignon (CS) and Pinot noir (PN) photographed at different levels of grape maturity assessed by total soluble solids (TSS). See Table 4.1 for TSS values.

The color changes were found to be most rapid in the first few days after veraison and gradually slowed down afterwards,(Fredes et al., 2017) which matches the timeline of the observations made in the present study. In most of the previous studies, seed color was compared to a self-developed scale separating seeds into three or more ripeness categories. Applying the proposed scheme from Fredes et al. (2010) here, Cabernet Sauvignon seeds from the first and second harvest dates were categorized as unripe and only seeds from the final harvest date were considered ripe. Pinot noir seeds were deemed unripe at the first and second harvest dates and partially ripe at the final harvest date, with only a few seeds showing signs of ripeness based on the observed color. However, the existing color descriptions available in the literature do not account for Pinot noir seeds because previous studies mainly focused on

varieties such as Cabernet Sauvignon, Shiraz, and Carmenere. Therefore, existing classifications might be unsuitable for Pinot noir. The assumably unripe Pinot noir seeds would potentially be reclassified as ripe with a new color scheme. This variety-specific difference highlights the necessity for customized tools.

Continuous polyphenol oxidation in the seed coat contributes to the increasingly brown coloration of the seed.(Kennedy et al., 2000b) After the berry has reached its maximum weight, dehydration and sclerification of the seed coat stabilize the brown oxidation products.(Coombe and McCarthy, 2000; Kennedy et al., 2000b; Ristic and Iland, 2005) Different researchers have identified correlations between the changing color of the seed coat and concentrations of anthocyanins and total phenolics in grape skins, or tannin extractability and seed flavanol concentrations.(Fredes et al., 2017; Ristic and Iland, 2005; Rodriguez-Pulido et al., 2014) However, it remains to be determined whether there is a direct correlation between seed color and the phenolic composition of the resulting wine.(Casassa et al., 2013) Additionally, the color varies widely between individual seeds, leading to a high margin of error when attempting to discern slight differences between harvest dates solely based on color assessment.

While the seed color does indicate a progression of phenolic ripeness, as it is associated with polyphenol oxidation, the seed colors' implication for the overall phenolic composition of the wine is relatively minor. Foremost, the color only changes because of the oxidation of polyphenols in the most outer layer of the seed, which contribute only a very limited proportion of the whole seed phenolic content. This is further supported by the fact that all other parameters, particularly phenolic measurements, revealed more significant developments in Pinot noir grapes compared to Cabernet Sauvignon grapes, despite the visibly greater change in seed color in the latter (Figures 4.4-6). Consequently, relying solely on seed color assessment may not provide a comprehensive measure of overall phenolic ripeness. Nevertheless, the observed changes in seed color offer valuable insights into the progression of phenolic development during berry ripening, contributing to a more comprehensive understanding of the grape maturation process.

### *3.3 Hardening of Cabernet Sauvignon and Pinot noir seeds during berry ripening*

Grape seeds harden during berry ripening due to lignification and water loss of the cells. Two key parameters, maximum break force and compression, can be extracted from force-time diagrams to quantify seed hardness and toughness, respectively.(Letaief et al., 2013) The maximum break force indicates seed hardness, whereas compression measures the toughness

of the seeds. Toughness is the total energy absorbed by the seed through compression. In our study, we observed an increase in Pinot noir seed hardness and toughness after each ripening stage (Figures 4.2 and 4.3). In contrast, Cabernet Sauvignon seed hardness and toughness remained relatively constant across all ripening stages. Notably, Cabernet Sauvignon seeds exhibited the same level of hardness and toughness that Pinot noir seeds achieved at the final ripening stage. These findings are consistent with earlier reports by Letaief et al. (2008a), who also measured a higher hardness of Cabernet Sauvignon seeds compared to Pinot noir seeds. The observed hardening phenomenon is a result of histological and histochemical changes within the seed cells throughout the ripening process.(Cadot et al., 2006)

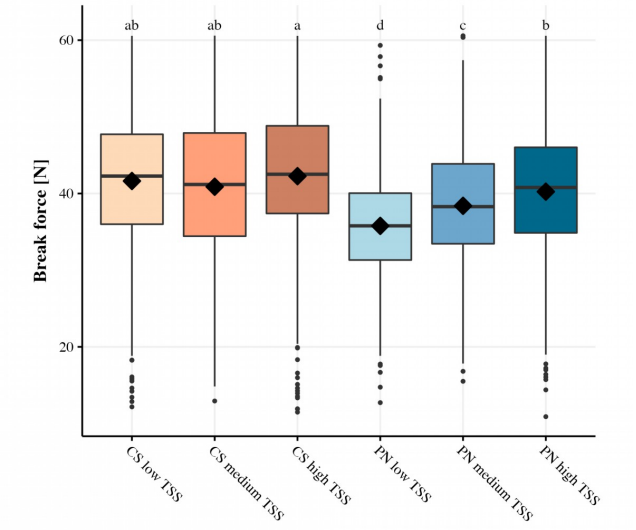



**Figure 4.2** Cabernet Sauvignon (CS) and Pinot noir (PN) seed hardness at different levels of grape maturity. Hardness was measured as seed break force. Significance was calculated by Kruskal Wallis test ($p \geq 0.05$) and Dunn's posthoc test ($p \geq 0.05$) and indicated by compact letter display. See Table 4.1 for TSS values.

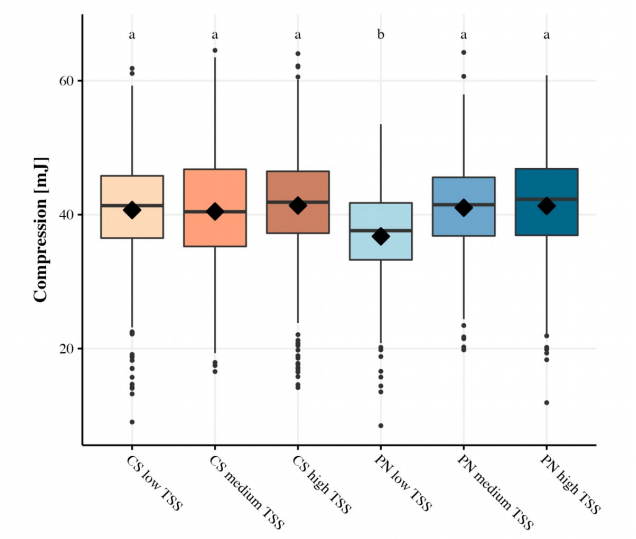



**Figure 4.3** Cabernet Sauvignon (CS) and Pinot noir (PN) seed toughness at different levels of grape maturity. Toughness was measured as compression. Significance was calculated by Kruskal Wallis test ($p \geq 0.05$) and Dunn's posthoc test ($p \geq 0.05$) and indicated by compact letter display. See Table 4.1 for TSS values.

Textural changes have been used as a ripening indicator of grapes in several studies.(Letaief et al., 2008a; Letaief et al., 2008b; Río Segade et al., 2011a; Río Segade et al., 2011b; Río Segade et al., 2008) However, these studies primarily focused on the skin or whole berry texture. Although winemakers often assess the grape quality by the feel and texture of the seeds, only few studies, such as the one by Letaief et al. (2013), included seed texture in their analysis. According to Letaief et al. (2013), ripeness is considered to be achieved when seed hardness or toughness reaches a point of stabilization after a logarithmic increase. In the case of Pinot noir seeds, we observed that the measured texture parameters continued to increase, suggesting that textural ripeness might not have been reached. Rolle et al. (2012) have shown that there is a weak correlation between the textural changes of the seeds and the extractability of their phenolic compounds, pointing out that there is a progressive development.

On the other hand, Cabernet Sauvignon seeds displayed constant textural characteristics and were considered ripe. The work of Letaief et al. (2013) focused on Cabernet Franc seeds and indicated that the seeds were mature three weeks after veraison, which corresponds to a similar ripening period observed in our study. However, it is crucial to acknowledge that varietal differences and specific weather conditions may significantly influence the ripening progress of seeds, as demonstrated for whole grapes as well.(Letaief et al., 2008a) An interesting hypothesis put forth by Letaief et al. (2008a) suggests that rain before sampling might increase the seed water content, potentially leading to decreased seed hardness. In our case, rainfall on the days immediately before the last harvest date did not change the water content and seed hardness of Cabernet Sauvignon samples.

Beyond their analytical significance, textural changes in seeds might also impact the extraction of polyphenols during winemaking. Typically, seeds remain intact during the winemaking process, limiting the extraction of polyphenols to those present in the outer layer of the seeds. Increased seed hardness might result in the release of even fewer polyphenols into the wine by preventing mechanical breaches that could otherwise expose more surface area to the surrounding liquid. Consequently, seeds from berries with higher concentrations of total soluble solids (TSS) are less likely to release their polyphenols, regardless of their sensorial quality or concentration, due to their tougher exterior. This aspect might be critical in understanding the influence of seed texture on the overall polyphenolic composition and mouthfeel characteristics of wines.

### *3.4 Phenolic composition of Cabernet Sauvignon and Pinot noir seeds during berry ripening*

The polyphenols found in grape seeds often contribute less desirable sensorial qualities to wine compared to those from the skins and flesh of the berry, as they tend to intensify astringency and introduce bitterness.(Bautista-Ortín et al., 2014) Winemakers typically aim for desirable changes, such as the polymerization of seed polyphenols during ripening, to produce wines with pleasant sensorial characteristics. Alternatively, a lower concentration of seed polyphenols can mitigate the potential negative impact on the wine. Consequently, grapes are considered ripe when polyphenols in the seeds have a low concentration or extractability. However, polyphenol analysis with protein precipitation showed no changes in tannin or total phenolic concentrations in the Cabernet Sauvignon seeds during berry ripening (Figure 4.4 and Supporting Figure 4.1S). Notably, the tannin concentrations were slightly higher than those reported by Ortega-Regules et al. (2008), even at the highest level of berry ripeness. Typically, seed tannin and flavanol concentrations have been reported to decrease during berry ripening in different varieties until they remain constant.(Downey et al., 2003; Kennedy et al., 2000a; Kennedy et al., 2000b) The constant polyphenol concentration in Cabernet Sauvignon seeds in our study indicates that this plateau phase had already been reached. It is worth noting that earlier studies have assumed more pronounced changes in polyphenol concentration during the first month after veraison.(Kennedy et al., 2000a) As our first harvest closely followed veraison, the reported changes in polyphenol concentration may not be universally applicable in all instances. The slightly higher concentration of polyphenols in the seeds compared to previously reported values might suggest a general lack of physiological ripeness, especially when compared to grapes from warmer climate regions. This deviation from previous reports might be specific to seed development in cooler climates.

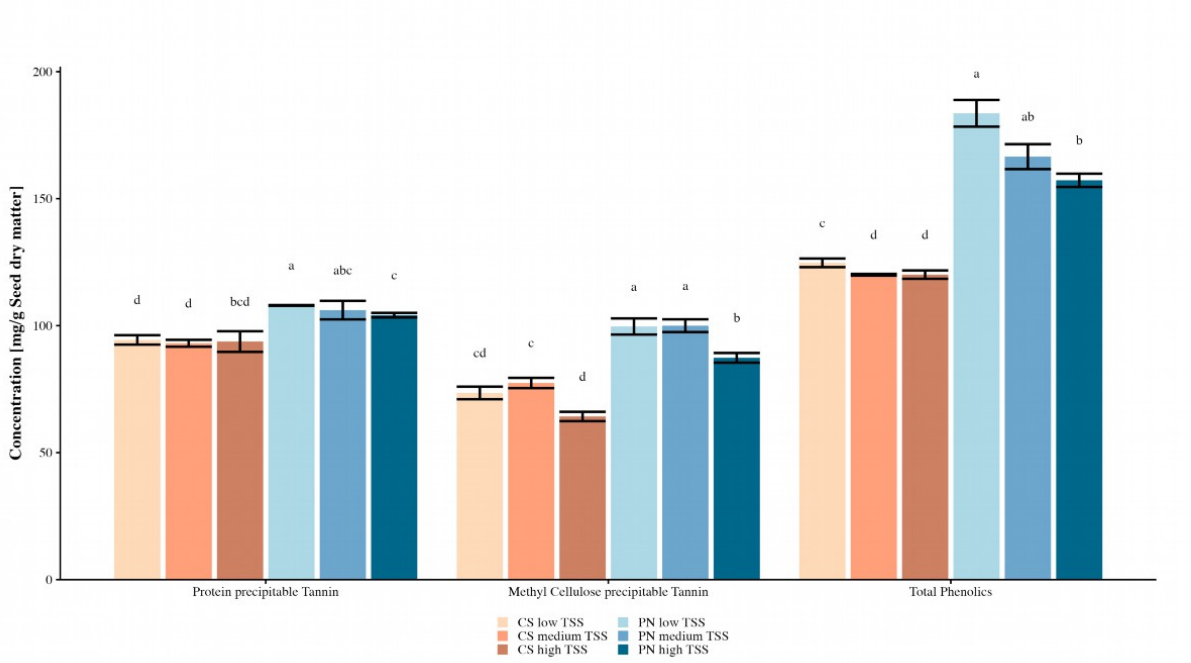



**Figure 4.4** Phenolic composition of extracts from Cabernet Sauvignon (CS) and Pinot noir (PN) seeds at different levels of grape maturity. Tannin concentration was measured as protein precipitable and methylcellulose precipitable tannin. Total phenolic concentration was measured as iron reactive polyphenols. Significance was calculated by a one-way ANOVA ($p \geq 0.05$) and Tukey's posthoc test ($p \geq 0.05$) for each analyte and indicated by compact letter display. See Table 4.1 for TSS values.

In Pinot noir seeds, the polyphenol concentration decreased and tannin concentration remained constant during ripening when measured with the protein precipitation assay. A study by Blank et al. (2019), who analyzed Pinot noir seed tannin concentrations in Geisenheim (Germany) covering ten vintages, reported values ranging from 1.3 to 2.3 mg/g berries. This range is lower than the tannin concentrations we observed in Pinot noir seeds, which decreased from 3.4 mg/g berry to 2.7 mg/g berry. Although both studies were conducted in Germany and employed the same method for tannin quantification, the differences in annual and regional weather, combined with variations in soil properties and possibly different vine clones, probably explain the higher tannin concentrations found in our study.

The tannin concentrations were higher in Pinot noir seeds than in Cabernet Sauvignon seeds. However, Cabernet Sauvignon wines are known for their high tannin concentrations,(Chira et al., 2011) which can be explained by the higher seed to berry ratio. Consequently, tannin concentrations per berry are higher in Cabernet Sauvignon seeds. Seed tannin concentrations per berry decreased in both varieties due to increased berry weight. During the observed ripening period, seed tannin concentration decreased by 22% in Pinot noir and 13% in Cabernet Sauvignon.

We additionally quantified seed tannin concentrations using the methylcellulose precipitation assay, which also revealed a decrease in tannin concentrations during berry ripening (Figure 4.4). In both varieties, seed tannin concentrations decreased after berries had reached medium ripeness, with consistent reductions in tannin concentrations per berry. The reduction was slightly higher when tannins were measured with the methylcellulose precipitation assay (29% in Pinot noir and 23% in Cabernet Sauvignon) compared to the protein precipitation assay. The protein precipitation assay, which uses bovine serum albumin, has been reported to precipitate flavanol dimers and trimers incompletely,(Adams and Harbertson, 1999) whereas methylcellulose has been shown to complex and precipitate them.(Vidal et al., 2003) This method-specific difference suggests a primary decrease in flavanol dimers and trimers in the development of seed tannin concentrations, which could be confirmed by further analysis with IMS TOF MS (Figure 4.5). Although both precipitation methods showed a high correlation between measured tannin concentrations in the seeds ($R^2$ = 0.87) during ripening, the concentrations were approximately 20% higher when measured with the protein precipitation assay. Both assays are widely used in wine tannin quantification. However, no comparative data on seed tannin determination by methylcellulose precipitation is available. Comparing studies present inconsistent differences in the results, showing higher measured concentrations for either assay.(Sarneckis et al., 2006; Seddon and Downey, 2008) There are multiple potential explanations for this, e.g., the use of different wavelengths for the tannin detection or varying precipitation of tannins depending on a variety of factors.

Both methods for tannin quantification indicated that the first harvest point exhibited potentially lower quality in both grape varieties compared to harvests with higher TSS levels, primarily due to the elevated tannin concentration. However, it is important to note that a higher concentration of tannins in grape seeds does not always correlate with an increased concentration in wine.(Casassa et al., 2013) By adjusting the maceration process during winemaking, seed tannin extraction can be controlled to achieve the desired level, allowing for the production of palatable red wines from grapes that may have lower seed ripeness.

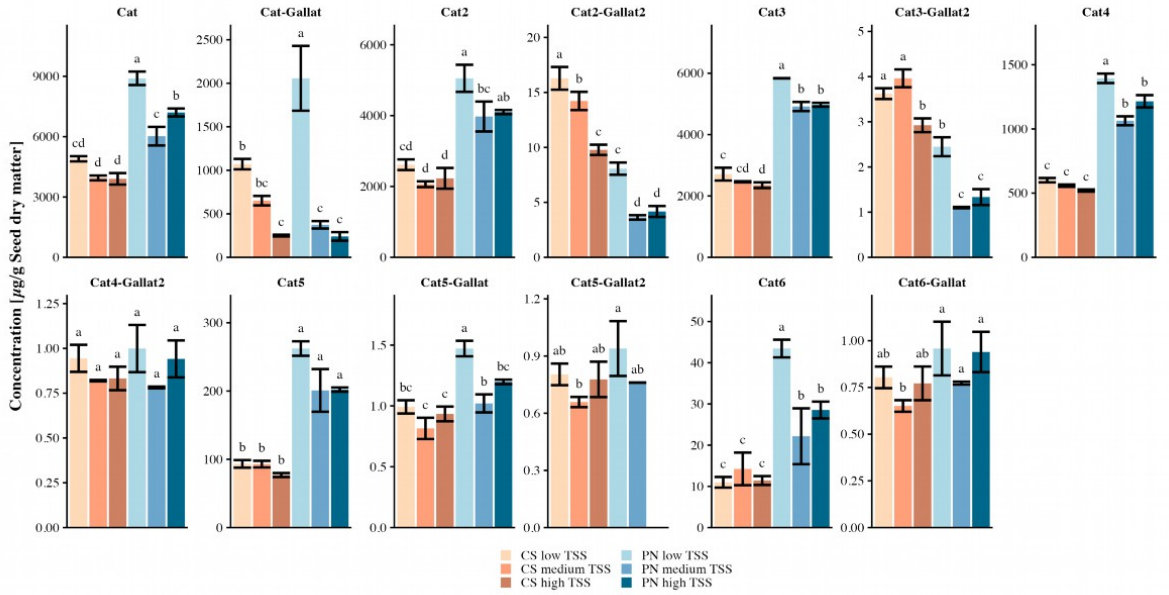



**Figure 4.5** Concentration of (epi)-catechin [Cat], (epi)-catechin dimers [Cat2], (epi)-catechin trimers [Cat3], (epi)-catechin tetramers [Cat4], (epi)-catechin pentamers [Cat5], (epi)-catechin hexamers [Cat6)], and their monogalloylated [Gallat] or digalloylated [Gallat2] derivatives in extracts from Cabernet Sauvignon (CS) and Pinot noir (PN) seeds at different levels of grape maturity. Significance was calculated by a one-way ANOVA ($p \geq 0.05$) and Tukey's posthoc test ($p \geq 0.05$) for each analyte and indicated by compact letter display. See Table 4.1 for TSS values.

### *3.5 Mean degree of polymerization of polyphenols in Cabernet Sauvignon and Pinot noir seeds during berry ripening*

The mDP of tannins calculated after phloroglucinolysis (Table 4.2) shows a distinct development in Pinot noir and Cabernet Sauvignon seeds. In Pinot noir seeds, the mDP remains constant throughout the ripening stages. In contrast, in Cabernet Sauvignon seeds, the mDP increases from the lowest to medium berry ripeness and subsequently decreases to its lowest value. This trend is consistent with findings by Bautista-Ortín et al. (2012), who observed a similar decrease and variability in mDP in Cabernet Sauvignon seeds. However, such variations in mDP seem to be less commonly found among different grape varieties. In fact, previous reports have suggested that the mDP tends to remain relatively stable in various grape varieties.(Bautista-Ortín et al., 2012; Downey et al., 2003) The same trend was observed for Pinot noir seed tannins in this study.

**Table 4.2** Mean degree of polymerization (mDP) of polyphenols in extracts from Cabernet Sauvignon and Pinot noir seeds at different levels of grape maturity. Significance was calculated by a one-way ANOVA and Tukey's posthoc test ($p \geq 0.05$).

| Variety | TSS [°Bx] | mDP |
|---|---|---|
| Cabernet Sauvignon | 17.6 | 8.95± 0.28 b |
| | 21.2 | 12.80± 0.61 a |
| | 22.7 | 5.91±0.48 c |
| Pinot noir | 17.6 | 8.78 ± 0.25 b |
| | 21.6 | 7.56 ± 0.33 bc |
| | 22.7 | 7.70 ±1.23 bc |

However, the mDP of Pinot noir seed tannins was much higher compared to previous findings by Mattivi et al. (2009) and Moreno et al. (2008), who reported mDP values between 2.7 and 3.3. In general, mDPs of seed tannins across different studies vary widely, ranging between 1 and 20.(Cheynier et al., 2006; Mattivi et al., 2009) Such discrepancies might arise due to the use of different analytical methods and variations during berry ripening, influenced by grape variety, maturity level, local and seasonal vegetation conditions. Despite these challenges in analysis and sampling, the mDP remains the most comprehensive parameter for tannin characterization as it covers larger molecules that cannot be determined otherwise. The lack of any variation of the mDP of Pinot noir tannins implies a steady development with simultaneous concentration changes of tannins of all sizes. Seemingly, the concentration of all compounds might decline in the same ratio, leaving polymerization not as a relevant ripening process within the evaluated time frame in Pinot noir seeds. This might result in consistent sensorial properties of the tannins because their mouthfeel is closely connected to the mDP. In Cabernet Sauvignon seeds, the variation of the mDP might indicate that an optimized harvest management based on the degree of polymerization may help improve the sensorial quality of the seed tannins and thereby increase the grape and wine quality.

### *3.6 Dynamics of oligomers: changes in flavanol concentrations and molecular composition during berry ripening*

Time of flight mass spectrometry was used to identify and quantify monomeric and oligomeric flavanols in grape seed extracts. The analysis identified (epi-)catechin and trihydroxylated (epi-)catechin and their galloylated derivatives with oligomers detected up to hexamers.

Pinot noir seed flavanol concentrations decreased between low and medium berry ripeness, whereas in Cabernet Sauvignon, the concentrations of the same polyphenols either remained constant or declined for a few analytes (Figure 4.5). As similarly reported in other studies,(Bautista-Ortín et al., 2012; Kennedy et al., 2000a) the epicatechin concentrations were slightly lower than the catechin concentrations in the sampled seeds. The decrease in monomeric flavan-3-ols observed after veraison has also been reported previously.(Bautista-Ortín et al., 2012; Downey et al., 2003; Kennedy et al., 2000a) Kennedy et al. (2000a) found that the main part of the flavanol concentration vanishes within the first month after veraison. Our studies show that this decrease is not limited to monomers but includes smaller oligomers, which supports the above-mentioned assumption made after analyzing tannins with both precipitation assays. Decreasing flavan-3-ol monomers and smaller oligomer concentrations during berry ripening are desired processes to enhance sensory qualities. The sensory qualities of tannins, such as chalky, dry, and overall unpleasant astringent mouthfeel, are closely connected to the degree of polymerization, particularly with these smaller proanthocyanidins.(Vidal et al., 2003) Besides the influence of the mDP, Vidal et al. (2003) found a connection between the degree of galloylation and a coarse mouthfeel of tannins that might be unwanted. Hence, galloylated tannins are particularly important for the sensory properties of seed polyphenols. In the samples, monogallates and digallates were identified, whereby Cabernet Sauvignon seeds had higher concentrations of digallates and Pinot noir seeds exhibited slightly higher concentrations of monogallate oligomers. The concentrations of all (epi-)catechin gallates in Pinot noir seeds reduced after the first harvest, whereas in Cabernet Sauvignon, reductions were more inconsistent and were observed mainly after the second harvest. Notably, the proportion of galloylated compounds with a higher degree of polymerization increased in both varieties, even though overall concentrations decreased (Table 4.3). Except for catechin-3-O-gallate, the concentrations of digallates were higher than monogallate oligomers. This might partly result from the higher degree of polymerization compared to monogallates. The decrease in concentrations of all galloylated analytes between

low and high berry ripeness was 31.6% in Cabernet Sauvignon and 56.2% in Pinot noir, which was higher compared to the non-galloylated flavanols with 18.7% and 21.6% respectively. Although overall concentrations decreased, the proportion of galloylated compounds with a higher degree of polymerization increased in both varieties. The results are indicative of a higher tendency of galloylated flavanols to polymerize during berry ripening.

**Table 4.3** Distribution of phenolic compounds extracted from Cabernet Sauvignon (CS) and Pinot noir (PN) seeds at different levels of grape maturity analyzed with IMS TOF MS. Significance was calculated by a one-way ANOVA and Tukey's posthoc test ($p \geq 0.05$).

| | Degree of Polymerization | CS low TSS [%] | CS medium TSS [%] | CS high TSS [%] | PN low TSS [%] | PN medium TSS [%] | PN high TSS [%] |
|---|---|---|---|---|---|---|---|
| Non-Galloylated | Monomer | 33.67±1.74 a | 32.75±0.74 a | 33.91±0.24 a | 34.20±0.40 a | 31.66±0.73 a | 34.96±0.10 a |
| | Dimer | 34.21±0.45 a | 33.56±0.77 ab | 32.52±1.21 abc | 29.79±1.09 bcd | 28.86±1.42 cd | 28.39±0.11 d |
| | Trimer | 25.54±1.01 b | 26.57±0.25 b | 26.36±0.87 b | 27.94±1.04 ab | 30.77±1.10 a | 28.45±0.26 ab |
| | Tetramer | 5.35±0.12 b | 5.89±0.27 ab | 5.89±0.40 ab | 6.63±0.29 ab | 6.99±0.65 a | 6.80±0.65 a |
| | Pentamer | 0.81±0.04 b | 0.89±0.02 ab | 0.92±0.13 ab | 1.10±0.00 ab | 1.34±0.26 a | 1.14±0.05 ab |
| | Hexamer | 0.28±0.07 a | 0.17±0.03 a | 0.27±0.01 a | 0.24±0.11 a | 0.24±0.09 a | 0.27±0.01 a |
| Galloylated | Monomer | 80.28±2.59 ab | 72.34±5.08 ab | 60.07±0.96 b | 86.43±5.79 a | 71.11±7.99 ab | 58.68±9.06 b |
| | Dimer | 10.40±0.29 b | 12.31±0.12 b | 18.29±0.62 a | 4.93±0.71 c | 11.86±0.59 b | 19.22±1.65 a |
| | Trimer | 2.19±0.13 a | 1.70±0.18 a | 2.94±0.31 a | 1.55±0.37 a | 2.18±0.01 a | 5.87±3.38 a |
| | Tetramer | 3.55±2.17 a | 5.32±1.04 a | 5.19±0.42 a | 2.92±0.83 a | 4.27±2.96 a | 9.24±3.26 a |
| | Pentamer | 0.14±0.01 a | 0.17±0.02 a | 0.34±0.02 b | 0.12±0.03 a | 0.34±0.02 b | 0.28±0.02 b |
| | Hexamer | 0.06±0.01 d | 0.07±0.00 cd | 0.15±0.02 b | 0.05±0.01 d | 0.14±0.00 bc | 0.24±0.04 a |

Among all samples, concentrations of trihydroxylated (epi-)catechin and their galloylated derivatives did not differ considerably (Figure 4.6). Some measurable concentration changes followed the described differences of all other compounds. However, their concentrations were much lower than those of other flavanols.

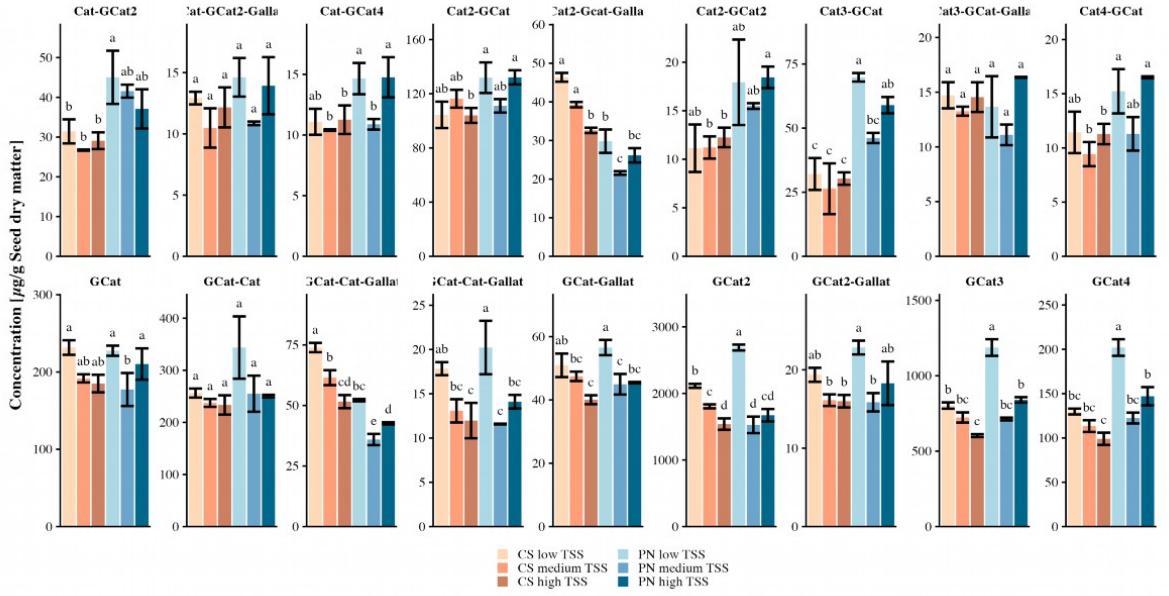



**Figure 4.6** Concentration of (epi)-gallocatechin [GCat], (epi)-galloatechin dimers [GCat2], (epi)-gallocatechin trimers [GCat3], (epi)-gallocatechin tetramers [GCat4], (epi)-gallocatechin pentamers [GCat5], (epi)-gallocatechin hexamers [GCat6], and their monogalloylated [Gallat] or digalloylated [Gallat2] derivatives in extracts from Cabernet Sauvignon (CS) and Pinot noir (PN) seeds at different levels of grape maturity. Significance was calculated by a one-way ANOVA ($p \geq 0.05$) and Tukey's posthoc test ($p \geq 0.05$) for each analyte and indicated by compact letter display. See Table 4.1 for TSS values.

Collision cross section values (CCS values) were calculated for the detected analytes from ion mobility spectrometry data, providing information on the rotational average of the conformation of an ion, also referred to as shape. With an increasing degree of polymerization, the number of different conformational or positional isomers increases, with potential implications for solubility and sensorial properties. The CCS values did not change significantly, indicating a consistent diversity of conformers for individual metabolites with a specific mass (Figures 4.7 and 4.8). However, the variability of CCS values increased with the degree of polymerization of the identified compounds. Galloylation further increased the variability of the CCS values among specific compounds. Accordingly, seed polyphenols with a higher mDP consist of more diverse molecular structures. There are indications that the range of CCS values for molecules with a specific mass is slightly increased in Cabernet Sauvignon samples, similar to the more diverse mDP of tannins during berry ripening. While the CCS

values for non-galloylated tannins generally increased with an increasing mDP, galloylated tannins revealed a broadening of CCS values with increasing mDP, maintaining also small CCS values observed for smaller oligomers. This dramatically increased the range of CCS values measured for galloylated molecules with the same mass. It may be speculated that this is related to differences between the ester bond and the inter-flavanic bond regarding their rotational rigidity.

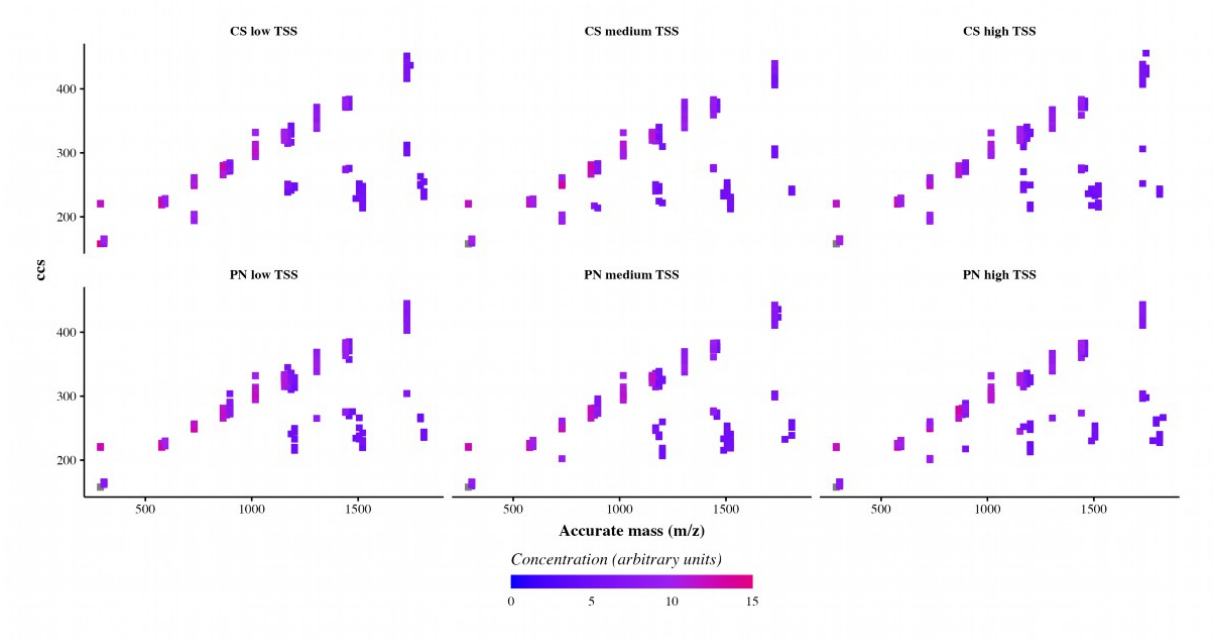



**Figure 4.7** Distribution of flavan-3-ols CCS values and mass-to-charge ratios *(m/z)* in grape seed phenolic extracts at different levels of grape maturity measured with TOF-MS. See Table 4.1 for TSS values.

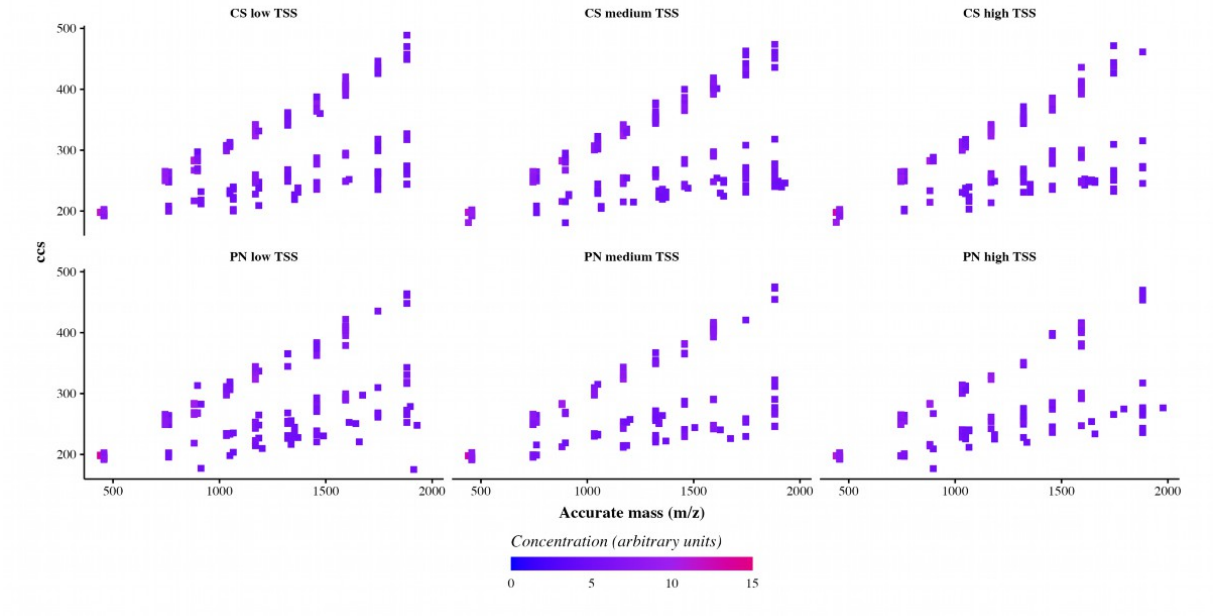



**Figure 4.8** Distribution of galloylated flavan-3-ols CCS values and mass-to-charge ratios *(m/z)* in grape seed phenolic extracts at different levels of grape maturity measured with TOF-MS. See Table 4.1 for TSS values.

Because the shape of tannins influences the potential for interactions with other molecules like proteins, CCS values might prospectively be useful to deduce the sensorial characteristics of tannins. Although the mouthfeel subqualities of tannins are not fully understood, their molecular structure can definitely be considered a major aspect.(Van der Weide et al., 2020; Weber, 2022) Because of their size, tannins interact with salivary proteins depending on steric and other factors like polarity, which potentially leads to precipitation of the aggregates formed. The specific information on the conformation of the tannins provided by the CCS values makes them a potential indicator for these interactions. While the molecular weight or the mDP have been reported to correlate with the ability to precipitate proteins, the conformation of the tannins of the same size might further impact the precipitability, potentially causing a variation in the mouthfeel properties. It can be concluded from the different results of the two precipitation assays that the linear structure of methylcellulose interacts better with rather linear tannin structures compared to the more compact BSA protein. This might be associated with a higher CCS value of the interacting tannins. Further analysis of CCS values, particularly of larger tannins and with adapted analytical tools, might offer deeper insights into the potential sensory impact of specific molecular shapes and compositions in seed polyphenols.

## 4 Enhancing the understanding of seed ripening: interplay of chemical and physical changes

The ripening process of grape seeds involves intricate changes in their chemical composition and physical attributes. The determination of the optimal seed ripeness has primarily relied on identifying the stage when seed characteristics stabilize during ripening. Pinot noir seeds exhibited more pronounced developments in nearly all analyzed parameters compared to Cabernet Sauvignon seeds, suggesting higher maturity levels in the latter when further progress becomes imperceptible. Consequently, the assessment of seed ripeness may carry greater significance in Pinot noir samples.

Although visual changes of the seeds are widely used to determine seed and berry ripeness, the presented results indicated that visible changes in the seeds differed from other analyzed parameters. Color assessment, primarily influenced by a small subset of polyphenols in the outer seed layers, might overlook the overall polyphenolic composition. Additionally, the presented results highlight that drawing the correct conclusions requires variety-specific color scales, in particular for late ripening varieties

To extract polyphenols underneath the outer layer, seeds need to be penetrated or crushed. Therefore, seed texture also influences the extractability of polyphenols. Because Pinot noir seeds became harder and tougher during ripening, the extractability of polyphenols might be reduced. These textural changes progressed similarly to the polyphenol concentration, thus, textural measurements might point to the changing flavanol concentration during ripening, aside from their direct impact on the extractability.

Phenolic measurements revealed declining tannin and total phenolic concentrations in the seeds. Notably, smaller flavanols, particularly monomers and dimers, experienced a more substantial reduction in concentration compared to larger oligomers. As a result, the overall distribution shifted towards larger oligomers, especially larger galloylated oligomers. However, the mDP showed different variations during grape ripening, suggesting polymerization and degradation reactions outside the range of the analyzed oligomers. The variability of conformational shapes, as reflected by CCS values, increased with the degree of polymerization, potentially influencing the sensorial characteristics of the polyphenols.

However, the proportion of seeds in a berry emerged as the most significant factor impacting seed polyphenol concentration in the wine. Berries in cool climate regions tend to increase in weight, particularly Pinot noir grapes, which usually do not lose water before harvest. This, combined with the steady decline of polyphenol concentrations in seeds that slightly lose weight, dilutes seed polyphenols in the wine. Although seemingly subtle, this factor may significantly impact wine quality. Winemakers may potentially improve wine quality by optimizing the seed-to-berry ratio, which can be achieved by either assessing seed and berry weight prior to harvest or alternatively during maceration by removing seeds. Seed removal, which is a well-established method in winemaking, is possibly the most efficient option, especially since other methods necessitate more sophisticated analyses.

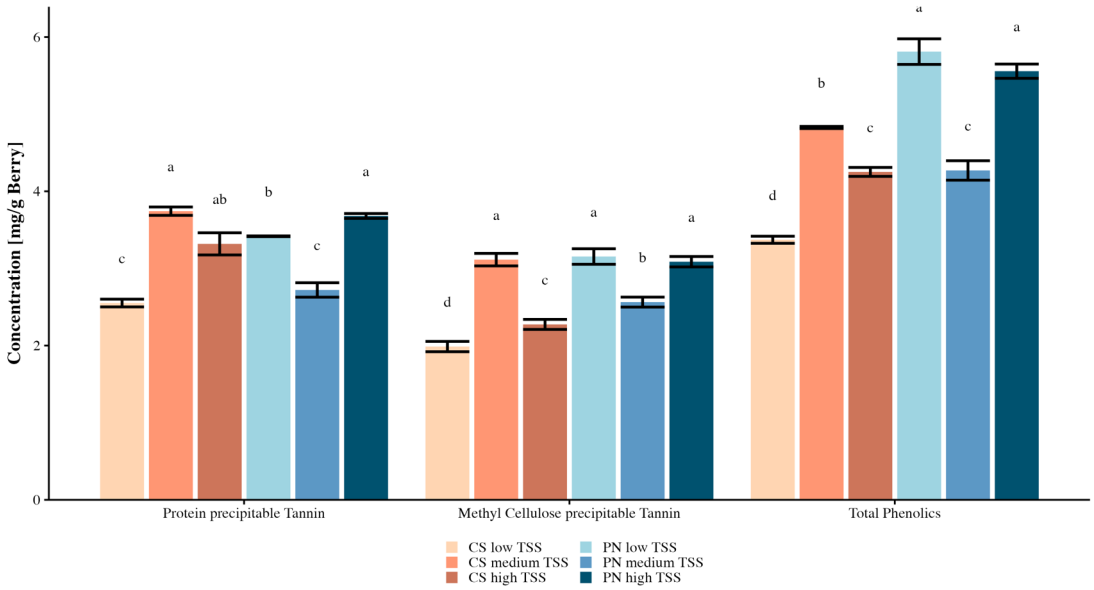
The fact that all these measurements aim to characterize seed or berry ripeness raises the question when seeds are ripe and which characteristics determine the perfect maturity. Pinot noir seeds would be considered ripe in the observed period beginning with the second harvest. Subsequently, only minor qualitative and quantitative changes in the analyzed characteristics were measurable, which would not necessarily suggest an improved ripeness. Cabernet Sauvignon seeds were considered ripe at the beginning of the harvest period, although the concentration of total soluble solids in the berries was still low. In both varieties, the potential of further polymerization reactions of galloylated flavanols was given. With the different measuring approaches, obvious connections were seen between some of the analyzed parameters and potentially highlight alternative methods for characterizing phenolic seed

ripeness. Because most of the analyzed characteristics followed a similar stagnation phase at the end, parameters developed similarly. Whether the morphological and chemical changes are coincidentally correlated or causally connected needs further assessment. Presently, however, texture analysis for assessing grape seed ripeness appears to be a useful alternative to more sophisticated polyphenol quantification methods. Nevertheless, the advantages of chemical measurements are obvious. Especially using both precipitation assays generated superior insights into the phenolic seed composition while still using only basic laboratory equipment.

**Funding**

This research project was financially supported by the German Ministry for Economic Affairs and Energy (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 20024N.

## Supporting information




**Supporting Figure 4.1S** Phenolic composition of seed polyphenols in Cabernet Sauvignon (CS) and Pinot noir (PN) at different levels of grape maturity. Tannin concentration was measured as protein precipitable and methylcellulose precipitable tannin. Total phenolic concentration was measured as iron reactive polyphenols. Significance was calculated by a one-way ANOVA ($p \geq 0.05$) and Tukey's posthoc test ($p \geq 0.05$) for each analyte and indicated by compact letter display. See Table 4.1 for TSS values.

**References**

Adams, D. O., and Harbertson, J. F. 1999. Use of Alkaline Phosphatase for the Analysis of Tannins in Grapes and Red Wines. *Am. J. Enol. Vitic.* **50**. 247-252.

Bautista-Ortín, A. B., Busse-Valverde, N., López-Roca, J. M., Gil-Muñoz, R., and Gómez-Plaza, E. 2014. Grape seed removal: effect on phenolics, chromatic and organoleptic characteristics of red wine. *Int. J. Food Sci. Technol.* **49**. 34-41.

Bautista-Ortin, A. B., Martinez-Hernandez, A., Ruiz-Garcia, Y., Gil-Munoz, R., and Gomez-Plaza, E. 2016. Anthocyanins influence tannin-cell wall interactions. *Food Chem.* **206**. 239-248.

Bautista-Ortín, A. B., Rodríguez-Rodríguez, P., Gil-Muñoz, R., Jiménez-Pasqual, E., Busse-Valverde, N., Martínez-Cutillas, A., López-Roca, J. M., and Gómez-Plaza, E. 2012. Influence of berry ripeness on concentration, qualitative composition and extractability of grape seed tannins. *Aust. J. Grape Wine R.* **18**. 123-130.

Blank, M., Hofmann, M., and Stoll, M. 2019. Seasonal differences in Vitis vinifera L. cv. Pinot noir fruit and wine quality in relation to climate. *OENO One*. **53**. 189-203.

Bogs, J., Downey, M. O., Harvey, J. S., Ashton, A. R., Tanner, G. J., and Robinson, S. P. 2005. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol.* **139**. 652-663.

Bordiga, M., Travaglia, F., Locatelli, M., Coïsson, J. D., and Arlorio, M. 2011. Characterisation of polymeric skin and seed proanthocyanidins during ripening in six Vitis vinifera L. cv. *Food Chem.* **127**. 180-187.

Cadot, Y., Minana-Castello, M. T., and Chevalier, M. 2006. Anatomical, histological, and histochemical changes in grape seeds from Vitis vinifera L. cv Cabernet franc during fruit development. *J. Agric. Food. Chem.* **54**. 9206-9215.

Casassa, L. F., Beaver, C. W., Mireles, M., Larsen, R. C., Hopfer, H., Heymann, H., and Harbertson, J. F. 2013. Influence of Fruit Maturity, Maceration Length, and Ethanol Amount on Chemical and Sensory Properties of Merlot Wines. *Am. J. Enol. Vitic.* **64**. 437-449.

Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J. M., Sarni-Manchado, P., and Fulcrand, H. 2006. Structure and properties of wine pigments and tannins. *Am. J. Enol. Vitic.* **57**. 298-305.

Chira, K., Lorrain, B., Ky, I., and Teissedre, P. L. 2011. Tannin composition of cabernet-sauvignon and merlot grapes from the bordeaux area for different vintages (2006 to 2009) and comparison to tannin profile of five 2009 vintage mediterranean grapes varieties. *Molecules*. **16**. 1519-1532.

Coombe, B. G., and McCarthy, M. G. 2000. Dynamics of grape berry growth and physiology of ripening. *Aust. J. Grape Wine R.* **6**. 131-135.

Downey, M. O., Harvey, J. S., and Robinson, S. P. 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine R.* **9**. 15-27.

Fredes, C., Mora, M., and Carrasco-Benavides, M. 2017. An Analysis of Seed Colour During Ripening of Cabernet Sauvignon Grapes. *S. Afr. J. Enol. Vitic.* **38**. 38-45.

Fredes, C., Von Bennewitz, E., Holzapfel, E., and Saavedra, F. 2010. Relation between Seed Appearance and Phenolic Maturity: A Case Study Using Grapes cv. Carménère. *Chil. J. Agric. Res.* **70**. 381-389.

Geny, L., Saucier, C., Bracco, S., Daviaud, F., and Glories, Y. 2003. Composition and cellular localization of tannins in grape seeds during maturation. *J. Agric. Food. Chem.* **51**. 8051-8054.

Hang, Y. D. 1988. Recovery of Food Ingredients from Grape Pomace. *Process Biochem*. **23**. 2-4.

Hanlin, R. L., Hrmova, M., Harbertson, J. F., and Downey, M. O. 2010. Review: Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust. J. Grape Wine R*. **16**. 173-188.

Harbertson, J. F., Kennedy, J. A., and Adams, D. O. 2002. Tannin in skins and seeds of Cabernet Sauvignon, Syrah, and Pinot noir berries during ripening. *Am. J. Enol. Vitic*. **53**. 54-59.

Harbertson, J. F., Mireles, M., and Yu, Y. 2015. Improvement of BSA Tannin Precipitation Assay by Reformulation of Resuspension Buffer. *Am. J. Enol. Vitic*. **66**. 95-99.

Harbertson, J. F., Mireles, M. S., Harwood, E. D., Weller, K. M., and Ross, C. F. 2009. Chemical and sensory effects of saignée, water addition, and extended maceration on high brix must. *Am. J. Enol. Vitic*. **60**. 450-460.

Harrison, R. 2017. Practical interventions that influence the sensory attributes of red wines related to the phenolic composition of grapes: a review. *Int. J. Food Sci. Technol*. **53**. 3-18.

Hensen, J. P., Hoening, F., Weilack, I., Damm, S., and Weber, F. 2022. Influence of Grape Cell Wall Polysaccharides on the Extraction of Polyphenols during Fermentation in Microvinifications. *J. Agric. Food. Chem*. **70**. 9117-9131.

Kennedy, J. A., and Jones, G. P. 2001. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food. Chem*. **49**. 1740-1746.

Kennedy, J. A., Matthews, M. A., and Waterhouse, A. L. 2000a. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry*. **55**. 77-85.

Kennedy, J. A., Troup, G. J., Pilbrow, J. R., Hutton, D. R., Hewitt, D., Hunter, C. R., Ristic, R., Iland, P. G., and Jones, G. P. 2000b. Development of seed polyphenols in berries from Vitis vinifera L. cv. Shiraz. *Aust. J. Grape Wine R*. **6**. 244-254.

Letaief, H., Maury, C., Symoneaux, R., and Siret, R. 2013. Sensory and instrumental texture measurements for assessing grape seed parameters during fruit development. *J. Sci. Food Agric*. **93**. 2531-2540.

Letaief, H., Rolle, L., and Gerbi, V. 2008a. Mechanical behavior of winegrapes under compression tests. *Am. J. Enol. Vitic*. **59**. 323-329.

Letaief, H., Rolle, L., Zeppa, G., and Gerbi, V. 2008b. Assessment of grape skin hardness by a puncture test. *J. Sci. Food Agric*. **88**. 1567-1575.

Mattivi, F., Vrhovsek, U., Masuero, D., and Trainotti, D. 2009. Differences in the amount and structure of extractable skin and seed tannins amongst red grape varieties. *Aust. J. Grape Wine R*. **15**. 27-35.

Mercurio, M. D., and Smith, P. A. 2008. Tannin quantification in red grapes and wine: comparison of polysaccharide- and protein-based tannin precipitation techniques and their ability to model wine astringency. *J. Agric. Food. Chem*. **56**. 5528-5537.

Moreno, J. J., Cerpa-Calderon, F., Cohen, S. D., Fang, Y., Qian, M., and Kennedy, J. A. 2008. Effect of postharvest dehydration on the composition of pinot noir grapes (Vitis vinifera L.) and wine. *Food Chem*. **109**. 755-762.

Obreque-Slier, E., Pena-Neira, A., Lopez-Solis, R., Zamora-Marin, F., Ricardo-da Silva, J. M., and Laureano, O. 2010. Comparative study of the phenolic composition of seeds and skins from Carmenere and Cabernet Sauvignon grape varieties (Vitis vinifera L.) during ripening. *J. Agric. Food. Chem*. **58**. 3591-3599.

Ortega-Regules, A., Romero-Cascales, I., Ros García, J. M., Bautista-Ortín, A. B., López-Roca, J. M., Fernández-Fernández, J. I., and Gómez-Plaza, E. 2008. Anthocyanins and tannins in four grape varieties (Vitis vinifera L.). Evolution of their content and extractability. *OENO One*. **42**. 147.

Peleg, H., Gacon, K., Schlich, P., and Noble, A. C. 1999. Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. *J. Sci. Food Agric.* **79**. 1123-1128.

Río Segade, S., Giacosa, S., Gerbi, V., and Rolle, L. 2011a. Berry skin thickness as main texture parameter to predict anthocyanin extractability in winegrapes. *LWT-Food Sci. Technol.* **44**. 392-398.

Río Segade, S., Orriols, I., Giacosa, S., and Rolle, L. 2011b. Instrumental Texture Analysis Parameters as Winegrapes Varietal Markers and Ripeness Predictors. *Int. J. Food Prop.* **14**. 1318-1329.

Río Segade, S., Rolle, L., Gerbi, V., and Orriols, I. 2008. Phenolic ripeness assessment of grape skin by texture analysis. *J. Food Compos. Anal.* **21**. 644-649.

Ristic, R., and Iland, P. G. 2005. Relationships between seed and berry development of Vitis Vinifera L. cv Shiraz: Developmental changes in seed morphology and phenolic composition. *Aust. J. Grape Wine R.* **11**. 43-58.

Robichaud, J. L., and Noble, A. C. 1990. Astringency and bitterness of selected phenolics in wine. *J. Sci. Food Agric.* **53**. 343-353.

Rodriguez-Pulido, F. J., Hernandez-Hierro, J. M., Nogales-Bueno, J., Gordillo, B., Gonzalez-Miret, M. L., and Heredia, F. J. 2014. A novel method for evaluating flavanols in grape seeds by near infrared hyperspectral imaging. *Talanta.* **122**. 145-150.

Rolle, L., Torchio, F., Lorrain, B., Giacosa, S., Río Segade, S., Cagnasso, E., Gerbi, V., and Teissedre, P.-L. 2012. Rapid methods for the evaluation of total phenol content and extractability in intact grape seeds of Cabernet-Sauvignon: instrumental mechanical properties and FT-NIR spectrum. *OENO One.* **46**. 29-40.

Rustioni, L., Cola, G., Van der Weide, J., Murad, P., Failla, O., and Sabbatini, P. 2018. Utilization of a freeze-thaw treatment to enhance phenolic ripening and tannin oxidation of grape seeds in red (Vitis vinifera L.) cultivars. *Food Chem.* **259**. 139-146.

Saint Cricq de Gaulejac, N., Vivas, N., and Glories, Y. 1998. Phenolic maturity: definition and control. *Rev. Fr. Oenol.* **173**. 22-25.

Saint-Cricq de Gaulejac, N., Augustin, M., Vivas, N., and Glories, Y. 2007. A biochemical approach to the evolution of procyanidins in grape seeds during the ripening of red grapes (Vitis vinifera l. cv. Merlot Noir). *J. Wine Res.* **8**. 159-167.

Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., Herderich, M. J., and Smith, P. A. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. *Aust. J. Grape Wine R.* **12**. 39-49.

Seddon, T. J., and Downey, M. O. 2008. Comparison of analytical methods for the determination of condensed tannins in grape skin. *Aust. J. Grape Wine R.* **14**. 54-61.

Strassmann, S., Passon, M., and Schieber, A. 2021. Chemical Hemisynthesis of Sulfated Cyanidin-3-O-Glucoside and Cyanidin Metabolites. *Molecules.* **26**.

Vallverdu-Queralt, A., Meudec, E., Eder, M., Lamuela-Raventos, R. M., Sommerer, N., and Cheynier, V. 2017. Targeted filtering reduces the complexity of UHPLC-Orbitrap-HRMS data to decipher polyphenol polymerization. *Food Chem.* **227**. 255-263.

Van der Weide, J., Forte, A., Peterlunger, E., Sivilotti, P., Medina-Meza, I. G., Falchi, R., Rustioni, L., and Sabbatini, P. 2020. Increase in seed tannin extractability and oxidation using a freeze-thaw treatment in cool-climate grown red (Vitis vinifera L.) cultivars. *Food Chem.* **308**. 125571.

Vidal, S., Francis, L., Guyot, S., Marnet, N., Kwiatkowski, M., Gawel, R., Cheynier, V. r., and Waters, E. J. 2003. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. *J. Sci. Food Agric.* **83**. 564-573.

Weber, F. 2022. Noncovalent Polyphenol-Macromolecule Interactions and Their Effects on the Sensory Properties of Foods. *J. Agric. Food. Chem.* **70**. 72-78.

# Chapter 5

## Concluding remarks

Polyphenols emerge as pivotal contributors to distinctive sensory attributes of wine. Because of their value for wine quality, winemakers always focus on polyphenol extraction from grape berries during fermentation. However, this pursuit begins even before fermentation, with winemakers striving to assess the inherent quality of their raw materials. The identification of effective methodologies and the comprehension of underlying principles governing the transfer of grape berry polyphenols to wines stand as the cornerstones for crafting exceptional wines. This thesis explored the impact of polysaccharide-polyphenol interactions during berry ripening on the wine composition (Chapters 2 and 3) and shed light on the importance of grape seeds in phenolic grape maturity (Chapter 4).

### 1 Evolution of the polysaccharide composition during berry ripening

During berry ripening, the cell wall structure and its constituent polysaccharides change (Gil et al., 2012). Enzymatic degradation during this process increases the solubility of polysaccharides and weakens the integrity of cell walls, resulting in berry softening (Nunan et al., 1998). The presented data unveils that the monomeric composition of polysaccharides remains relatively unaltered across diverse degrees of berry maturity. Similarly, the proportions of pectin, hemicellulose, and cellulose exhibit negligible changes throughout the ripening phase (Chapter 2). These findings imply a potential constancy in the polysaccharide composition of cell walls during berry ripening, as reported in the literature (Nunan et al., 1998; Vicens et al., 2009).

Nonetheless, the larger macromolecular structure of cell wall polysaccharides is undoubtedly impacted by the ripening process. Interestingly, the presented results suggest an increase in polysaccharide size with advanced grape maturity—a phenomenon not necessarily anticipated, given the general enzymatic degradation of cell wall structures during ripening. While pectic polysaccharides reportedly undergo depolymerization and structural

modifications (Barnavon et al., 2001; Nunan et al., 2001), leading to side chain loss and de-esterification of homogalacturonans, the presented measurements indicate an expansion in size within the assessed molecular size range. This counterintuitive observation might be attributed to the possibility that the measurements captured depolymerized polysaccharides originating from larger cell wall fragments. This, in turn, could have inflated the proportion of the measured large polysaccharide fraction.

Given that the polysaccharide structure significantly influences the interactions with polyphenols, structural changes of polysaccharides during berry ripening inevitably impact the extractability of polyphenols during fermentation. Surprisingly, interactions of the different polysaccharides with polyphenols varied immensely in Chapter 2, despite their molecular composition being so similar. With the only measurable differences being the size distribution of polysaccharides in the extract, the specific impact of the macromolecular structure may cause the variable interaction. Nevertheless, even the slightest variation of the molecular composition can change the structure of polysaccharides. Pectic polysaccharides, due to their branching structure, are particularly prone to structural variations that substantially affect polyphenol interactions.

However, the overall homogeneity observed in measurable molecular compositions underscores the stability of the cell wall's foundational structure. Although enzymatic processes exert modifications on polysaccharides during grape ripening, the fundamental molecular constituents remain largely unaltered. Moreover, enzymatic action might release previously unextracted soluble fragments, augmenting the entirety of soluble polysaccharides. Consequently, while grape ripening induces modifications in concentrations and sizes, the extractable grape cell wall polysaccharide composition remains relatively steady, particularly in the late growth stage.

## 2 Polyphenol-polysaccharide interactions

The extractability of polyphenols from grape berries during winemaking varies depending on grape maturity. Multiple factors impact the extraction of polyphenols, but polysaccharide-polyphenol interactions are likely the strongest. Although various publications analyzed the mechanisms of these interactions, their real-world consequences still need to be determined. This was specifically explored in Chapter 2 and Chapter 3.

### *2.1 Impact of maturity of polysaccharides on polyphenol-polysaccharide interactions*

The extractability of polyphenols from grape berries during winemaking is known to vary throughout the ripening process. Numerous factors affect the extraction of polyphenols, with polysaccharide-polyphenol interactions emerging as one of the most significant contributors. While multiple publications have delved into the mechanisms of these interactions, their real-world implications remain uncertain. Therefore, Chapter 2 specifically explores the influence of grape maturity on polysaccharide-polyphenol interactions and their subsequent impact on the phenolic composition of red wines.

These experiments revealed a pivotal role played by the structural variations in grape cell wall polysaccharides, contingent upon grape variety and maturity. These structural disparities exert distinct effects on different polyphenol classes. Notably, proanthocyanidins and monomeric flavan-3-ols exhibited substantial interactions with grape cell wall polysaccharides across both grape varieties. In contrast, anthocyanin concentrations were minimally affected by polysaccharide interactions, and this influence was primarily observed in Cabernet Sauvignon. Surprisingly, even though pectin-anthocyanin interactions are well documented, they did not significantly impact anthocyanin extraction or stability during the winemaking process.

In unpublished experiments that utilized an ultrasound-based extraction method, variations in tannin and anthocyanin extractability were observed, ranging from 50% to 90% during berry ripening. These variations were analyzed across different years and at various stages of berry maturity. In the same vintage when polysaccharides were extracted for later use (as discussed in Chapter 2), tannin extractability increased while anthocyanin extractability remained constant. Similarly, one year later, when the extracted polysaccharides were added to new grape must (as outlined in Chapter 2), the extractability of anthocyanins remained constant, while tannin extractability changed in relation to the added polysaccharides. This finding strongly indicates that the changing composition of polysaccharides during grape ripening significantly influences the extractability of polyphenols. Essentially, extractability involves both the physical extraction process limited by cell structure and the stability of polyphenols influenced by interactions with polysaccharides.

The extraction and stability of polyphenols during winemaking are significantly influenced by cell wall polysaccharides. The addition of varying polysaccharides alters the measurable polyphenol concentrations, leading to varying consequences for the analyzed wines.

Consequently, the macromolecular structure of polysaccharides affects the affinity and effects of interactions with polyphenols. This mirrors the well-documented formation of stable complexes between anthocyanins and pectin (Buchweitz et al., 2013; Larsen et al., 2019).

The stability of polysaccharide-polyphenol complexes hinges on attributes like side chains and branching within polysaccharides (Larsen et al., 2019). Similarly, the evolving size of extractable polysaccharides during berry ripening substantially influences interactions, either fortifying or precipitating polyphenols. In Pinot noir, characterized by fewer large soluble polysaccharides (≥162.1 kDa), flavan-3-ols and tannins experience stabilization, whereas in Cabernet Sauvignon wines—enriched in larger polysaccharides—precipitation tendencies arise. It is possible that added polysaccharides interact with tannins or flavan-3-ol monomers in a way that shields them from oxidation or polymerization. Additionally, as demonstrated by Watrelot et al. (2017), tannin-polysaccharide complexes are more likely to precipitate when subjected to BSA in the assay used for tannin quantification. This raises the question whether grape cell wall polysaccharides not only alter the observable concentration of tannins but further affect the sensory impact of newly formed complexes. Interestingly, these protective interactions were only observed in Pinot noir wines. It is conceivable that the initially higher concentration of monomeric flavan-3-ols extracted from seeds, as seen in Chapter 4, may account for this effect, but further research is necessary to confirm this hypothesis.

Although it is commonly assumed that the extractability of anthocyanins increases with higher grape berry maturity (del Llaudy et al., 2007), with the analyzed samples, this assumption could not be confirmed. Anthocyanin concentrations were primarily influenced by their concentration in the grapes. However, a small proportion of anthocyanins did form complexes with added cell wall polysaccharides that precipitated during Cabernet Sauvignon must fermentation. By the end of fermentation, this initial effect was offset by the natural precipitation or oxidation of anthocyanins. Therefore, this temporary effect merely accelerated the naturally stable proportion of anthocyanins extracted during winemaking.

In the case of Pinot noir wines, their composition remained consistent regardless of grape berry maturity. This suggests that winemakers could produce Pinot noir wines with similar polyphenol concentrations at each stage of berry ripening. However, ethanol concentrations were adjusted across samples. Without additional sugar to achieve higher ethanol concentrations, some tannins and monomeric flavan-3-ols may not have been adequately extracted. Furthermore, while the concentrations of the analyzed polyphenols were similar, proanthocyanidins likely varied in molecular size and structure between the different wines. On

the other hand, the composition of Cabernet Sauvignon wines exhibited the expected changes during berry ripening, including a decrease in flavan-3-ol monomer concentrations, consistent with the declining seed concentrations discussed in Chapter 4. Anthocyanin and tannin concentrations increased as berry ripening progressed, along with the non-precipitable polyphenol concentration, which correlated with the anthocyanin concentration. In terms of polyphenol concentration, Cabernet Sauvignon wine quality improved during berry ripening. However, the artificially increased ethanol concentration could not fully compensate for the lower concentration or extractability of polyphenols from Cabernet Sauvignon berries. Surprisingly, the grape variety at different ripening stages did not have an observable effect on polyphenol-polysaccharide interactions. Nevertheless, there were indications that a higher concentration of specific polyphenols, with a greater affinity for interactions with polysaccharides, may occupy those polysaccharides, potentially influencing the concentration of other polyphenols.

For winemakers, the management of polysaccharide composition may hold the key to influencing polyphenol extractability and stability. The use of enzymes or other methods to alter polysaccharide structure, such as ultrasonication (Larsen et al., 2019; Nemetz et al., 2023), or the adjustment of ethanol concentrations during winemaking, could prove valuable for improving wine composition. Furthermore, enhanced harvest management practices can mitigate the impact of polysaccharide maturity on polyphenol extractability. However, this approach has its limitations. Firstly, interactions are specific to grape variety, and they may vary between vintages. Secondly, analytical assessment is imperative for informed decision-making during winemaking. Unfortunately, there are no accessible approaches for polysaccharide analysis, necessitating the development of specific methods capable of revealing variations in extractability. Lastly, there is a substantial research gap regarding the sensory impact of stable polyphenol-polysaccharide complexes (Weber, 2022). Even if a method were developed to alter polyphenol extractability influenced by interactions with polysaccharides, the resulting changes in wine sensory experiences might diverge significantly from expectations.

### *2.2 Formation of polymeric pigments and the role of pectic polysaccharides in wine color stability*

The color profile of red wines is inextricably linked to the formation of polymeric pigments over the aging process. As wines mature, the vibrant red hues attributed to anthocyanins tend

to fade. However, through a series of complex reactions, anthocyanins can give rise to pigments that not only maintain a red hue but also exhibit remarkable stability over time. These polymeric pigments are primarily composed of anthocyanins and other polyphenols, particularly proanthocyanidins. Recent research has shed light on the formation of stable complexes between anthocyanins and pectic polysaccharides, sharing chemical characteristics akin to non-precipitable polymeric pigments (Graves and Sommer, 2021; Larsen et al., 2019). These findings broaden the conventional definition of polymeric pigments in wines, previously categorized solely based on chemical attributes.

Crucially, the formation of these pigments hinges on the presence of pectic polysaccharides. Notably, cellulose fibrils did not exhibit interactions with anthocyanins in fermenting must, as suggested by unpublished data. However, in the presence of pectic polysaccharides, anthocyanins form various complexes during the winemaking process. Initially, the strong affinity between anthocyanins and pectic polysaccharides, coupled with limited competition, results in the formation of an abundance of complexes with attributes known from solely phenolic polymeric pigments (Chapter 3). However, not all of these complexes are stable. The evolving matrix conditions during fermentation lead to a significant reduction in the concentration of these complexes. Whether this decrease is attributed to the heightened ethanol concentration, increased extraction of other polyphenols, particularly proanthocyanidins, or other condition changes such as temperature fluctuations, warrants further investigation. Nonetheless, a smaller yet noteworthy concentration of anthocyanin-pectin complexes remains stable and soluble in wines even after a one-year bottle aging period. The experiments revealed that purified commercial pectin as well as grape-derived polysaccharide extracts form polymeric pigments with anthocyanins. Similar observations were made by Weilack et al. (2023), who showed that polysaccharides impact the wine pigment composition.

It is noteworthy that polymeric pigments can only be quantified completely through spectrophotometric methods that distinguish them based on physico-chemical characteristics rather than their actual structure. Consequently, distinguishing between various polymeric pigments remains a challenge at present. Both types of polymeric pigments share specific traits, such as resistance to protein precipitation, bisulfite bleaching, and, notably, their red coloration. Therefore, future research in the realm of polymeric pigments should prioritize the exploration of distinctive differences among them, particularly focusing on their stability and sensory characteristics. To achieve a distinctive analysis, measuring polymeric pigments might

necessitate additional purification steps. One plausible approach could involve the use of pectinase to disrupt anthocyanin-pectin complexes.

In practical winemaking applications, the addition of pectic polysaccharides could emerge as a valuable tool for controlling the formation of polymeric pigments and ensuring long-term color stability. However, it's important to note that a reduction in tannin concentration might be an unintended consequence. For slightly underripe grape berries, which already contain elevated anthocyanin levels but lack the desired sensory attributes of proanthocyanidins, pectic polysaccharides could aid in precipitating unwanted tannins while stabilizing anthocyanins. Therefore, in cooler climate zones, in particular, the influence of pectic polysaccharides could prove highly beneficial.

## 3 The impact of grape seed polyphenols on wine composition

Grape seeds play a pivotal role in shaping the polyphenolic profile of wine, as they contain most of the polyphenols in a grape berry. However, only a fraction of these polyphenols is extracted during winemaking. Most seeds remain intact during winemaking, prohibiting the extraction of their polyphenols. Winemakers generally welcome this limited extraction as seed polyphenols often exhibit less desirable sensory characteristics. Nonetheless, a portion of these compounds does find their way into the final wine, prompting winemakers to carefully consider the optimal harvest alignment for achieving the desired seed polyphenol composition.

Throughout berry ripening, the composition and the concentration of seed polyphenols undergo significant changes. In the final growth phase, which is the focus of this thesis, the concentration of seed polyphenols experiences a decline before stabilizing. The expression of genes responsible for proanthocyanidin synthesis is primarily confined to the early ripening stages (Bogs et al., 2005). Subsequently, pronthocyanidin concentrations decrease as seen here or even more drastically as reported by Kennedy et al. (2000a). There have not been any reports on whether there is a specific minimum polyphenol concentration in grape seeds, but it is consensus that a stagnating polyphenol concentration marks the end of phenolic maturation (Downey et al., 2003; Kennedy et al., 2000a; Kennedy et al., 2000b).

In the analyzed Pinot noir seeds, polyphenol concentrations decreased during berry ripening, whereas in Cabernet Sauvignon seeds, they remained nearly constant. Although the changes in Pinot noir seeds were more pronounced, concentrations of nearly all analyzed polyphenols were higher than in Cabernet Sauvignon seeds. Seed polyphenol composition is inherently specific to grape variety (Chira et al., 2011), and as evidenced here, so are the changes that occur during berry ripening. Specific to Cabernet Sauvignon seeds was a

comparatively high concentration of digallates, while Pinot noir wines contained much higher concentrations of all non-galloylated flavan-3-ols. In both varieties, the concentrations of galloylated proanthocyanidins and proanthocyanidins with a degree of polymerization below three decreased most notably. Given that both galloylated and smaller proanthocyanidin oligomers are associated with negative bitter and astringent sensory attributes, (Ferrer-Gallego et al., 2010) the overall quality of grape seed polyphenols improved during berry ripening.

However, Bautista-Ortín et al. (2014) raised concerns, that it is not the composition or concentration of all proanthocyanidins in the seeds but only the extractable proanthocyanidins, that impact the sensorial perception. Extracting seed flavan-3-ols requires sufficient time and ethanol concentrations. While skin flavan-3-ol monomers are completely extracted after 24 h, their extraction from the seeds continues for three more weeks (González-Manzano et al., 2004). A higher ethanol concentration (> 15 % v/v) in the must improves further the extraction of seed proanthocyanidins (Harbertson et al., 2009). Despite seeds contributing the majority of flavan-3-ols in wines, transferring the seed polyphenol composition directly to the wine composition is unfeasible due to this limited extractability. Bindon et al. (2010a) reported an extractable polyphenol proportion of only 30 % from seeds. Grape cell wall material binds up to 47 % of seed proanthocyanidins and especially galloylated proanthocyanidins (Bindon et al., 2010b). During ripening the extractability reduces further (del Llaudy et al., 2007) possibly caused by changing interactions with cell wall polysaccharides as seen in Chapter 2.

Based on the idea that the phenolic maturity of grape berries improves when the extractable concentration of grape seed polyphenols, which mainly consists of larger flavan-3-ol oligomers, is lower, the findings indicate that phenolic maturity tends to increase during later ripening stages. However, these observed changes are not as pronounced as reported in some studies, and they might not be substantial enough to significantly impact the overall sensory perception of wines.

Despite the negative associations that winemakers may have about grape seed polyphenols, they can implement strategies to optimize both the harvest and winemaking processes. This optimization ensures a controlled concentration and extraction of seed polyphenols, potentially addressing concerns and maintaining the desired quality of the final wine product.

### *3.1 Analyzing grape seeds as a tool for assessing phenolic maturity*

Grape seeds obtained a prominent yet controversial role among winemakers. According to Glorie's initial proposition for assessing phenolic maturity, achieving grape phenolic maturity was linked to low concentrations of seed polyphenols and high levels of skin tannins. As a result, harvest management accounted for the seeds' polyphenols. Then, with the introduction of Ristic and Iland (2005)'s concept proposing a connection between grape phenolic maturity and the seed's physiological characteristics, seeds became probably the most used indicator for phenolic maturity in practice. Because, analyzing seeds was such a simple approach, winemakers diverted toward only analyzing seeds, disregarding the important skin tannins. This overemphasis on seed analysis was motivated by the high concentrations of monomeric and small oligomeric flavan-3-ols found in seeds, whose extraction during winemaking could significantly impact wine characteristics, often in a negative way.

Nevertheless, whether the physiological characteristics of seeds can accurately reflect phenolic composition and extractability remained relatively unexplored, particularly in cool climate zones. Despite this, winemakers in these regions continued to rely on seed analysis as a primary tool for determining phenolic maturity.

The results obtained in this thesis shed light on the dynamics of seeds' physiological characteristics during the late growth phase of grape berries. It is worth noting that these characteristics may develop more significantly earlier in the grape's growth, but at that point, the grapes are not yet considered technologically mature and are thus not considered for harvest. Detecting these subtler changes requires specialized equipment, raising questions about the practicality of such precise assessments for practitioners who typically rely on sensory evaluation. Additionally, the high variability between seeds from different berries underscores the necessity of a meticulous sampling method. As discussed in Chapter 4, these physiological changes may indicate a reduced extractability of seed polyphenols due to the hardening of the seed coat or an increased oxidation of polyphenols in the coat, as evidenced by browning. However, both characteristics developed differently in the presented results, making it challenging for winemakers to make clear decisions solely based on seed analysis.

While the phenolic composition within the seeds exhibited minimal variation, with more pronounced changes observed in Pinot noir seeds than in Cabernet Sauvignon seeds, no definitive date emerged that could pinpoint the optimum phenolic maturity.

Spectrophotometric analysis of tannins remains the most practical method for obtaining quantitative measurements. The choice between bovine serum albumin or methylcellulose for

tannin precipitation may vary from case to case. However, the use of methyl cellulose may provide a more comprehensive overview as it precipitates smaller flavan-3-ol oligomers, which are particularly associated with negative sensory characteristics. Advanced analytical methods involving MS offer a more detailed insight into grape seed polyphenols, but these methods come with significant trade-offs. Firstly, these measurements are not all-encompassing and are limited by the degree of polymerization of the polyphenols and the structural variability in their results. Secondly, these methods are not readily accessible to the wine industry at large. While these analyses may not be a practical option for winemakers, they do offer valuable insights into the structural diversity of phenolic compounds in grape seeds. The incorporation of ion mobility separation emerges as a valuable tool for gaining deeper insights into proanthocyanidins and their evolving impact on astringency throughout berry ripening. My findings highlight notable changes in ion mobility, particularly observed in larger oligomers. The variation in ion mobility is contingent on the ion's conformation, potentially influencing the interactions of proanthocyanidins and, consequently, impacting sensory characteristics among different proanthocyanidins. While this approach is relatively novel in proanthocyanidin characterization, the results suggest the potential utility of ion mobility chromatography in enhancing our understanding of the correlation between proanthocyanidin structure and its sensory effects.

Although the phenolic composition exhibited consistent changes in the same direction as the physiological characteristics, the two progressed at different rates. Because the proportion of grape seeds per berry reduces during ripening, the impact of grape seeds diminishes. This fundamental yet crucial change underscores that relying solely on grape seeds to determine overall grape phenolic maturity becomes increasingly inaccurate as maturity advances. Though there are a number of published methods that measure phenolic grape maturity by grape seed analysis, those might just not suit the development in cool climate zones. As an alternative, I propose analyzing the entire berry using specific extraction methods. However, these methods necessitate specialized laboratory equipment and may not be feasible for every winemaker. For a more straightforward and practical approach, a simple weight measurement that assesses the proportion of seeds in the berries could be valuable. The presented results indicate a dramatic change in this proportion, directly affecting the seed polyphenol concentration in the berry, which, in comparison, remained relatively stagnant.

# 4 Conclusions

Defining phenolic grape maturity has been a longstanding challenge in grape and wine research, spanning several decades. This thesis has centered its focus on understanding the concentrations of polyphenols in grape berries during the ripening process and their subsequent extraction during winemaking. It is my contention that the combination of these two critical parameters should serve as the foundation for defining phenolic grape maturity. This holistic perspective is crucial because the interplay between polyphenol concentrations and their extractability profoundly influences wine composition.

The extractability of polyphenols is a complex phenomenon influenced by intricate interactions between polyphenols and other cellular components during maceration and fermentation. The vast diversity of molecular structures within polyphenols and their interaction partners, such as polysaccharides, results in a myriad of possibilities that can impact the extraction and stability of polyphenols. Interactions between polysaccharides and polyphenols can both enhance and restrict the polyphenol concentrations in wines, as I have demonstrated.

The structural nuances of grape cell wall polysaccharides have a significant influence on their interactions with polyphenols, a topic thoroughly explored in this research. Even minor variations in molecular composition lead to substantial changes in polysaccharide structure, subsequently affecting their interactions with polyphenols. The impact of grape maturity on polysaccharide-polyphenol interactions provided intriguing insights. The structural differences in grape cell wall polysaccharides, influenced by grape variety and maturity, had distinct effects on different polyphenol classes. Proanthocyanidins and monomeric flavan-3-ols displayed substantial interactions with grape cell wall polysaccharides in both grape varieties, while anthocyanin concentrations were less affected by these interactions. However, the role of pectic polysaccharides in the formation of polymeric pigments from anthocyanins was discovered as a new contribution to wine color formation and stabilization. The stable complexes between anthocyanins and pectic polysaccharides, sharing characteristics with non-precipitable polymeric pigments, challenged conventional definitions of pigment formation in red wines, contributing to an extended understanding of polyphenol interactions during winemaking and aging. Because the relationship between polyphenols and polysaccharides is a complex, multifaceted interplay that significantly shapes the quality and characteristics of wines. As our understanding of these interactions continues to evolve, winemakers have the potential to employ innovative strategies to enhance wine quality, harnessing polyphenols and polysaccharides to craft wines that captivate the senses and fascinate wine enthusiasts.

Grape seeds have emerged as pivotal contributors to the intricate polyphenolic composition of wine. However, the process of extracting these polyphenols during winemaking remains a nuanced challenge. A limited extraction is generally welcomed by winemakers, given that seed polyphenols often exhibit sensory characteristics that are less desirable. Nevertheless, a fraction of these compounds ends up in the final wine, necessitating careful consideration of the optimal harvest conditions to achieve the desired seed polyphenol profile.

The investigation into the dynamics of seed polyphenols throughout the ripening of grape berries has unveiled a complex narrative. During the late growth phase, which has been the focal point of this research, I observed a decline in seed polyphenol concentrations, followed by a stabilization period. The genetic expression governing proanthocyanidin synthesis primarily manifests in the early ripening stages, leading to subsequent reductions in proanthocyanidin levels.

This comparative analysis of Pinot noir and Cabernet Sauvignon seeds highlighted varietal-specific responses during berry ripening. Pinot noir seeds displayed more pronounced changes, albeit maintaining higher concentrations of nearly all analyzed polyphenols compared to Cabernet Sauvignon seeds. The inherent specificity of seed polyphenol composition to grape variety is evident in these findings. Specifically, Cabernet Sauvignon seeds exhibited a notably high concentration of digallates, whereas Pinot noir seeds contained considerably higher levels of non-galloylated flavan-3-ols. Both varieties demonstrated significant reductions in the concentrations of galloylated proanthocyanidins and proanthocyanidins with a degree of polymerization below three. Given the associations of both galloylated and smaller proanthocyanidin oligomers with negative sensory attributes, my research underscores the overall enhancement in the quality of grape seed polyphenols during berry ripening.

Nevertheless, it is not only the composition or concentration of all proanthocyanidins within seeds that influences wine sensory perception, but rather the subset of extractable proanthocyanidins. Extracting flavan-3-ols from seeds entails specific conditions, including sufficient time and ethanol concentrations. My findings corroborate previous research, indicating an extractable polyphenol proportion of approximately 30% from seeds, with grape cell wall material binding a substantial portion of seed proanthocyanidins, particularly the galloylated variants. This reduced extractability is further compounded during ripening, possibly due to evolving interactions with cell wall polysaccharides. Under the premise that phenolic maturity of grape berries can be optimized with lower extractable concentrations of

grape seeds, primarily containing larger flavan-3-ol oligomers, the presented results suggest that phenolic maturity is more pronounced in later ripening stages.

While there exists considerable interest among researchers in unraveling the complexities of phenolic grape maturity and elucidating the underlying mechanisms, it is crucial to emphasize that this concept should primarily serve as a practical tool for winemakers. Much like the measurement of technological maturity, the concept of phenolic maturity should be harnessed to guide grape harvest and processing decisions. Therefore, I advocate that phenolic grape maturity be defined through the measurement of both total polyphenol concentrations within grape berries and the proportion of these polyphenols that can be easily extracted through consecutive extractions. This approach reveals the balance between potential interactions that may limit extraction and the opportunities for intervention through specific winemaking techniques. Ultimately, this pragmatic approach empowers winemakers to make informed decisions, enhancing their ability to craft wines of exceptional quality.

**References**

Barnavon, L., Doco, T., Terrier, N., Ageorges, A., Romieu, C., and Pellerin, P. 2001. Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. *Phytochemistry*. **58**. 693-701.

Bautista-Ortín, A. B., Busse-Valverde, N., López-Roca, J. M., Gil-Muñoz, R., and Gómez-Plaza, E. 2014. Grape seed removal: effect on phenolics, chromatic and organoleptic characteristics of red wine. *Int. J. Food Sci. Technol.* **49**. 34-41.

Bindon, K. A., Smith, P. A., Holt, H., and Kennedy, J. A. 2010a. Interaction between grape-derived proanthocyanidins and cell wall material. 2. Implications for vinification. *J. Agric. Food. Chem.* **58**. 10736-10746.

Bindon, K. A., Smith, P. A., and Kennedy, J. A. 2010b. Interaction between grape-derived proanthocyanidins and cell wall material. 1. Effect on proanthocyanidin composition and molecular mass. *J. Agric. Food. Chem.* **58**. 2520-2528.

Bogs, J., Downey, M. O., Harvey, J. S., Ashton, A. R., Tanner, G. J., and Robinson, S. P. 2005. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol.* **139**. 652-663.

Buchweitz, M., Speth, M., Kammerer, D. R., and Carle, R. 2013. Stabilisation of strawberry (Fragaria x ananassa Duch.) anthocyanins by different pectins. *Food Chem.* **141**. 2998-3006.

Chira, K., Lorrain, B., Ky, I., and Teissedre, P. L. 2011. Tannin composition of cabernet-sauvignon and merlot grapes from the bordeaux area for different vintages (2006 to 2009) and comparison to tannin profile of five 2009 vintage mediterranean grapes varieties. *Molecules*. **16**. 1519-1532.

del Llaudy, M. C., Canals, R., Canals, J. M., and Zamora, F. 2007. Influence of ripening stage and maceration length on the contribution of grape skins, seeds and stems to phenolic composition and astringency in wine-simulated macerations. *Eur. Food Res. Technol.* **226**. 337-344.

Downey, M. O., Harvey, J. S., and Robinson, S. P. 2003. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine R.* **9**. 15-27.

Ferrer-Gallego, R., Garcia-Marino, M., Hernandez-Hierro, J. M., Rivas-Gonzalo, J. C., and Escribano-Bailon, M. T. 2010. Statistical correlation between flavanolic composition, colour and sensorial parameters in grape seed during ripening. *Anal. Chim. Acta*. **660**. 22-28.

Gil, M., Kontoudakis, N., Gonzalez, E., Esteruelas, M., Fort, F., Canals, J. M., and Zamora, F. 2012. Influence of grape maturity and maceration length on color, polyphenolic composition, and polysaccharide content of Cabernet Sauvignon and Tempranillo wines. *J. Agric. Food. Chem.* **60**. 7988-8001.

González-Manzano, S., Rivas-Gonzalo, J. C., and Santos-Buelga, C. 2004. Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Anal. Chim. Acta*. **513**. 283-289.

Graves, J., and Sommer, S. 2021. Polysaccharides Influence the Results of Polymeric Pigment Analysis in Red Wines. *Food Sci. Technol.* **1**. 1770-1775.

Harbertson, J. F., Mireles, M. S., Harwood, E. D., Weller, K. M., and Ross, C. F. 2009. Chemical and sensory effects of saignée, water addition, and extended maceration on high brix must. *Am. J. Enol. Vitic.* **60**. 450-460.

Kennedy, J. A., Matthews, M. A., and Waterhouse, A. L. 2000a. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry*. **55**. 77-85.

Kennedy, J. A., Troup, G. J., Pilbrow, J. R., Hutton, D. R., Hewitt, D., Hunter, C. R., Ristic, R., Iland, P. G., and Jones, G. P. 2000b. Development of seed polyphenols in berries from Vitis vinifera L. cv. Shiraz. *Aust. J. Grape Wine R.* **6**. 244-254.

Larsen, L. R., Buerschaper, J., Schieber, A., and Weber, F. 2019. Interactions of anthocyanins with pectin and pectin fragments in model solutions. *J. Agric. Food. Chem.* **67**. 9344-9353.

Nemetz, N. J., Winter, A. R., Hensen, J.-P., Schieber, A., and Weber, F. 2023. Toward gentle chokeberry juice production by ultrasound-assisted enzymatic maceration. *Curr. Res. Food Sci.* 100518.

Nunan, K. J., Davies, C., Robinson, S. P., and Fincher, G. B. 2001. Expression patterns of cell wall-modifying enzymes during grape berry development. *Planta*. **214**. 257-264.

Nunan, K. J., Sims, I. M., Bacic, A., Robinson, S. P., and Fincher, G. B. 1998. Changes in cell wall composition during ripening of grape berries. *Plant Physiol*. **118**. 783-792.

Ristic, R., and Iland, P. G. 2005. Relationships between seed and berry development of Vitis Vinifera L. cv Shiraz: Developmental changes in seed morphology and phenolic composition. *Aust. J. Grape Wine R.* **11**. 43-58.

Vicens, A., Fournand, D., Williams, P., Sidhoum, L., Moutounet, M., and Doco, T. 2009. Changes in polysaccharide and protein composition of cell walls in grape berry skin (cv. Shiraz) during ripening and over-ripening. *J. Agric. Food. Chem.* **57**. 2955-2960.

Watrelot, A. A., Schulz, D. L., and Kennedy, J. A. 2017. Wine polysaccharides influence tannin-protein interactions. *Food Hydrocoll*. **63**. 571-579.

Weber, F. 2022. Noncovalent Polyphenol-Macromolecule Interactions and Their Effects on the Sensory Properties of Foods. *J. Agric. Food. Chem.* **70**. 72-78.

Weilack, I., Mehren, L., Schieber, A., and Weber, F. 2023. Grape-derived pectic polysaccharides alter the tannin and pigment composition of Cabernet Sauvignon red wines. *Curr. Res. Food Sci.* **6**. 100506.

## Summary

Red wines contain a variety of polyphenols that majorly impact sensorial characteristics. Because the quality of red wines depends on their polyphenol content, winemakers focus significantly on polyphenol extraction. Polyphenols are extracted from grape berries during the maceration and fermentation processes during winemaking. However, the extraction is always limited by the concentration of polyphenols in the grape and their interactions during and after the extraction. Both parameters change during berry ripening, propelling winemakers to optimize the harvest schedule toward an ideal availability of polyphenols from the grape berry. Wine researchers established phenolic grape maturity as the measure combining the extractability and composition of polyphenols in grape berries. Various complex factors influence phenolic grape maturity, but interactions between polyphenols and macromolecules potentially have the most significant impact on the extraction of polyphenols.

This thesis analyzes multiple factors that change phenolic maturity in Cabernet Sauvignon and Pinot noir berries. Grape seeds were analyzed as a significant contributor to the grape phenolic content to reveal their impact on phenolic grape maturity. Besides, the effect of grape cell wall polysaccharides on polyphenol extraction during fermentation was studied.

Overall, seeds changed very little in the last stage of berry ripening. Despite their widely accepted use as indicators for phenolic grape maturity, there were only slight changes in measurable physiological characteristics that would have been related to changes in the phenolic composition. Thus, at least in cool climate zones, using grape seeds to determine grape phenolic maturity is not recommended based on the presented results. The concentration of polyphenols generally decreased. Especially the concentrations of galloylated flavan-3-ols in Pinot noir seeds were reduced. However, the overall proportion of the seeds in the berry decreased even more significantly, thereby limiting the broad impact seeds had on the overall berry composition. Because seed polyphenols are widely considered to detriment wine quality, the changing phenolic composition and seed proportion in the berry could benefit an improved wine quality.

Polymeric pigments are thought to be reaction products of anthocyanins and flavan-3-ols, mostly proanthocyanidins. They are essential in red wines because they preserve the red color for years. Anthocyanins are generally unstable in the conditions present in wines. Polymerization with other polyphenols is thought to increase their stability while mostly

keeping the color. Because anthocyanins also have a high affinity for interactions with pectic polysaccharides, the formation of anthocyanin-pectin complexes during fermentation is likely and might stabilize anthocyanins similarly. In the presented studies, a high concentration of these complexes initially formed at the beginning of fermentation, but only a fraction remained stable until the end. Due to an increasing ethanol concentration or competition with other compounds in the must, anthocyanins were again released from the complex. However, a limited proportion of stable complexes remained in the wine after one year of aging.

Phenolic maturity describes the concentration and extractability of polyphenols from grape berries. Polysaccharide interactions with polyphenols significantly impact the extractability during grape ripening. The changing macromolecular composition and the grape varietal differences of cell wall polysaccharides during ripening affect the extractability. Generally, monomeric flavan-3-ol and tannin concentrations were more sensitive to interactions with grape cell wall polysaccharides than anthocyanins. In Pinot noir wines, their measurable concentrations were increased, and decreased in Cabernet Sauvignon wines due to polysaccharide-polyphenol interactions.

This thesis proves that a changing grape cell wall composition during berry ripening causes variance in polyphenol extractability depending on the grape variety. Polysaccharides are, therefore, the main factor that impacts phenolic maturity, which relies on the extractability of polyphenols from the berry. Despite the popularity of using seeds to determine grape phenolic maturity, the present studies recommend focusing less on seed characteristics but on the proportion of seeds in the berry to assess their contribution to the wine.

## Zusammenfassung

Rotweine enthalten eine Vielzahl von Polyphenolen, die einen großen Einfluss auf die sensorischen Eigenschaften haben. Da die Qualität von Rotweinen von ihrem Polyphenolgehalt abhängt, legen Winzer großen Wert auf die Polyphenolextraktion. Polyphenole werden während der Mazeration und der Gärung bei der Weinherstellung aus den Traubenbeeren extrahiert. Die Extraktion wird jedoch immer durch die Konzentration der Polyphenole in der Traube und Wechselwirkungen während und nach der Extraktion begrenzt. Beide Parameter ändern sich während der Reifung der Beeren, was Winzer dazu veranlasst, den Lesezeitpunkt hinsichtlich einer idealen Verfügbarkeit von Polyphenolen aus der Beere zu optimieren. Weinforscher haben als Parameter für Extrahierbarkeit und Konzentration von Polyphenolen in den Traubenbeeren die phenolische Traubenreife festgelegt. Verschiedene komplexe Faktoren beeinflussen die phenolische Traubenreife, wobei Wechselwirkungen zwischen Polyphenolen und Makromolekülen möglicherweise den größten Einfluss auf die Extraktion von Polyphenolen haben.

In dieser Arbeit werden mehrere Faktoren analysiert, die den phenolischen Reifegrad von Cabernet Sauvignon- und Spätburgunderbeeren beeinflussen. Traubenkerne wurden als Faktor für den Phenolgehalt der Trauben analysiert, um ihren Einfluss auf die phenolische Reife der Trauben zu ermitteln. Außerdem wurde der Einfluss der Zellwandpolysaccharide der Trauben auf die Polyphenolextraktion während der Gärung untersucht.

Insgesamt haben sich die Kerne in der letzten Phase der Beerenreife nur wenig verändert. Trotz ihrer weithin akzeptierten Verwendung als Indikator für die phenolische Traubenreife gab es nur geringe Veränderungen bei messbaren physiologischen Merkmalen, die mit Veränderungen in der phenolischen Zusammensetzung in Zusammenhang stehen könnten. Daher wird zumindest in kühlen Klimazonen die Verwendung von Traubenkernen zur Bestimmung der phenolischen Reife von Trauben in dieser Arbeit nicht empfohlen. Die Konzentration der Polyphenole nahm allgemein ab. Insbesondere die Konzentrationen der galloylierten Flavan-3-ole in den Kernen des Spätburgunders nahmen ab. Der Gesamtanteil der Kerne an der Beere nahm jedoch noch deutlicher ab, so dass der Einfluss der Kerne auf die Gesamtzusammensetzung der Beeren zunehmend reduziert wurde. Da die Polyphenole in den Kernen weithin als nachteilig für die Weinqualität angesehen werden, könnten die veränderte phenolische Zusammensetzung und der Anteil der Kerne in der Beere einer verbesserten Weinqualität zugutekommen.

Man geht davon aus, dass polyphenolische Pigmente Reaktionsprodukte von Anthocyanen und Flavan-3-olen, hauptsächlich Proanthocyanidinen, sind. Sie sind in Rotweinen unerlässlich, da sie die rote Farbe über Jahre hinweg bewahren. Anthocyane sind im Allgemeinen unter den im Wein herrschenden Bedingungen instabil. Es wird angenommen, dass die Polymerisation mit anderen Polyphenolen ihre Stabilität erhöht, während die Farbe weitgehend erhalten bleibt. Da Anthocyane auch eine hohe Affinität zu Wechselwirkungen mit pektischen Polysacchariden haben, ist die Bildung von Anthocyan-Pektin-Komplexen während der Gärung wahrscheinlich und könnte die Anthocyane in ähnlicher Weise stabilisieren. In den vorgestellten Studien bildete sich zu Beginn der Fermentation eine hohe Konzentration dieser Komplexe, aber nur ein Teil blieb bis zum Ende der Fermentation stabil. Mit zunehmender Ethanolkonzentration oder durch Konkurrenz mit anderen Verbindungen im Most wurden die Anthocyane wieder aus dem Komplex freigesetzt. Ein begrenzter Anteil an stabilen Komplexen blieb jedoch nach einem Jahr Reifung im Wein erhalten.

Die Wechselwirkungen zwischen Polysacchariden und Polyphenolen beeinflussten die Extrahierbarkeit von Polyphenolen während der Traubenreifung erheblich. Die sich während der Reifung verändernde makromolekulare Zusammensetzung und die sortentypischen Unterschiede der Zellwandpolysaccharide beeinflussen die Extrahierbarkeit und damit die phenolische Reife. Im Allgemeinen reagierten die Konzentrationen monomerer Flavan-3-ole und Tannine empfindlicher auf Wechselwirkungen mit den Zellwandpolysacchariden als die Anthocyane. In Spätburgunderweinen waren ihre messbaren Konzentrationen erhöht und in Cabernet Sauvignonweinen aufgrund von Polysaccharid-Polyphenol-Wechselwirkungen verringert.

Diese Arbeit zeigt, dass eine veränderte Zusammensetzung der Traubenzellwand während der Beerenreife zu einer unterschiedlichen Extrahierbarkeit von Polyphenolen je nach Rebsorte führt. Polysaccharide haben daher einen Einfluss auf die phenolische Reife, die von der Extrahierbarkeit der Polyphenole aus der Beere abhängt. Trotz der Beliebtheit der Verwendung von Kernen zur Bestimmung der phenolischen Reife legen die vorliegenden Studien nahe, sich weniger auf die Merkmale der Kerne als vielmehr auf den Anteil der Kerne in der Beere zu konzentrieren, um ihren Beitrag zum Wein zu bewerten.

## Acknowledgement

I would like to express my sincere thanks to everyone who has supported me throughout the last few years.

First and foremost, I extend my deepest gratitude to my supervisor, Prof. Dr. Andreas Schieber. Your guidance, support, and expertise have been invaluable. I am profoundly grateful for the opportunity to work at the institute and for all the knowledge I gained during this time.

I also wish to wholeheartedly thank my second supervisor, Prof. Dr. Fabian Weber. Regardless of the effort required, I could always count on your unwavering support, enthusiasm, and belief in me. With your guidance and the right amount of freedom, I was able to grow both personally and professionally.

I extend my thanks to Prof. Dr. Matthias Wüst and Prof. Dr. Ralf Pude for being part of my examination committee.

I am grateful to all the amazing students who contributed their time, effort, and commitment to this thesis. Your dedication has been an invaluable asset.

To my colleagues and friends, thank you for your input, feedback, and encouragement. Your willingness to share your experiences, knowledge, and perspectives has deeply enriched my journey and created so many joyful moments.

A special thank you to Ingrid for your companionship throughout this journey. The shared laughs and mutual growth have created special memories that I will always cherish.

I also express my deep gratitude to my family and loved ones for their unwavering support, love, and encouragement.

Thank you, Malea, for showing me what is truly important in life.

Finally, I owe my greatest thanks to my wife, Alida. You have been my constant companion, always by my side, cheering me on and believing in me. Your love has been the cornerstone of my achievements. I could not have accomplished all that I have without you.

*Danke Papa*