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Study of the mechanism of interaction between red varietal wines anthocyanins and proteins rich in proline and sensory consequences

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Proučavanje mehanizma interakcija antocijanina crvenih sortnih vina i proteina bogatih prolinom i senzornih posledica

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Study of the mechanism of interaction between red varietal wines anthocyanins and proteins rich in proline and sensory consequences

Abstract

In this study, the reactivity of procyanidins and anthocyanins in young and aged 'Prokupac', 'Merlot' and 'Cabernet Sauvignon' wines toward salivary proteins is investigated via SDS-PAGE and UHPLC-QTOF-MS to determine the differences between the phenolic compounds of red wine in relation to the aging process of wine. SDS-PAGE analysis revealed that procyanidins, flavanol-anthocyanin polymers, and ellagitannins in aged wine have strong affinities for salivary proteins, leading to the formation of insoluble complexes. By contrast, young wine contained predominantly procyanidins with high salivary protein affinity, as well as monomeric flavan-3-ols and anthocyanins, which mainly form soluble aggregates, while polymeric phenolics were less represented. Electrophoretic patterns further showed that seed-derived procyanidins mainly formed insoluble complexes with salivary proteins, whereas skin-derived anthocyanins tended to form soluble ones. The total content of all phenolic compounds quantified by UHPLC-QTOF-MS was 2.5 times higher in young wine than in aged wine, primarily due to the significantly greater abundance of malvidine-3-*O*-glucoside in young wine (eightfold higher level in young wine). Targeted UHPLC-QTOF-MS analysis of selected phenolics confirmed the electrophoretic results and showed a higher binding affinity of procyanidins in aged wine compared to young wine, as well as a higher percentage of procyanidin binding compared to anthocyanins, independent of the age of the wine. Sensory evaluation showed that aged wine had higher tannin quality scores, whereas young wine exhibited greater acidity and astringency, with bitterness being comparable between them. These results highlight the influence of wine aging on the interaction between phenolic compounds and salivary proteins and emphasize the dominant role of procyanidins in protein binding and the potential synergistic contribution of anthocyanins to mouthfeel perception.

Keywords : young wine; aged wine; salivary proteins; procyanidins; anthocyanins

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Scientific field: Technological engineering
Scientific subfield: The science of food preservation and fermentation

Résumé

Dans cette étude, la réactivité des procyanidines et des anthocyanes dans les vins jeunes et vieillis de 'Prokupac', 'Merlot' et 'Cabernet Sauvignon' vis-à-vis des protéines salivaires est examinée par SDS-PAGE et UHPLC-QTOF-MS afin de déterminer les différences entre les composés phénoliques du vin rouge en relation avec le processus de vieillissement du vin. L'analyse par SDS-PAGE a révélé que les procyanidines, les polymères flavanol-anthocyanes et les ellagitannins présents dans le vin vieilli présentent de fortes affinités pour les protéines salivaires, conduisant à la formation de complexes insolubles. En revanche, le vin jeune contenait principalement des procyanidines à forte affinité pour les protéines salivaires, ainsi que des flavan-3-ols monomériques et des anthocyanes, qui forment principalement des agrégats solubles, tandis que les phénoliques polymériques étaient moins représentés. Les profils électrophorétiques ont montré en outre que les procyanidines d'origine graine formaient principalement des complexes insolubles avec les protéines salivaires, tandis que les anthocyanes d'origine pelliculaire avaient tendance à en former des solubles. La teneur totale de tous les composés phénoliques quantifiés par UHPLC-QTOF-MS était 2,5 fois plus élevée dans le vin jeune que dans le vin vieilli, principalement en raison de l'abondance nettement plus importante de malvidine-3-*O*-glucoside dans le vin jeune (niveau huit fois plus élevé dans le vin jeune). L'analyse ciblée par UHPLC-QTOF-MS des phénoliques sélectionnés a confirmé les résultats électrophorétiques et a montré une affinité de liaison plus élevée des procyanidines dans le vin vieilli par rapport au vin jeune, ainsi qu'un pourcentage de liaison des procyanidines supérieur à celui des anthocyanes, indépendamment de l'âge du vin. L'évaluation sensorielle a montré que le vin vieilli présentait des scores de qualité des tanins plus élevés, tandis que le vin jeune exprimait une acidité et une astringence plus marquées, l'amertume étant comparable entre les deux. Ces résultats soulignent l'influence du vieillissement du vin sur l'interaction entre les composés phénoliques et les protéines salivaires et mettent en évidence le rôle dominant des procyanidines dans la liaison aux protéines ainsi que la contribution synergique potentielle des anthocyanes à la perception des sensations en bouche.

Mots clefs : vin jeune, vin vieilli, protéines salivaires, procyanidines, anthocyanes

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Domaine scientifique: Génie technologique
Sous-domaine scientifique: Science de la conservation des aliments et de la fermentation

Apstrakt

U ovoj studiji ispitivana je reaktivnost procijanidina i antocijanina u mladim i odležalim vinima sorti 'Prokupac', 'Merlo' i 'Kaberne Sovinjon' prema pljuvačnim proteinima, primenom SDS-PAGE i UHPLC-QTOF-MS, radi utvrđivanja razlika između fenolnih jedinjenja crnog vina u odnosu na proces starenja vina. Analiza metodom SDS-PAGE pokazala je da procijanidini, flavanol-antocijanin polimeri i elagitanini u odležalom vinu imaju visoku afinitetnost ka pljuvačnim proteinima, što dovodi do formiranja nerastvorljivih kompleksa. Nasuprot tome, mlado vino je sadržalo uglavnom procijanidine sa visokom afinitetnošću ka pljuvačnim proteinima, kao i monomerne flavan-3-ole i antocijanine, koji pretežno formiraju rastvorljive agreate, dok su polimerni fenoli manje zastupljeni. Elektroforetski obrasci su dalje pokazali da procijanidini poreklom iz semena uglavnom formiraju nerastvorljive komplekse sa pljuvačnim proteinima, dok antocijanini poreklom iz pokožice imaju tendenciju da formiraju rastvorljive komplekse. Ukupni sadržaj svih fenolnih jedinjenja kvantifikovanih metodom UHPLC-QTOF-MS bio je 2,5 puta veći u mladom nego u odležalom vinu, uglavnom zbog značajno veće zastupljenosti malvidin-3-*O*-glukozida u mladom vinu (osam puta veći nivo u mladom vinu). Ciljana UHPLC-QTOF-MS analiza odabranih fenola potvrdila je elektroforetske rezultate i pokazala veću afinitetnost vezivanja procijanidina u odležalom vinu u odnosu na mlado, kao i veći procenat vezivanja procijanidina u odnosu na antocijanine, nezavisno od starosti vina. Senzorska procena pokazala je da odležalo vino ima više ocene kvaliteta tanina, dok je mlado vino ispoljavalo izraženiju kiselost i oporost, dok je gorčina bila uporediva između njih. Ovi rezultati naglašavaju uticaj starenja vina na interakciju između fenolnih jedinjenja i pljuvačnih proteina i ističu dominantnu ulogu procijanidina u vezivanju proteina, kao i potencijalni sinergistički doprinos antocijanina u percepciji senzacija u ustima.

Ključne reči : mlado vino, odležalo vino, salivarni proteini, procijanidini, antocijani

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Naučna oblast: Tehnološko inženjerstvo
Naučna uža oblast: Nauka o konzervisanju i vrenju

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List of Abbreviations

aPRP	acidic proline rich proteins
ASTM	American Society for Testing and Materials
ATP	aqueous two-phase
B1	epicatechin-(4β→8)-catechin
B2	epicatechin-(4α→8)-epicatechin
B3	catechin-(4α→8)-catechin
B4	catechin-(4α→8)-epicatechin
BCA	bicinchoninic acid
bPRP	basic proline rich proteins
BSA	bovine serum albumin
CAT	catechin
CATA	check-all-that-apply
CD	circular dichroism
COSMO-RS	Conductor-like screening model for real solvents
cyan-3-glc	cyanidin *3-O*-glucoside
DAD	diode-array detection
del-3-glc	delphinidin *3-O*-glucoside
df	Degrees of freedom
DF	Dilution factor
EGCG	epigallocatechin gallate
EPICAT	Epicatechin
FC	Folin-Ciocalteu Index
Gln	Glycine
Glu	Glutamine
gPRP	Glycosylated proline rich proteins
HPLC	high-performance liquid chromatography
HPPLC	high-performance preparative liquid chromatography
HPTLC	high-performance thin-layer chromatography
HSCCC	high-speed counter-current chromatography
IDPs	Intrinsically disordered proteins
ITC	isothermal titration calorimetry
K	Kadarka
kDa	Kilodalton
LC	liquid chromatography
Leu	Leucine
LOD	limit of detection
LOQ	limit of quantification
Lys	Lysine
MAE	microwave assisted extraction
mal-3-glc	Malvidin-*3-O*-glucoside
mal-acet	Malvidin-*3-O*-(6-*O*-acetyl)-glucoside
mal-coum	Malvidin-*3-O*-(6-*O*-*p*-coumaroyl)-glucoside
MALDI	matrix assisted laser desorption/ionization
md	Mean difference
mDa	Mean mass accuracy
mDP	Mean degree of polymerization
Met	Methionine
MF	Micro-filtration
μ-LC	Micro-liquid chromatography
MS/MS	Tandem mass spectrometry
MW	Molecular weight standard
NADES	Natural deep-eutectic solvents
NF	Nano-filtration
P	Prokupac
peo-3-glc	Peonidin-*3-O*-glucoside

peo-acet	Peonidin-*3-O*-(6-*O*-acetyl)-glucoside
peo-coum	Peonidin-*3-O*-(6-*O-p*-coumaroyl)-glucoside
pet-3-glc	Petunidin-*3-O*-glucoside
PGG	pentagalloyl glucose
Phe	Phenylalanine
Pro	Proline
PRP	Proline rich protein
PSP	Polarized sensory positioning
QAMS	Quantitative analysis of multiple components by single marker
QqQ/MS	Triple quadrupole mass spectrometry
QTOF	Quadrupole time-of-flight
RP-LC	Reversed-phase liquid chromatography
RT	Retention time
$SCCO_2$	Supercritical carbon dioxide
SCDE	Supercritical carbon dioxide extraction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Solvent extraction method
Ser	Serine
SPE	Solid-Phase Extraction
SPR	surface plasmon resonance
TA	Total Anthocyanins
TFA	Three Fluor-acetic acid
TPI	Total Polyphenols Index
Trp	Tryptophan
TT	Total Tannins
Tyr	Tyrosine
UF	Ultra-filtration
UHPLC	Ultra high-performance liquid chromatography
UV/VIS	Ultra-violet/visible
Val	Valine
UAE	Ultra-sound assisted extraction
UV	Ultra-violet

1. Introduction

Anthocyanins are a class of water-soluble natural pigments found extensively across various plant tissues, including both vegetative and reproductive organs. These pigments are primarily responsible for imparting red, blue, purple, and intermediate hues in plant structures, contributing to the coloration of leaves, stems, flowers, and fruits. In addition to their role in coloration, anthocyanins are hypothesized to serve multiple functional roles within plants (Winefield *et al.*, 2009). These include photoprotection, defensive mimicry, camouflage, shielding against ultraviolet (UV) radiation, scavenging of free radicals, and facilitating seed dispersal by attracting pollinators and seed-dispersing organisms (He *et al.*, 2010).
Biochemically, anthocyanins are phenolic compounds classified within the flavonoid group. The aglycone form of anthocyanins, known as anthocyanidins, consists of two aromatic rings (A and B) connected by a heterocyclic ring (C) that carries a positive charge due to the presence of double bounds within its structure (Clifford, 2000). There are six most known anthocyanins – malvidin, cyanidin, delphinidin, pelargonidin, petunidin, and peonidin. Through an *O*-glycosylation reaction, occurring at the third carbon atom of the C ring, anthocyanidins are converted into anthocyanins, forming the glycosylated derivatives. The most observed sugars involved in the glycosylation of anthocyanidins are glucose, galactose, rhamnose, and arabinose. These sugars typically attach to the anthocyanidin molecule as 3-*O*-glycosides or 3,5-diglycosides, forming stable glycosidic linkages. Additionally, changes in the anthocyanidin structure occur through reactions of hydroxylation, acylation, and methylation.
Acylation of anthocyanins occurs at the sugar moiety through the addition of various acids as acylation agents, including cinnamic acids (such as caffeic, *p*-coumaric, ferulic, and sinapic) and aliphatic acids (such as acetic, malic, malonic, oxalic, and succinic). Due to their highly reactive and unstable nature, anthocyanins are sensitive to a wide range of physical and chemical factors. In solution, they often undergo co-pigmentation reactions with both phenolic and non-phenolic compounds (Boulton, 2001). The typically unstable anthocyanidin molecules gain enhanced stability through glycosylation, acylation, methylation, and co-pigmentation reactions (Li *et al.*, 2021).
Anthocyanins are recognized for their wide range of health-promoting properties. These include potent antioxidant activity, antibacterial effects, anti-inflammatory properties, and their roles in mitigating diabetes, cancer, and cardiovascular disease (Zia Ul Haq *et al.*, 2016; Khoo *et al.*, 2017). Additionally, anthocyanins have gained considerable interest in the food industry, particularly for their application in smart and intelligent food packaging systems. This is facilitated through advanced techniques such as micro- and nano-encapsulation, which enhance the stability and functionality of these bioactive compounds within packaging materials (Luo *et al.*, 2022).
In the wine industry, anthocyanins are one of the most important bio-compounds, responsible for the red color of the wine, directly influencing the sensory properties and quality of red wine. In black grapes, they are found mostly in the skin of black grapes, but in some grapevine varieties (teinturiers) they can be found in the flesh of the berries (Korosi *et al.*, 2022). In some varieties, they can also be found in the leaves and canes at the end of the season. During the winemaking process, anthocyanins from the skin of berries go into the grape juice. Anthocyanins undergo different chemical reactions and transformations, through maceration, pressing, and fermentation (Ribereau-Gayon *et al.*, 2006). In young wines, anthocyanins are mostly found as free and unstable, in forms of anthocyanin-3-*O*-glycosides. Their content largely depends on characteristics of variety but is influenced also by the meteorological conditions at the vineyard site, the winemaking technique, yeasts used, etc., (Ntuli *et al.*, 2023; Kuchen *et al.*, 2018). After the fermentation, young wine proceeds to the process of aging. Wine aging is one of the most important winemaking steps since the anthocyanins are undergoing co-pigmentation reactions, becoming more stable (Wang *et al.*, 2023). In a sensory sense, other than directly influencing the color of the red wine, anthocyanins might indirectly be involved in the mouthfeel of red wine, affecting astringency, and bitterness (Paissoni *et al.*, 2020).
Astringency is a peculiar sensation occurring in the oral cavity of the human mouth, during wine tasting, usually connected to the feeling of tightening of the mouth tissue (Green, 1993). This sensation is proven to arise due to the precipitation of salivary proteins, after their interaction with tannins (Breslin *et al.*, 1993). It is known that anthocyanins form different complexes with tannins and other phenolic

compounds, giving new polyphenolic compounds that are directly involved in the development of astringency perception (pyranoanthocyanins) (de Freitas and Mateus, 2001). Also, the saliva-anthocyanin complexes have been reported (Ferrer-Gallego *et al.*, 2015), as well as the specific mouthfeel during the sensory analysis of anthocyanin extracts which gives the basis for setting up a hypothesis about the influence of anthocyanins on the perception of astringency (Ferrero-del-Teso *et al.*, 2022).

The primary mechanism underlying astringency development is well established as the interaction between proline-rich salivary proteins (PRPs) and astringent compounds (Pires *et al.*, 2020). PRPs are categorized into acidic (aPRP), basic (bPRP), and glycosylated (gPRP) groups, each characterized by their high reactivity. Their occurrence in saliva has been previously associated with the intake of tannin-rich foods (Bennick, 1982). Biochemically, astringency arises from non-covalent interactions, including hydrophobic interactions and hydrogen bonding between PRPs and tannins (Garcia-Estevéz *et al.*, 2018; Delić *et al.*, 2023). Beyond tannins, anthocyanins – key polyphenolic constituents – are crucial for red wine quality, particularly affecting visual and sensory attributes (Boulton, 2001). Current studies are increasingly focusing on the role of anthocyanins in modulating taste and astringency through interactions with tannins and PRPs (Paissoni *et al.*, 2018; Ferrer-Gallego *et al.*, 2015; Paissoni *et al.*, 2020). As anthocyanins interact with tannins during winemaking to form anthocyanin-tannin complexes, an intriguing question emerges: is there a distinct interaction mechanism between anthocyanins and salivary proteins? (Paissoni *et al.*, 2018; Ferrer-Gallego *et al.*, 2014; Ferrer-Gallego *et al.*, 2015; Mattioli *et al.*, 2020). Evidence of anthocyanin-saliva protein complexes has been observed, though their interaction mechanisms remain elusive (Ferrer-Gallego *et al.*, 2015). Paissoni *et al.* (2018) explored the influence of anthocyanins on enhancing or diminishing red wine astringency. Studies show that anthocyanins form polymeric pigments during aging, which, when interacting with salivary proteins, may reduce astringency levels (Villamor *et al.*, 2009). Additionally, polysaccharides in aged wines can associate with anthocyanins and tannins, further attenuating astringency (Escot *et al.*, 2001). Molecular research is thus essential to deepen our understanding of anthocyanin-protein interactions. Previous studies indicate that the affinity between plant pigments and salivary proteins may depend on pigment functional groups and molecular weights (Yao *et al.*, 2011). Studies of different anthocyanin fractions (glucoside, acylated, and coumaroyl) have revealed that coumaroyl anthocyanins exhibit the highest reactivity with PRPs (Paissoni *et al.*, 2018). Soares *et al.* (2019) investigated whether the co-pigmentation of malvidin-3-*O*-glucoside with epicatechin could modulate flavonol interactions with PRPs. The epicatechin-malvidin-3-*O*-glucoside mixture demonstrated similar affinity for PRPs as individual components, where epicatechin exhibits both hydrophobic and hydrophilic interactions, while malvidin-3-*O*-glucoside predominantly engages in electrostatic interactions. However, Torres-Rochera *et al.* (2023) found that isolated malvidin-3-*O*-glucoside showed the strongest affinity for salivary mucins over catechin, epicatechin, and quercetin-3-β-glucopyranoside, suggesting that co-pigmentation might significantly impact astringency by modifying mucin and phenolic compound interactions. Similarly, Mao *et al.* (2024) studied cyanidin-3-*O*-glucoside interactions with oral mucins, finding that anthocyanins oxidize to quinones, which form covalent bonds with mucins, intensifying astringency perception. Further studies have developed *in vitro* oral models that simulate human oral cavity conditions to analyze phenolic compound interactions. Soares *et al.* (2020) created an oral epithelium model with human saliva and mucosal pellicle to study anthocyanin interactions from red wine and green tea extracts. Their findings showed that anthocyanins primarily interact with oral epithelial cells, with comparable binding potential across different anthocyanin types (delphinidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside).

In analytical terms, prior research has focused on isolating individual anthocyanin fractions and their derivatives via chromatography, coupled with monitoring their interactions with salivary proteins to detect the formation of anthocyanin-salivary protein complexes. However, further investigation is required to elucidate the biochemical interactions of anthocyanins within complex matrices, specifically in the presence of salivary proteins, to assess the relative affinities of distinct anthocyanin derivatives. Analytical methods employed thus far have largely comprised liquid chromatography-mass spectrometry

(LC-MS). Notably, electrophoretic techniques have yet to be utilized in this area, despite their significant advantages in examining proteinaceous and protein-associated matrices.
This study introduces a novel approach to exploring the mechanisms underlying the interaction between red wine anthocyanins, procyanidins and salivary proteins, offering valuable insights into the biochemical pathways that contribute to the perception of astringency. To conduct a comprehensive study on a mechanism of interaction between red wine phenolics and salivary proteins, with emphasis on potential sensorial consequences, this thesis is structured with specific objectives as follows:

- Identification and quantification of skin, seed, and red wine phenolics of indigenous Serbian and international French varieties.
- Identification and quantification of red wine phenolics, with special emphasis on molecular anthocyanins and procyanidins.
- Investigation of the interaction mechanisms between anthocyanins and procyanidins from black grape varieties and Serbian red wines and salivary proteins, with an accent on proline-rich proteins, focusing on the molecular and physicochemical pathways involved.
- Assessment of the sensory consequences of these anthocyanin-salivary protein interactions on wine astringency.
- Evaluation of the influence of wine aging and its composition on their affinity for salivary proteins.
- Development of practical recommendations for winemakers based on the findings to optimize wine characteristics and improve the sensory quality of red wine.

2. Literature review

2.1. Grape and wine anthocyanins

Anthocyanins are among the primary polyphenolic compounds in black grapes, playing a crucial role in determining the diverse colors of red wines and significantly influencing their sensory characteristics (Flamini *et al.*, 2013). These compounds exhibit notable health benefits, functioning as antioxidants and bioactive agents with anti-cancer, anti-diabetic, anti-inflammatory, and cardioprotective properties (Zhou *et al.*, 2022; Sabra *et al.*, 2021). Beyond health, anthocyanins have applications in smart/intelligent food packaging (da Silva *et al.*, 2022; Duan *et al.*, 2021; Forghani *et al.*, 2021; Almasi *et al.*, 2022), natural food colorants (Alvarez Gaona *et al.*, 2022; Montibeller *et al.*, 2018; Bridle and Timberlake, 1997), and the development of functional foods (Khoo *et al.*, 2017; Ferrer-Gallego and Silva, 2022).
Biochemically, anthocyanins belong to the flavonoid class, synthesized alongside other phenolic compounds via the phenylpropanoid (flavonoid) pathway, originating from the amino acid phenylalanine (He *et al.*, 2010). Structurally, they exist as glycosylated and acyl-glycosylated derivatives of anthocyanidins (their aglycone forms). Glycosylation typically occurs at the 3 and 5 positions of the C ring, with glucose, rhamnose, and arabinose as common sugar moieties (Clifford *et al.*, 2000). Additionally, acylation of the sugar moieties is often mediated by acids such as acetic, p-coumaric, and caffeic acids (Grotewold, 2006) (Figure 2.1.). Anthocyanins predominantly accumulate in the skins of grape berries (Figure 2.2a); however, in teinturier grape varieties, they are also found in the flesh (Figure 2.2b) (Roubelakis-Angelakis, 2009).

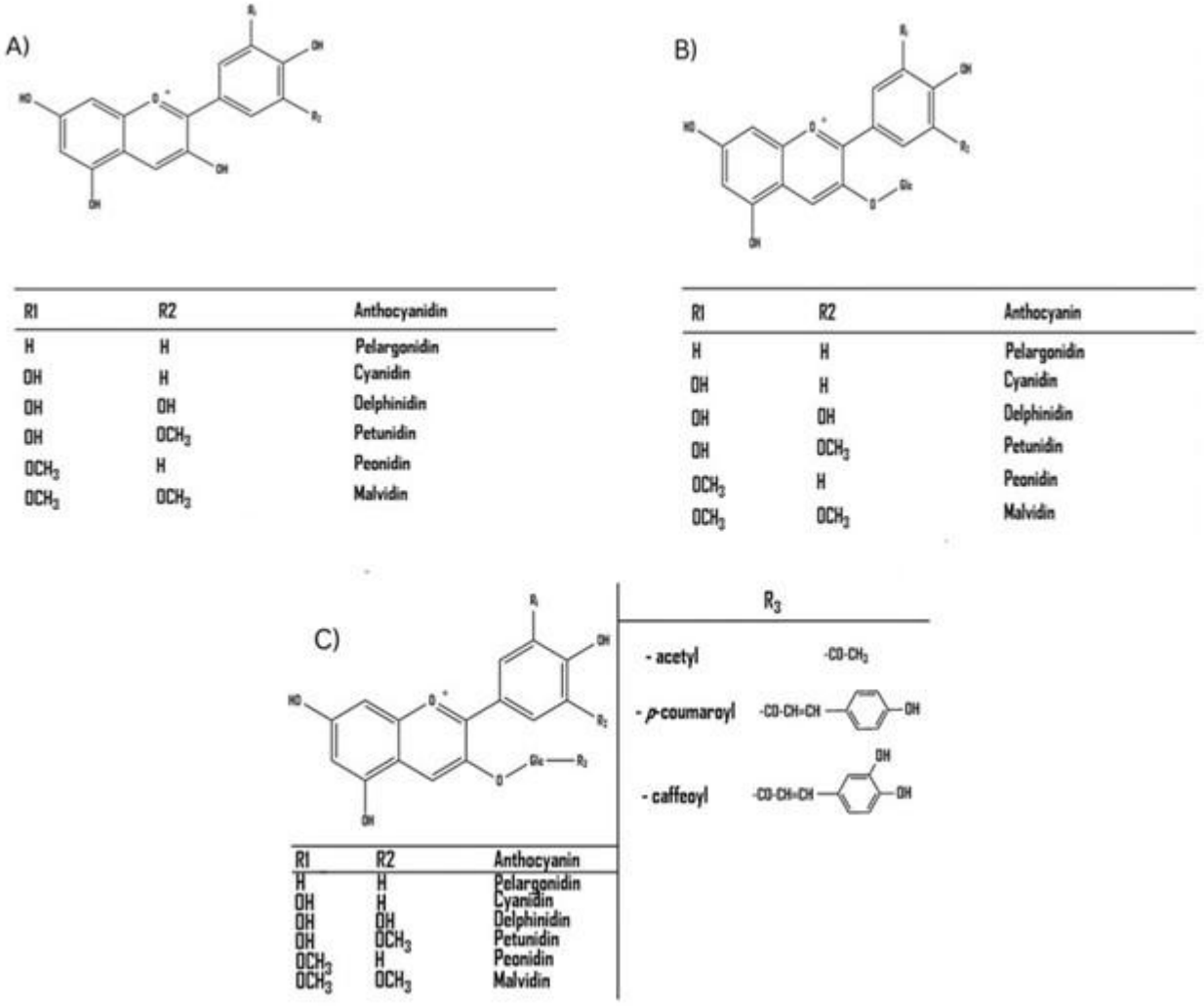

Figure 2.1. Structural formulas of A) Anthocyanidins, B) Anthocyanins, and C) Acylated anthocyanins (Delić *et al.*, 2024).

The content of anthocyanins in grape skins and wines is primarily determined by genetic factors, making them valuable for chemotaxonomic classification (Keller, 2020; Liang *et al.*, 2008). Nonetheless, environmental conditions and viticultural practices have been shown to influence their biosynthesis and accumulation (Bouzas-Cid *et al.*, 2016; Blanco-Vega *et al.*, 2014; Giacosa *et al.*, 2015). The impact of winemaking techniques on the composition and concentration of anthocyanins in red wines remains a topic of active research, with diverse and sometimes contradictory findings reported in the literature (El Darra *et al.*, 2016; Portu *et al.*, 2023; Aleixandre-Tudo and du Toit, 2018; Ortega-Heras *et al.*, 2012; Aguilar *et al.*, 2016; López *et al.*, 2008).

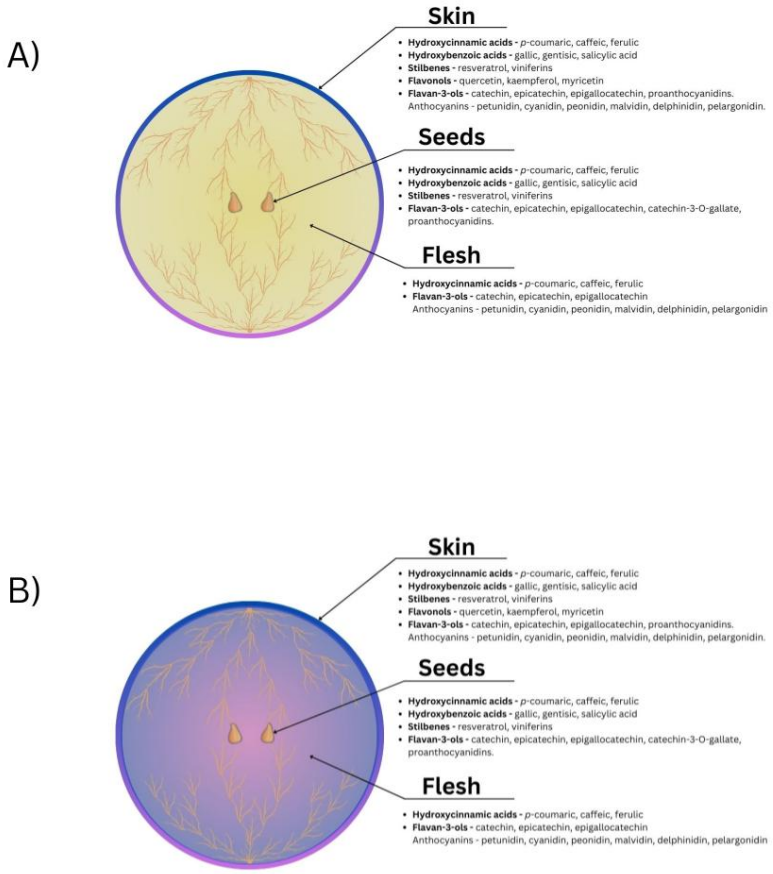

Figure 2.2. Anatomy of the black grapevine berry: A) varieties without anthocyanins in the mesocarp, and B) 'teinturier' varieties (Delić *et al.*, 2024).

Anthocyanins are unstable molecules susceptible to biochemical changes. External factors such as light, temperature, oxygen, metals, the presence of different organic and inorganic particles, and the pH of their medium can alter their structure and color, highlighting their sensitivity and the challenges associated with their preservation. Anthocyanins in grape skin are present as both aglycone forms, namely malvidin, cyanidin, petunidin, peonidin, delphinidin, and pelargonidin, and their glycosides. In *Vitis vinifera* L. varieties and their wines, anthocyanins predominantly occur as monoglycosides. In contrast, hybrids of European, American, and East Asian grape species typically exhibit 3,5-diglycosides, a trait believed to be genetically determined (Laminakra, 1989; Zhao *et al.*, 2010; Teissedre, 2018).

During winemaking, anthocyanins are extracted from grape skin into grape juice through maceration and fermentation processes (Ribereau-Gayon *et al.*, 2006). Research indicates that the length of maceration and winemaking techniques significantly influence anthocyanin concentration (Alencar *et al.*, 2018; Maza *et al.*, 2020). As previously noted, anthocyanins are unstable biomolecules and undergo various interactions with phenolic and non-phenolic compounds during winemaking and aging, leading to the formation of organic complexes (Bindon *et al.*, 2014; Campbell *et al.*, 2021). These interactions involving several mechanisms, termed co-pigmentation, enhance wine color stability (Liao *et al.*, 1992; Boulton, 2001).

In young wines, anthocyanins predominantly exist in their free forms, glycosides, and acylated glycosides. Over time, as the wine ages, anthocyanins undergo further reactions, forming tannin-anthocyanin complexes and other derivatives through interactions with tannins and phenolic acids (Zhang *et al.*, 2021; Ćurko *et al.*, 2021). These transformations contribute to the evolving chemical and sensory profile of wine (Ivanova *et al.*, 2012). It has been hypothesized that anthocyanins may modulate the perception of tannin astringency by forming tannin-anthocyanin complexes. These interactions could reduce the perceived harshness of tannins, rendering them softer and silkier in texture, thereby enhancing their sensory quality (Paissoni *et al.*, 2018; Paissoni *et al.*, 2020). This mechanism represents a potential indirect pathway through which anthocyanins influence astringency. Studies on anthocyanins have explored their potential impact on the perception of astringency (Soares *et al.*, 2019). Research has shown that glycosylated, acylated, and *p*-coumaroylated anthocyanin derivatives exhibit reactivity with salivary proteins, leading to the formation of soluble and insoluble anthocyanin-salivary protein complexes (Ferrer-Gallego *et al.*, 2015). To advance this understanding, further investigations are required to determine the affinity and binding capacity of individual anthocyanin derivatives with proline-rich salivary proteins, which play a critical role in modulating astringency perception.

2.1.1. Anthocyanins biosynthesis

Anthocyanins are synthesized via the flavonoid biosynthetic pathway (He *et al.*, 2010), which is governed by two major gene groups: structural and regulatory. This biosynthetic process involves a complex network of intermediates that serve as precursors for various related compounds, requiring the coordinated activity of numerous enzymes (Ivanova *et al.*, 2011b). The structural genes encode enzymes directly involved in anthocyanin biosynthesis (Yang *et al.*, 2023), while regulatory genes, primarily transcription factors, modulate the expression of structural genes, regulating anthocyanin production (Holton and Cornish, 1995; Moreno-Arribas and Polo, 2009).

Anthocyanin accumulation primarily occurs during the phenological ripening phase and is regulated by the VvMYBA1 transcription factor, which controls the expression of the anthocyanin-specific gene encoding UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) (Cutanda-Perez *et al.*, 2009) (Figure 2.3.). While anthocyanin accumulation typically increases after veraison, recent findings indicate that this pattern is highly variety-dependent (Wang *et al.*, 2024). In addition to genetic control, environmental conditions, particularly temperature and light, significantly influence gene expression related to anthocyanin biosynthesis (Azuma, 2018). Nonetheless, emerging evidence suggests that in certain cultivars, flesh pigmentation may occur independently of light exposure, following skin pigmentation (Lu *et al.*, 2023).

Recent studies have advanced our understanding of the metabolic pathways and gene networks involved in anthocyanin biosynthesis. Some reports highlight the substantial impact of these pathways on different developmental stages of grape berries (Zia Ul Haq *et al.*, 2016), suggesting that various phases of berry development may depend on the availability of anthocyanin precursors and the activity of associated biosynthetic genes and enzymes. In teinturier grape varieties, a pronounced tendency toward cyanidin methylation has been observed, alongside a limited capacity for hydroxylation (Papouskova *et al.*, 2011). Moreover, it has been conclusively demonstrated that anthocyanin biosynthesis in the grape flesh can occur independently of that in the skin (Lu *et al.*, 2023). These findings have been validated in newly developed teinturier cultivars such as 'ZhongShan-HongYu' (Yang *et al.*, 2023), 'Mio Red' (Lu *et al.*, 2023), and the medicinal *Vitis vinifera* L. variety 'SuoSuo' (Wang *et al.*, 2023). Following synthesis, the

anthocyanidin aglycones unstable in their native form undergo various modifications including glycosylation, methylation, and acylation to enhance stability (He *et al.*, 2010). Glycosylation increases both hydrophilicity and molecular stability, converting anthocyanidins into anthocyanins. In *Vitis vinifera* L., glycosylation occurs exclusively at the C3 position, while methylation of hydroxyl groups typically takes place at the C3' or both C3' and C5' positions of the B ring (Grotewold, 2006). Acylation, which occurs at the C6" position of the glycosyl moiety, involves the attachment of aromatic and/or aliphatic groups (He *et al.*, 2010; Holton and Cornish, 1995; Grotewold, 2006). These chemical modifications play a crucial role in stabilizing anthocyanins, particularly during intracellular transport, which primarily takes place in the cytosol where anthocyanins are otherwise unstable (Wang *et al.*, 2024).

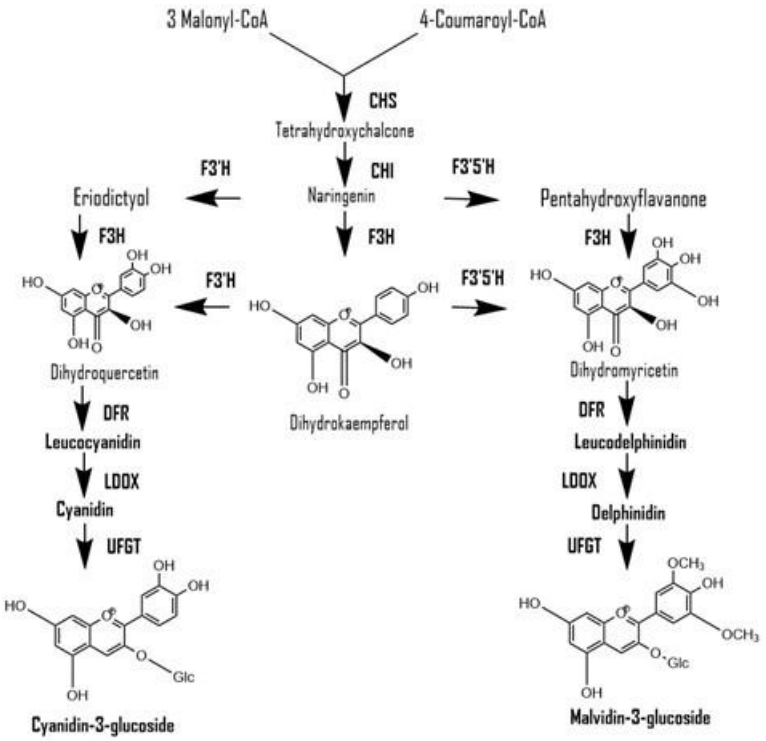

Figure 2.3. Biosynthesis pathway of anthocyanins in black grapes (Delić *et al.*, 2024).

The biosynthesis of anthocyanins is localized to the endoplasmic reticulum (Alfenito *et al.*, 1998), after which the compounds are actively transported into the vacuole (Grotewold, 2004). Two distinct cellular transport mechanisms have been identified: ligandin transport (LT) and vesicular transport (VT) (Zhao and Dixon, 2010). The final step of anthocyanin accumulation involves their sequestration within vacuoles, where their visible coloration is determined not only by their molecular structure but also by vacuolar pH, and the presence of metal ions and co-pigments (Grotewold, 2006). These mechanistic insights contribute significantly to our understanding of the molecular architecture, stability, and bioavailability of anthocyanins.

2.1.2. Anthocyanins in black grapes

The mature black grape berry exhibits a complex anatomical structure composed of several distinct tissues, each with specific physiological roles. The outermost layer, known as the exocarp or skin, consists of multiple layers of epidermal cells that are notably rich in anthocyanins. Beneath this layer lies the mesocarp, or flesh, which is composed predominantly of parenchyma cells. These cells serve as reservoirs for sugars, organic acids, and water, all of which contribute to the juiciness and flavor profile of the berry. In contrast to most grape varieties, teinturier cultivars possess red flesh, as anthocyanins are present not only in the skin but also in the mesocarp tissue (Kőrösi *et al.*, 2022). Despite extensive study, the precise biological function of anthocyanins in grapevines remains incompletely understood (Roubelakis-

Angelakis, 2009). Besides their presumed roles in seed dispersal and ultraviolet (UV) protection, anthocyanins are chiefly responsible for the red and black pigmentation of grape berries, making them key contributors to wine coloration. The concentration, composition, and spatial distribution of anthocyanins and their derivatives differ markedly among red grape (*Vitis vinifera* L.) cultivars. Furthermore, these traits also vary significantly across other species such as *Vitis labrusca*, *Vitis rotundifolia*, *Vitis amurensis*, and interspecific hybrids involving *V. vinifera* (Teissedre, 2018). Numerous studies have aimed to identify and quantify the polyphenolic profiles of red grape varieties, encompassing both vinifera and non-vinifera types. Substantial differences in anthocyanin composition and concentration have been reported. According to Laminakra (1989), *V. vinifera* L. cultivars predominantly accumulate both acylated and non-acylated anthocyanins. In contrast, *V. rotundifolia* and its hybrids primarily contain non-acylated 3,5-*O*-diglucosides, while *V. labrusca* cultivars exhibit a mixture of acylated and non-acylated mono- and di-glucosides of various anthocyanidins (Ehrhardt *et al.*, 2014; Wojdyło *et al.*, 2018; Tassoni *et al.*, 2019; Milinčić *et al.*, 2021a; Forino *et al.*, 2022; Tampaktsi *et al.*, 2023; Milinčić *et al.*, 2021b). The anthocyanin profiles of the internationally cultivated French varieties 'Cabernet-Sauvignon' and 'Merlot' have been extensively characterized across global winegrowing regions (Chira *et al.*, 2011; Chira *et al.*, 2012; Zhao *et al.*, 2023). Both cultivars are rich in malvidin-3-O-glucoside, making this compound a potential chemotaxonomic marker (Garcia-Beneytez *et al.*, 2003). Additionally, 'Cabernet-Sauvignon' exhibits 1.5- to 3-fold higher levels of acetylated anthocyanins compared to 'Merlot' (Lorrain *et al.*, 2011). Cinnamoyl derivatives, including *p*-coumaroylglucosides and caffeoylglucosides, have also been identified in both varieties (Costa *et al.*, 2014).

Other *V. vinifera* L. cultivars have been similarly investigated. Garcia-Beneytez *et al.* (2003) analyzed a variety of Spanish red grape cultivars such as 'Alicante Bouschet', 'Bobal', 'Carinena', 'Crujidera', 'Garnacha Peluda', 'Monastrell', 'Moristel', 'Morrastel-Bouschet', 'Petit Bouschet', 'Prieto Picudo', 'Tempranillo', and 'Vitidillo'. Using HPLC-MS, they confirmed the presence of 3-*O*-glucoside derivatives, acetylglucosides, and cinnamoyl derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin. Malvidin-3-*O*-glucoside was identified as the dominant anthocyanin in most cultivars, except in teinturier types such as 'Alicante Bouschet', 'Morrastel Bouschet', and 'Petit Bouschet', where peonidin-3-*O*-glucoside predominated, a characteristic specific to teinturier cultivars. Similar patterns were observed in Portuguese varieties. Costa *et al.* (2014) examined native *V. vinifera* L. cultivars (e.g., 'Camarate', 'Monvedro', 'Moreto Boal', 'Negro Mole', 'Alfrocheiro', 'Bastardo', 'Tinta Amarela', 'Tinta Barroca', and 'Tinto Cão') alongside international varieties (e.g., 'Gewürztraminer', 'Aramon', 'Cabernet Franc', 'Carignan Noir', 'Gamay', and 'Grenache'). Malvidin-3-*O*-glucoside was consistently the predominant anthocyanin, followed by its cinnamoyl derivative, malvidin-3-*p*-coumaroylglucoside. These subtle differences in anthocyanin content and profile are believed to be genetically determined, as supported by several studies (Obreque-Slier *et al.*, 2013; Guerrero *et al.*, 2009; Arozarena *et al.*, 2002; Lingua *et al.*, 2016; Ivanova *et al.*, 2011a; Šuković *et al.*, 2020; Milinčić *et al.*, 2021a; Milinčić *et al.*, 2021b).

Non-vinifera cultivars, including hybrids and PIWI (fungus-resistant) varieties, are gaining attention as sustainable alternatives in viticulture due to their resistance to common grapevine pathogens and phylloxera. These cultivars often exhibit phenolic compositions distinct from those of *V. vinifera* L. The relevance of these differences is underscored by growing industrial demand for natural colorants and the need for enhanced color stability in grape juice and wine. One notable feature of non-vinifera grapes is the presence of anthocyanidin-3,5-diglucosides, compounds generally absent or found only in trace amounts in *V. vinifera* (Lamikanra, 1989). Although a few studies have reported their occurrence in certain *V. vinifera* cultivars (Pantelić *et al.*, 2016), their concentrations are negligible compared to those found in non-vinifera varieties.

Recent studies on *Vitis amurensis* and its hybrids have identified a novel class of anthocyanins, proposed to be 3,5,7-*O*-triglucosides (Zhu *et al.*, 2021). These cultivars typically contain high levels of diglucoside anthocyanins and low levels of acylated derivatives, aligning with earlier findings (Zhao *et al.*, 2010). To further explore the oenological and health-promoting potential of non-vinifera grapes, red hybrids such as 'Rondo,' 'Regent,' and 'Cabernet-Cortis' have been studied. Wojdyło *et al.* (2018) identified delphinidin, cyanidin, petunidin, peonidin, and malvidin 3,5-diglucosides in 'Rondo' and 'Regent.' Ehrhardt *et al.*

(2014) additionally reported the presence of pelargonidin-3,5-diglucoside in both 'Cabernet-Cortis' and 'Regent,' a compound typically detected only in trace amounts and potentially serving as a distinguishing marker for PIWI anthocyanin profiles.
From a technological standpoint, diglucoside anthocyanins (particularly 3,5-diglucosides) are preferred in winemaking due to their superior thermal and photostability relative to monoglucosides (Lamikanra, 1989). This property makes hybrid cultivars increasingly attractive for both grape juice and wine production.

2.1.3. Anthocyanins in red wine

Red winemaking is a multifaceted process involving the biochemical transformation of red grapes into wine through several sequential stages. These include grape harvesting and crushing, maceration, fermentation, aging, and bottling, each contributing significantly to the final wine's colour, flavour, and aromatic profile. Among these, maceration is considered a critical phase, during which grape skins, seeds, and pulp remain in contact with the fermenting must for a defined period. This contact facilitates the extraction of key phenolic compounds, notably anthocyanins, tannins, and various volatile constituents, from the solid grape material into the wine matrix. The duration of maceration is influenced by both grape variety and winemaking decisions. Anthocyanins, primarily extracted from the skins, are responsible for the characteristic red colour of the wine, while tannins influence its structural attributes, including astringency and aging capacity (Razungles, 2022).
Through the combined effects of maceration, fermentation, aging, and bottling, red wines gradually develop their distinctive sensory characteristics. The complexity and diversity of red wines are largely shaped by the interplay between grape solids, phenolic compounds (particularly anthocyanins), yeast activity, and specific winemaking practices, all of which impact the wine's overall phenolic composition. The anthocyanin profile of red wine is primarily determined by the concentration and composition of anthocyanins inherent to the grape cultivar. However, other influential factors include environmental conditions, as well as viticultural and agrotechnical practices applied in the vineyard, all of which can significantly modulate anthocyanin levels and stability in the resulting wine (Morgani *et al.*, 2023; Haselgrove *et al.*, 2000; Sivilotti *et al.*, 2020).

2.1.3.1. Anthocyanins transformations during winemaking and wine aging processes

2.1.3.1.1. Winemaking transformations

During winemaking, anthocyanins are subjected to various biochemical reactions and structural transformations, which alter both their chemical and physicochemical properties. The extraction of anthocyanins, alongside other phenolic and non-phenolic compounds, as well as the stability of colour, is strongly influenced by maceration duration and fermentation conditions (Ribéreau-Gayon *et al.*, 2006). As a critical extraction phase in red winemaking, maceration directly impacts anthocyanin levels, prompting extensive investigation into varying maceration durations and techniques. Despite its central role, maceration remains an area of ongoing research, particularly in the pursuit of more sustainable and economically viable practices. Shortening maceration time without compromising wine quality could significantly reduce energy consumption and production expenses.
Previous studies have demonstrated that maceration time affects wine colour and both chemical and sensory attributes, with anthocyanin concentrations and colour intensity typically decreasing with prolonged maceration (Gil *et al.*, 2012). Notably, anthocyanin levels tend to peak within the first five to six days of maceration (Ribéreau-Gayon, 1982). To our knowledge, the kinetics of anthocyanin extraction are predominantly governed by the physicochemical properties of grape skins, as well as the intrinsic anthocyanin content specific to each variety (Otteneder *et al.*, 2004). Several studies have reported that extended maceration may result in a decline in total anthocyanin content (Sipiora *et al.*, 1998). For instance, Jagatić Korenika *et al.* (2023) confirmed that maximum anthocyanin concentrations and colour metrics are generally reached between three and six days of maceration. In contrast, Alencar *et al.* (2017) observed a continual increase in anthocyanin levels up to the 20th day in 'Syrah' must and wine. This was

attributed to the high anthocyanin content in 'Syrah' grape skins, likely due to favourable adaptation to the agroecological conditions of northeastern Brazil.

Anthocyanin extraction efficiency, overall phenolic composition, and colour characteristics can also be modulated by additional factors such as grape ripeness, the presence of seeds and solids, oak and tannin additions, fermentation temperature, and various chemical and physical treatments. A growing body of research focuses on optimizing extraction, enhancing co-pigmentation and complexation processes, and improving colour stability, all within a framework of environmentally conscious winemaking. Wines produced from fully ripened grapes tend to have higher levels of skin proanthocyanidins and require shorter maceration times (Gil *et al.*, 2012). Conversely, early seed removal has been shown to reduce monomeric anthocyanins, gallic acid, and flavan-3-ol concentrations (Jagatić Korenika *et al.*, 2023).

Emerging technologies have demonstrated potential in enhancing phenolic extraction. For example, the application of high hydrostatic pressure (HHP) in combination with oak chip maceration has been shown to increase both phenolic content and colour intensity (Tao *et al.*, 2016). Giacosa *et al.* (2023) investigated the influence of seed presence on anthocyanin extraction kinetics in four Italian varieties ('Aglianico', 'Nebbiolo', 'Primitivo', and 'Sangiovese') and found that seeds promoted polymerization, a key factor in long-term colour stability. Fermentation temperature is another critical variable, with divergent findings across studies. Reynolds *et al.* (2022) reported improved anthocyanin extraction and concentration under higher fermentation temperatures (25 °C for 14 days) in three 'Pinot noir' clones. They hypothesized that elevated temperatures enhance phenolic extraction, increase tannin release, and promote the formation of polymeric pigments. In contrast, numerous studies have demonstrated that cold maceration tends to reduce anthocyanin levels (Leong *et al.*, 2020; Casassa *et al.*, 2019).

Various maceration techniques including carbonic maceration, thermovinification, cryomaceration (cold maceration), pulsed electric field (PEF), microwave-assisted maceration, ohmic heating, and enzymatic treatments have been explored as alternatives or complements to traditional approaches (Tong *et al.*, 2023; Portu *et al.*, 2023; Zhang *et al.*, 2019; Pace *et al.*, 2014). Being water-soluble, anthocyanins are rapidly extracted early in maceration, whereas other phenolic compounds are increasingly solubilized as alcohol levels rise during fermentation (Bautista-Ortín *et al.*, 2004). While anthocyanin content is primarily governed by grape variety characteristics, the extraction of other phenolic and co-pigmented compounds depends more heavily on the winemaking technique employed. Pretreatment strategies have been shown to significantly influence anthocyanin levels (Wojdyło *et al.*, 2021).

Carbonic maceration, a method involving anaerobic fermentation of whole grape clusters in a carbon dioxide-saturated environment, initiates intracellular fermentation and induces unique biochemical modifications. This process impacts the phenolic profile, aroma, and flavour of the wine. Studies consistently report that carbonic maceration leads to reduced levels of individual anthocyanins, particularly monoglucosides, and total phenolics. However, this technique enhances the polymerization potential of anthocyanins, resulting in wines with higher hue intensity (Chinnici *et al.*, 2009). Gonzalez-Arezana *et al.* (2020) compared 84 commercial 'Tempranillo' wines made via carbonic maceration with conventionally produced counterparts and found increased colour intensity, higher polymerization rates, and elevated concentrations of vitisins A and B and coumaroyl derivatives in the carbonic maceration group findings supported by other studies (Chinnici *et al.*, 2009; Portu *et al.*, 2023). Despite lower total phenolic and anthocyanin content, carbonic maceration wines exhibit brighter, more saturated colour due to higher chroma values, elevated catechin concentrations, and increased levels of oligomeric and polymeric proanthocyanidins (Zhang *et al.*, 2019). These wines also display enhanced red hues and a distinctive phenolic composition, including hydroxycinnamic acids, flavanols, and ethyl-bridged anthocyanin isomers (Shmigelskaya *et al.*, 2021).

Thermovinification involves heating the must to temperatures below 85°C prior to fermentation to enhance the extraction of skin- and seed-derived phenolic compounds such as anthocyanins and tannins. This process aims to intensify colour, improve pigment stability, and alter tannin structure. As a temperature- and time-dependent approach, thermovinification has proven effective in elevating phenolic concentrations relative to conventional techniques (Aguilar *et al.*, 2016). Nevertheless, as highlighted by Maza *et al.* (2019), thermal treatments can introduce quality challenges due to heat-induced

alterations in grape components. The effectiveness of thermovinification also appears to be variety-dependent, with some studies noting positive effects on anthocyanin extraction in cultivars like 'Pinot noir' (Girard *et al.*, 1997).

Flash détente (FD), another thermal maceration technique, was examined by Ntuli *et al.* (2023) for its effects on 'Merlot' wine. The method entails heating grape must to 85°C, followed by rapid vacuum cooling to 32°C. The treatment significantly increased concentrations of caftaric acid, malvidin-3-*O*-glucoside, and quercetin glycoside. While FD facilitated rapid pre-fermentation anthocyanin extraction, approximately 40% of the colour was lost during fermentation. However, the authors noted that FD may improve wine body and astringency by enhancing polysaccharide and proanthocyanidin extraction.

Ohmic heating is a relatively novel pre-fermentative maceration technique that relies on the application of an electric current directly through the grape must. This process, also referred to as Moderate Electric Fields (MEF) treatment, induces rapid and uniform heating by exploiting the electrical conductivity of the must, leading to the electroporation of plant cell membranes. As a result, cellular permeability increases, facilitating the release of intracellular compounds such as anthocyanins, tannins, and other phenolics.

The mechanism of ohmic heating differs from conventional heating processes in that it allows a more controlled and homogeneous temperature increase without the need for external heat exchange surfaces, thereby reducing thermal degradation of sensitive compounds. Studies have demonstrated that ohmic heating can significantly enhance the extraction efficiency of anthocyanins and other colour-contributing molecules, while preserving the sensory quality of the wine (Zhang *et al.*, 2021).

Additionally, ohmic heating has been associated with the increased formation of stable polymeric pigments and improved colour stability during wine ageing. The electroporation effect enhances not only the release of anthocyanins but also their interaction with other macromolecules, including flavanols and polysaccharides, contributing to the formation of pigmented polymers that are more resistant to oxidation and sulfur bleaching. Furthermore, the treatment has been shown to alter the kinetics of fermentation, potentially accelerating yeast metabolism and shortening overall vinification time, thereby offering both qualitative and economic benefits.

Despite these advantages, the practical application of ohmic heating in commercial winemaking still faces challenges. These include the need for specialized equipment, optimization of voltage and frequency parameters based on grape variety and maturity, and careful monitoring to avoid undesirable changes in must composition or microbial stability. Nevertheless, the potential of ohmic heating to enhance phenolic extraction and support more sustainable, energy-efficient winemaking practices continues to draw significant research interest (Río Segade *et al.*, 2015).

2.1.3.1.2. Wine aging transformations

It has been already emphasized that during winemaking and aging anthocyanins undergo a series of chemical reactions and structural transformations. Influenced by multiple factors including grape variety, maceration duration, yeast strains, vinification techniques, sulfur dioxide (SO_2) concentration, aging conditions, use of fining agents, and micro-oxygenation, anthocyanins form a more complex polyphenolic structures through mechanisms such as copigmentation, condensation, and polymerization (Boulton *et al.*, 2001). The resulting compounds include condensed tannins (flavan-3-ol derivatives), collectively referred to as polymeric pigments, and various classes of pyranoanthocyanins such as vitisin A, vitisin B, vinylphenolic pyranoanthocyanins, vitisin A derivatives, oxovitisins, methylpyranoanthocyanins, and pyranoanthocyanin dimers.

Vinylphenolic pyranoanthocyanins include compounds like pinotins and flavanol-pyranoanthocyanins, while portisins represent a distinct group of pigments predominantly found in Port wines (Waterhouse and Zhu, 2019). These reaction products significantly alter the phenolic profile, colour intensity and hue, colour stability, and sensory attributes of red wines, as reviewed comprehensively by Quaglieri *et al.* (2017). During fermentation, anthocyanins interact with other molecules, such as other anthocyanins (self-association), tannins, proteins, and metal ions, leading to copigmentation, which enhances colour

intensity via hyperchromic and bathochromic effects (Moreno-Arribas and Polo, 2009; Zhang *et al.*, 2022).

In young red wines, intermolecular copigmentation may account for 30–50% of observed colour, while monomeric anthocyanins (in the form of flavylium cations) contribute 30–70% (Boulton *et al.*, 2001; Brouillard *et al.*, 1982). As wines age, polymerized pigments become the dominant contributors to colour, responsible for 35–63% of the total (Han *et al.*, 2008). Wine colour is frequently assessed using CIELAB parameters: lightness (L*), redness (a*), blueness (b*), chroma (C*), hue angle (H*), and colour difference (ΔE*). Young red wines are typically dark with high colour density and a violet-red hue. Han *et al.* (2008) demonstrated via principal component analysis that monomeric anthocyanins negatively correlate with L*, b*, and H*, but positively with a* and C* values in young 'Cabernet Sauvignon' wines. Over time, aging results in increased L*, b*, and H* values due to decreased colour density, loss of violet hues, and the development of tawny tones (McRae *et al.*, 2012). The chromatic evolution of red wines thus shifts from bright red and deep purple to pale red (Apolinar-Valiente *et al.*, 2016), making colour a useful proxy for wine age (Wang *et al.*, 2023). Pyranoanthocyanins, particularly pinotins (though not vitisin B), have been implicated in the tawny characteristics of aged wines (Zhang *et al.*, 2021). Formed via direct cycloaddition of malvidin and caffeic acid, pinotins are stable phenolic pigments that contribute to colour intensity and enhance the complexity of aged wine hues. Tawny and brick-red tones have been specifically linked to these compounds. Colour density during aging correlates closely with vitisin A and flavanyl-pyranoanthocyanin content (Zhang *et al.*, 2021). Notably, different anthocyanin derivatives exhibit varying stabilities. Pinotins are the most stable, followed by flavanyl-pyranoanthocyanins, vitisin A, monomeric anthocyanins, and direct anthocyanin–flavan-3-ol condensation products. The least stable compounds include vitisin B and anthocyanin–ethyl-linked flavan-3-ol products. These findings stem from an analysis of chromatic and phenolic characteristics in 234 red wines from various vintages and grape varieties ('Cabernet Sauvignon', 'Syrah', 'Merlot', 'Cabernet Franc', 'Tempranillo', 'Zinfandel', 'Pinotage', 'Carmenere', and 'Marselan') across 13 countries. Chromatic changes reflect underlying biochemical shifts, including modifications in the ratio of non-acylated to acylated anthocyanins. Acylation, a process that continues during aging, is crucial in forming pyranoanthocyanins and polymeric pigments, influencing colour stability and intensity (Wang *et al.*, 2023). Younger wines exhibit higher levels of acylated anthocyanins, which degrade more slowly. As aging progresses, the concentrations of both anthocyanin types decline, concurrent with the formation of pyranoanthocyanins and polymeric pigments. These changes correspond to a reduction in a* and increases in b* and H* values.

Post-fermentation, anthocyanins remain highly reactive, continuing to interact with other polyphenols and tannins extracted from grape skins and seeds (Boulton, 2001). Every subsequent step in winemaking introduces new opportunities for phenolic transformations, highlighting the importance of carefully selecting aging treatments and vessel types. Various studies have explored the effects of oak barrel aging, oak chip addition, and micro-oxygenation on the phenolic profile and colour of red wines. Barrel aging, often conducted in wooden casks or bottles, represents a pivotal stage in wine maturation (Teissedre and Jourdes, 2013), and ongoing monitoring of physicochemical parameters during this period is critical.

Watrelot and Waterhouse (2018) investigated the degradation of monomeric anthocyanins and the formation of pigmented tannins in 'Cabernet Sauvignon' wines aged for 8 and 12 months in barrels with different toasting intensities (low, medium, and high). Malvidin coumaroyl glucoside, a highly reactive monomeric anthocyanin, was particularly susceptible to degradation. This loss was attributed to ester hydrolysis or precipitation, rather than oxidation. Wines aged in lightly toasted barrels exhibited higher ellagitannin concentrations, as high toasting levels degrade these compounds. Furthermore, a lower ellagitannin concentration was associated with greater monomeric anthocyanin loss, suggesting a protective, stabilizing role of ellagitannins in anthocyanin preservation (Chassaing *et al.*, 2010). However, oak barrel aging entails significant financial investment due to the cost of barrels and the extended time required. Thus, alternative aging methods have been developed to reduce costs while preserving wine quality (Ferreiro-Gonzales *et al.*, 2019). Among these are oak chip addition, micro-oxygenation, and high hydrostatic pressure (HHP) treatment. The influence of oak chips on wine characteristics depends on the timing of their incorporation. When added during fermentation, they have minimal effect on ellagitannin

extraction and anthocyanin stabilization but may introduce wood-derived volatiles such as lactones, ethyl esters, and acetates. Conversely, post-fermentation addition may enhance wine aging potential by promoting tannin–anthocyanin condensation reactions (Kyraleou *et al.*, 2016).
Comparative studies have examined the effects of oak barrels, oak chips, micro-oxygenation, and HHP on wine colour and phenolic complexity. For example, Cano-López *et al.* (2010) found that micro-oxygenation improved colour quality in 'Monastrell' wines over a three-month period, comparable to barrel aging. However, six months of subsequent bottle aging resulted in increased yellow tint, likely due to differences in the anthocyanin–tannin complexes formed during barrel aging, driven by ellagitannins, phenolic acids, and wood aldehydes. González- Sáiz *et al.* (2014) conducted similar investigations on 'Tempranillo' wines, analyzing the effects of varying oxygen dosages, oak chip concentrations, wood origin (French vs. American oak), toasting degree, and maceration time. These studies aimed to replicate the conditions of barrel aging and demonstrated that anthocyanin content during aging is governed by competing reactions involving shared substrates, primarily monomeric anthocyanins. The final anthocyanin composition is thus the result of a complex dynamic equilibrium, highly sensitive to processing parameters.

2.1.4. Flavan-3-ols and procyanidins in grape and wine

Flavan-3-ols are polyphenols belonging to the flavonoid family of phenolic compounds, just like anthocyanins (Padilla-Gonzalez *et al.*, 2022). Since they contribute vastly to the color stabilization and sensory properties of red wine, i.e. astringency and bitterness, they are perceived as polyphenols of great significance in red winemaking. In grapes, they are mostly present in seeds, skins, and stalks, as monomers, oligomers and polymers. Key monomers are (+)-catechin and (-)-epicatechin, formed by a benzopyran unit (rings A and C) with an aromatic cycle (ring B) linked to the carbon C-2 of the pyranic cycle (ring C). Each of the monomers may have 4 possible configurations due to the presence of two chiral centers on the molecule (C2 and C3). These molecules are structurally diverse and reactive, interacting with each other and forming dimers, oligomers and polymers. All the polymeric structures derived from flavan-3-ol are collectively referred to as condensed tannins or proanthocyanidins (Hornedo-Ortega *et al.*, 2020).
Procyanidins are formed by (+)-catechin and (-)-epicatechin and their gallic esters, mostly located in grape seeds. Recent findings reported grape seeds glycosylated procyanidins, although glycosylation position and the structure of these molecules have not yet been illuminated (Zerbib *et al.*, 2018).
Flavan-3-ols and their polymers are synthesized via flavonoid pathway, more specifically phenylpropanoid metabolic pathway. Key differences between the anthocyanin and flavan-3-ols synthesis is in the last steps of flavonoid pathway: flavan-3-ols are synthesized by direct reduction of leucoanthocyanidins by enzyme leucoanthocyanidin reductase (LAR), resulting with corresponding flavan-3-ol. In contrast, anthocyanins go through synthesis reactions induced by anthocyanidin synthase (ANS) (Nabavi *et al.*, 2020). Both pathways are shown on Figure 2.4. (Ashihara *et al.*, 2010).
The concentration of the flavan-3-ols and condensed tannins in solid grape parts changes during the phenology, with a decreasing trend, with the concentrations highest at veraison and rapidly declining until becoming stable around maturity. Degree of polymerization is increasing with grape maturity, due to the fast decomposition of monomeric flavan-3-ols. The content of phenolic compounds in grape, especially seeds, can be modulated by external (sun radiation, temperature, water availability, location of the vineyard site, viticultural practices) and intrinsic (variety) factors. The concentration of the flavan-3-ols and procyanidins in wine will differ among varieties and depend on the winemaking technique (Ribereau-Gayon *et al.*, 2006).
The chemical dynamic of winemaking drives both degradation and polymerization of flavan-3-ols, shaping their functional and sensory impact in the final product. Firstly, the extraction is facilitated by elevated ethanol concentrations and prolonged maceration time. Gonzalez-Manzano *et al.* (2004) stated that the maximum content of flavanols from skin can be reached after the maceration of 24 h in 12.5% vol of ethanol, while for the seed extraction the needed conditions are long maceration and higher ethanol percentage. Their investigation of flavan-3-ols from grape seed and skin in simulated maceration showed

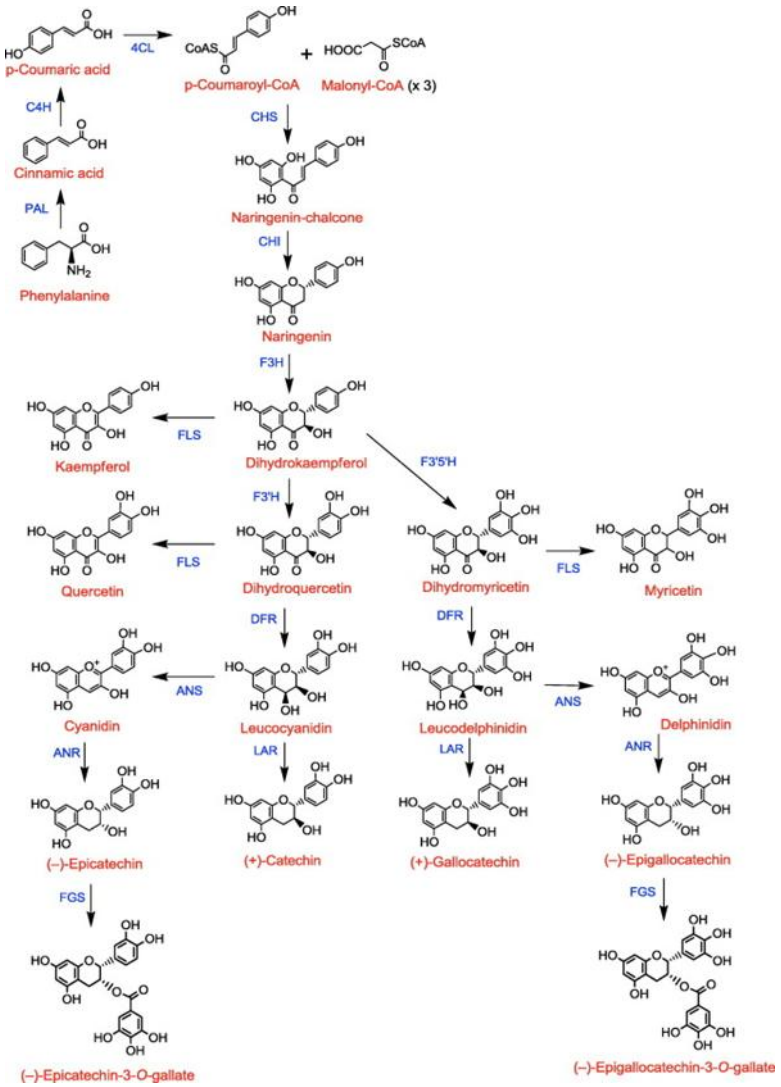

Figure 2.4. Possible biosynthetic pathways of flavan-3-ols in *Camellia sinensis* leaves. Abbreviations of enzymes are as follows: PAL, phenylalanine ammonia-lyase (EC 4.3.1.24); C4H, cinnamic acid 4-hydroxylase (EC 1.14.13.11); 4CL, 4-coumarate-CoA ligase (EC 6.2.1.12); CHS, chalcone synthase (EC 2.3.1.74); CHI, chalcone isomerase (EC 5.5.1.6); F3H, flavanone 3-hydroxylase (EC 1.14.11.9); F30,50H, flavonoid 30,50-hydroxylase (EC 1.14.13.88); F30H, flavonoid 30-hydroxylase (EC 1.14.13.21); FLS, flavonol synthase (EC 1.14.11.23); DFR, dihydroflavanol 4-reductase (EC 1.1.1.219); ANS, anthocyanidin synthase (EC 1.14.11.19); ANR, anthocyanidin reductase (EC 1.3.1.77); LAR, leucocyanidin reductase (EC 1.17.1.3); FGS, flavan-3-ol gallate synthase (EC number not assigned). (Ashihara *et al.*, 2010)

that the concentration of seed flavanols increased for 50% after 3 weeks of maceration. This has been confirmed by Ribereau-Gayon *et al.*, 2006. The extraction of condensed tannins during winemaking is slower than that of anthocyanins and increases with the increase of alcohol, during maceration with fermentation included, left on the skins. Numerous factors significantly influence the concentration and composition of flavan-3-ols in red winemaking. Through oxidation and condensation reactions, particularly in the presence of acetaldehyde and anthocyanins, flavan-3-ols undergo different structural changes. These reactions result in formation of polymeric and pigmented tannins, while enzymatic activity and pH conditions can facilitate the cleavage or rearrangement of interflavan bonds, leading to structural modifications.

2.1.5. Biochemical interactions of anthocyanins and procyanidins in red wine

During wine aging, flavan-3-ols undergo a series of complex biochemical transformations, primarily driven by oxidation reactions, polymerization, and interactions with other phenolic and non-phenolic compounds. Monomeric flavan-3-ols gradually polymerize into higher molecular weight procyanidins or

interact with anthocyanins through co-pigmentation reactions influencing the color stability, and forming new, complex pigments which have distinct roles in regard to red wine quality and sensorial properties, modulating the astringency over time (Gonzalez-Manzano *et al.* 2009). The resulting polymeric pigments exhibit increased resistance to pH, sulfite bleaching, and oxidative degradation compared to their monomeric precursors.

One of the primary pathways of their interactions involves direct condensation between anthocyanins (usually malvidin-3-*O*-glucoside) and flavan-3-ol units. These reactions occur either through nucleophilic attack of the procyanidin C6 or C8 position on the electrophilic carbon of the anthocyanin's flavylium ring. Additionally, acetaldehyde, formed during ethanol oxidation, acts as a bridging molecule in ethyl-linked condensation reactions, enhancing the formation of polymeric pigments. The concentration of free flavan-3-ols is reducing due to these reactions, but more stable and less reactive tannin structures are formed. Reducing tannin reactivity as a result has decreased astringency perception, whilst simultaneously enriching the wine's color intensity and hue. Interactions between anthocyanins and procyanidins may be the key mechanism underlying the evolution of red wine phenolic composition and sensory quality during aging.

2.1.6. Analytical techniques for investigating grape and wine phenolics

Research investigating the interactions between anthocyanins and proteins has been conducted to address various scientific objectives. Owing to their antioxidant properties and associated health benefits, anthocyanins have been utilized as bioactive compounds and encapsulated using various encapsulation techniques with carriers composed of diverse materials (Arroyo-Maya and McClemens 2015; Garcia-Tejeda *et al.*, 2016; Cai *et al.*, 2019; Chi *et al.*, 2019; da Silva Carvalho *et al.*, 2019; Carra *et al.*, 2022). Due to their sensitivity as bioactive compounds with diverse health benefits and susceptibility to structural changes and biochemical transformations, the stability of anthocyanins in combination with other compounds has been extensively studied (Cai *et al.*, 2022; Chamizo-Gonzalez *et al.*, 2023). In parallel with these studies, research on the mechanisms of interaction between grape and wine anthocyanins and salivary proteins has also been advanced, employing analytical techniques and methodologies developed for diverse research purposes (Ferrer-Gallego *et al.*, 2015; Paissoni *et al.*, 2018; Paissoni *et al.*, 2020). Regarding the development of analytical techniques and methods for investigating mechanisms of interactions between grape/wine anthocyanins and salivary proteins, it is essential to approach the research from various perspectives within the field of analytical chemistry.

2.1.6.1. Extraction, separation and purification of grape anthocyanins

Extraction of the anthocyanins from different plant materials, including black grapes, is one of the main postulates in preparation of the research. Efficiency, yield, stability and quality of the anthocyanins' crude extract will depend on the methodology used. As natural water-soluble flavonoids, containing unsaturated double bonds and easily oxidized groups, anthocyanins are highly unstable (Li *et al.*, 2021). Therefore, their biochemical structure can be easily modified (Tan *et al.*, 2022). The main goal during the extraction part of the research is that the biochemical structure of the anthocyanins remains highly intact, coupled with low costs, high extraction yield, high extraction rate, and reduced pollution potential. Some of the extraction methods widely employed are solvent extraction method (SEM), ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), supercritical carbon dioxide extraction (SCDE), and combined extraction method involving the simultaneous integration of multiple methods. SEM is one of the most widely used methods for anthocyanin extraction due to its simplicity and cost-effectiveness. The process of SEM involves preparation of plant material such as homogenization in the solvent, followed by extraction and filtration for removing the soluble compounds. Usual solvents for anthocyanins extraction are ethanol, methanol, acidified water, acidified methanol or acidified ethanol (with mineral or organic acids). Acidification of the solvent prevents anthocyanins' degradation maintaining the pH of medium appropriate for flavylium cation form of the pigment. One of the disadvantages of this method is co-extraction of undesirable compounds, such as tannins, demanding the

additional purification of the extract. Additionally, recent investigations have been conducted using ionic liquids as solvents. Ionic liquid solvents are a class of unique, environmentally friendly solvents composed entirely of ions, typically a bulky organic cation and an inorganic or organic anion. These solvents are liquid at temperatures below 100℃ and exhibit exceptional physicochemical properties, including negligible vapor pressure, high thermal and chemical stability, tunable polarity, and excellent solvation capabilities. In anthocyanin extraction, ionic liquids have shown promise due to their ability to disrupt plant cell walls and stabilize anthocyanins, enhancing yield and preserving pigment integrity. Despite their advantages, challenges such as high production costs, limited biodegradability, and potential toxicity must be addressed to enable their widespread adoption in industrial processes. Allendes *et al.* (2024) investigated different ionic liquid and their influence on the molecular structure of extracted anthocyanins from grape pomace of *Vitis vinifera* L. cv 'Carmenere'. Conductor-like Screening Model for Real Solvents (COSMO-RS) has been used to predict chemical potentials of solvents for the extraction of anthocyanins. Based on the COSMO-RS calculations they reported that acetate-based ionic liquids displayed the highest affinity for anthocyanins. Nevertheless, the best yields were noticed with hydrogen sulfate anion-based ionic liquids, with 4 mg/g of total anthocyanins, maintaining stability of anthocyanin molecules due to low pH.

UAE uses high-frequency sound waves to disrupt plant cell walls, enhancing the release of anthocyanins into the extraction solvent. Reducing extraction time, lower solvent usage and higher yields in comparison to SEM are some of the advantages of this method. UAE is energy efficient and scalable but may require optimization of parameters such as sonication time, power and temperature to prevent anthocyanin degradation. Xie *et al.* (2022) investigated the advantages of ultrasound-assisted aqueous two-phase (ATP) extraction method on the extraction of polyphenols of grape pomace (European-American hybrid Xiahei). As solutions for ATP were used ethanol and ammonium sulfate. Based on the phenolics yield, the ethanol-ammonium sulfate ATP gave best results and extraction performance at 30% of ethanol concentration and 20% of ammonium concentration, respectively. Additionally, the results showed that higher extraction temperatures (40℃) are in correlation with higher yields of phenolics, increasing solubility and diffusing coefficient of phenolics, promoting softening and swelling of particles and reducing the viscosity of the solvent. Although improved yields, excessive temperatures are not recommended since anthocyanins are sensitive to higher temperatures and susceptible to degradation. Zhao *et al.* (2020) reported that during the extraction of anthocyanins from 50 g of grape pomace (*Vitis vinifera* L. cv 'Merlot'), they obtained 56.15 mg of total anthocyanins. The extraction was conducted in 2% formic acid methanolic solution (MeOH), in combination with ultrasonication of 59 kHz frequency for 10 min at ambient temperature 25-35℃.

Decker *et al.* (2024) investigated the optimization conditions of ultrasound-assisted anthocyanins' extraction of *Vitis labrusca* grape pomace, utilizing acidified water as the solvent. They previously dried pomace in the oven, which helped preparation of the extraction material. The operative conditions encompassed a power density range of 8.3–16.7 W/mL, pulse intervals of 0–2 seconds, and extraction durations of 1–5 minutes, resulting in an extraction yield of up to 2.56 mg/g, suggesting that acidified water can be sustainable alternative to commonly utilized organic solvents.

MAE is an extraction method that utilizes microwave radiation to heat the solvent and plant material, enhancing mass transfer and anthocyanin solubilization. Microwaves are provoking rapid heating effects, disrupting cellular structures, and facilitating the release of intracellular compounds. This method is highly efficient, significantly reducing extraction time and energy consumption. Challenges occurring regarding this method are potential thermal degradation of anthocyanins in excessive microwave power or prolonged exposure. Therefore, optimizing process variables such as power, time and solvent composition is crucial for achieving high yields and preserving pigment integrity. Crescente *et al.* (2023) compared microwave hydro-diffusion and gravity (MHG) and UAE techniques for the extraction of phenolic compounds of grape pomace extract, *Vitis vinifera* L. cv 'Aglianico'. They documented varying extraction conditions for both techniques, specifically regarding extraction time, yield, and the preservation of compounds. For MHG, optimal power density in atmospheric pressure condition was set at 2 W/g (Huma *et al.*, 2009), and the humidifying of plant material was on 80%, since plant material

moisture is efficiency dependent factor in MHG (Ferreira *et al.*, 2020). For UAE, solvent concentration, frequency, temperature and sonication time were carefully observed. As an extracting solvent they used MeOH/H_2O (1:1 v/v), ultrasonic baths were at 40 kHz frequency, they set the ultrasonic bath temperature at 20℃ as not to induce degradation of anthocyanins, and the extraction cycle lasted for 30 min. These conditions were in accordance with previous research (Kumar *et al.*, 2021; Llobera *et al.*, 2009; Soria *et al.*, 2010; Tao *et al.*, 2014) Alternative solvents such as natural deep eutectic solvents have been investigated in combination with UAE and MAE, to facilitate the development of environmentally sustainable techniques for the extraction of biomolecules. Panić *et al.* (2019) investigated the scale-up NADES extraction of anthocyanins from the *Vitis vinifera* L. cv 'Plavac mali', utilizing choline chloride:citric acid as a solvent, and comparing microwave and ultrasound-assisted extraction results. The research results showed that the best anthocyanins' extraction was achieved with use of both UAE and MAE irradiation systems simultaneously. Recovering of anthocyanins from NADES solvent was more efficient when the NADES molecule structures were broken down by addition of > 50% (v/v) water (99.46%), with high solvent recycling yield (96.8%). The utilization of NADES may be particularly advantageous for the preparation of extracts intended for applications in the food and pharmaceutical industries, as they often eliminate the need for additional purification steps (Radošević *et al.*, 2016).

Supercritical carbon dioxide ($SCCO_2$) uses supercritical CO_2 as a solvent, often modified with polar co-solvents like ethanol, to extract anthocyanins. Supercritical CO_2 is an excellent solvent due to its low viscosity, high diffusivity, and tunable solvating power, which can be adjusted by changing pressure and temperature. $SCCO_2$ is a green and sustainable technique since CO_2 is non-toxic, recyclable, and leaves no solvent residue. Nevertheless, pure CO_2 is not practical for polar anthocyanins, and co-solvents are needed to enhance the solubility. This method requires specialized equipment and isn't cost-effective for certain applications, despite its high selectivity and environmentally friendly nature. Pazir *et al.* (2020) stated that $SCCO_2$ extraction can be efficient for industrial applications, due to preserving stability of the extracted molecules, cost reduction and optimization of the extraction time. This is in accordance with other findings (Vatai *et al.*, 2009; Machado *et al.*, 2022).

Given that anthocyanins in plant tissues, such as grapes, coexist with other organic and bioactive compounds which may accelerate anthocyanin degradation during storage and interfere with the research results (phenolic compounds, flavonoids, organic acids, proteins, carbohydrates), purification and separation of the crude extract are necessary. The best separation techniques for anthocyanins are solid-phase extraction (SPE), column chromatography, membrane separation, high-performance liquid chromatography (HPLC), high-speed counter-current chromatography (HSCCC), high-performance preparative liquid chromatography (HPPLC), and centrifugal partition chromatography (CPC).

Solid-phase extraction (SPE) is a widely used method for purification of anthocyanins due to its efficiency and ability to remove impurities such as sugars, acids, and other phenolic compounds. This technique implies that a sample solution is passed through a stationary phase, typically polymeric resin or reversed-phase silica, which selectively binds anthocyanins. Polar contaminants are washed away using aqueous solvents, while anthocyanins are eluted with acidified organic solvents like ethanol and methanol. Advantages of SPE lie in its simplicity, scalability, and precision, but its efficiency depends on careful optimization of parameters such as resin type, solvent composition, and pH value. While effective, SPE may require multiple steps for high-purity anthocyanin recovery. This has been confirmed by Crescente *et al.* (2023), who reported the presence of sugars and polysaccharide components in their extracts after SPE purification. Maciel-Silva *et al.* (2023) used an inline purification system of SPE which performed cleaning and allowed the fractionation and concentration of anthocyanins and other biocompounds.

Column chromatography is a versatile technique that employs a packed column with stationary phases, such as ion-exchange resins, reversed-phase materials, or Sephadex gels, separating anthocyanins based on their chemical properties. The sample is loaded onto the column, and a gradient of solvents is used to elute the anthocyanins. This technique allows for the fractionation of anthocyanins based on polarity, molecular weight, or charge, making it ideal for purifying specific anthocyanin derivatives. Column chromatography offers high resolution and purity, but can be time-consuming, requiring large volumes of solvents, and involve significant manual effort, making it less practical for large-scale applications

without automation. In analytical purposes, column chromatography still is widely used, since it allows working with small-scale applications, assuring great analytical precision. Sadilova *et al.* (2006) while investigated thermal degradation of acylated and nonacylated anthocyanins of strawberry, elderberry, and black carrot used XAD-16-HPand Sephadex-LH-20 columns, for removing amino acids, sugars, salts and phenolic compounds, respectively. They obtained the anthocyanins extract of great purity, which Paissoni *et al.* (2018) confirmed obtaining the total anthocyanins extract purity > 95%.

Membrane separation or membrane filtration uses semi-permeable membranes to separate anthocyanins based on their molecular size and charge (Kalbasi and Cisneros-Zevallos, 2007). Techniques such as ultra-, micro- or nanofiltration can concentrate and purify anthocyanins by selectively retaining large molecules like polysaccharides or proteins while allowing smaller molecules of anthocyanins to pass through (Patil and Raghavarao, 2007). Membrane filtration is energy-efficient, scalable, and does not require organic solvents, making it an environmentally friendly option (Chung *et al.*, 1986). The choice of membrane material and pore size is crucial to achieving high-purity anthocyanin fractions (Naveen *et al.*, 2006). Yammine *et al.* (2019) investigated several organic membranes for the fractionation of biocomounds of grape pomace extracts, with different molecular weights (2 – 100 kDa). They reported that polysulfone membranes not able to fractionate phenolic classes, except polymeric and monomeric proanthocyanidins. Furthermore, retention percentages of phenolic acids, stilbenes, anthocyanins, monomeric flavan-3-ols, and polymeric flavan-3-ols were determined using various membrane filtration systems. Tamires Vitor Pereira *et al.* (2020) investigated the recovering and concentration of monomeric anthocyanins from grape marc, using membrane nanofiltration (NF), in combination with previously conducted micro- (MF) and ultrafiltration (UF). The combination of microfiltration, followed by nanofiltration gave best results regarding retention coefficients of monomeric anthocyanins (78.2%), and total phenolics (71%), while maintaining high antioxidant capacity (52%). Munoz *et al.* (2021) investigated the recovery of anthocyanins and monosaccharides from grape marc (*Vitis vinifera* L. cv 'Carmenere'), using three different nanofiltration membranes (from 150 to 800 Da), evaluating their performance. Target compounds regarding anthocyanins were malvidin-3-*O*-glucoside, malvidin 3-*O*-(acetyl)-glucoside, and malvidin 3-*O*-(coumaroyl)-glucoside. The nanomembrane with the lowest molecular weight cut-off (150–300 Da) exhibited the least flux decay. However, all tested membranes demonstrated a rejection rate exceeding 99.42% for quantified anthocyanins.

Angela *et al.* (2024) reported that ultrafiltration could be employed to accelerate and reduce the costs associated with the finishing process of wines. However, its application was deemed suitable only for white wines, as red wines underwent significant depletion of essential characteristics, rendering them commercially unacceptable. This was attributed to the retention of anthocyanins and other phenolic compounds by the membrane. This method demonstrates considerable industrial potential for the separation of anthocyanins, enabling the production of natural pigments and antioxidant bioactive compounds. However, its application may be limited by challenges such as membrane fouling or clogging, which could lead to increased maintenance costs and operational inefficiencies.

High-performance liquid chromatography (HPLC) is a standard technique for anthocyanin purification, due to its exceptional resolution and precision. This method uses a high-pressure system to pass the sample through a column containing a high-performance stationary phase, such as reversed-phase C18 material, which separates anthocyanins based on their hydrophobic interactions. Acidified water and organic solvents like methanol and acetonitrile are used as mobile phases in a gradient system to achieve optimal separation. HPLC enables the isolation of individual anthocyanin compounds with high purity and quantification capability, perfect for analytical research purposes.

Preparative chromatography is a scaled-up version of analytical chromatography, used for isolating anthocyanins in larger quantities. It employs similar principles to HPLC but uses larger columns and higher flow rates to handle greater sample loads. This method is ideal for obtaining gram-scale purified anthocyanins for research or industrial use. Although preparative chromatography provides high-purity fractions, it shares the limitations of HPLC, including high costs, significant solvent consumption, and the need for sophisticated equipment. The method is most effective when coupled with preliminary purification steps like SPE to reduce the complexity of the sample matrix.

High-speed counter-current chromatography (HSCCC) is a liquid-liquid chromatography technique widely used for the purification of anthocyanins due to its high efficiency, scalability, and ability to maintain bioactivity. HSCCC operates without a stationary phase, instead it's utilizing two immiscible liquid phases: one serves as the stationary phase retained by centrifugal force, while the other acts as the mobile phase. Anthocyanins are separated based on their partitioning behavior between these two phases, driven by difference in polarity, hydrophobicity, and molecular interactions. This method eliminates the risk of irreversible binding and degradation associated with solid-phase methods, making it particularly suitable for sensitive compounds like anthocyanins. Acidified aqueous and organic solvent mixtures, such as water and ethyl acetate and butanol, are commonly used to optimize separation. HSCCC provides high recovery and purity, minimizes solvent consumption, and can handle complex sample matrices. However, the technique requires careful optimization of phase systems and operational parameters to achieve efficient separation, and the initial setup cost can be high. Li *et al.* (2013) have stated that this technique might find its purpose in industrial application for automatic extraction and separation of unstable compounds such as anthocyanins. This group of authors have investigated the application of HSCCC in anthocyanins isolation from the petals of Chaenomeles sinensis, in combination with supercritical fluid extraction. In a short period of 300 minutes, they successfully separated six anthocyanins (delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, delphinidin, peonidin, and malvidin). Previous research showed that anthocyanins separation from plant material via HSCCC might be successfully conducted without previous purification on SPE or column chromatography (Du *et al.*, 2004).

As a support free preparative chromatographic method, CPC has a biphasic system which is used to partition compounds between two immiscible liquid phases (stationary and mobile phases) according to their partition coefficient, where the phases remain separated in the column by centrifugal forces (Bouju *et al.*, 2015). CPC has numerous advantages for separation of phenolic compounds from grape, compared to previously mentioned HPLC. During separation process via HPLC sample losses occur, deterioration of column with the extract, where additionally above 50% of material weight never gets eluted because it sticks onto the solid support (Delaunay *et al.*, 2002). Previous investigation showed that gram-scaled amounts of pure anthocyanins extract could be obtained via CPC method (Renault *et al.*, 1997). Lima *et al.* (2021) stated that the choice of the best operational condition in CPC depends on the application of anthocyanins. Conducting research on CPC purification of anthocyanins from grape pomace, they used aqueous two-phase system (ATPS) and scaled-up centrifugal chromatography, using protic ionic liquids based on ethanol ammonium and sulfuric acid as solvent. Two different tie-line lengths (TLL) have been used for the experiments, 46.45 and 88.17, and descending and ascending operational modes. Results showed that for analytical purposes where high purity of the extracts is needed the best operational modes are long TLL in ascending mode (purification factor of 41.88 and recovery of 24.85%). Contrary, for the high recovery and moderate purification the best mode would be short TLL and ascending mode (purification factor 29.61-fold and recovery of 73.61%). Paissoni *et al.* (2018) reported that CPC enables efficient separation of anthocyanins based on the esterification of the glucoside moiety. They also stated that this method offers significant advantages over alternative techniques, including the ability to process large quantities of crude extracts and to isolate substantial amounts of acetylated and cinnamoylated derivatives, facilitated by the removal of the more abundant glucosides.

2.1.6.2. Qualitative and quantitative analysis of anthocyanins extracts

Following extraction, the anthocyanin crude extract must be characterized both qualitatively and quantitatively. For this purpose, various analytical techniques have been developed, investigated and employed. Research over the past decade highlights that the most employed chromatographic techniques for anthocyanin analysis include high-performance liquid chromatography (HPLC) integrated with various detection methods, such as photodiode array (PDA) or diode array detection (DAD), ultraviolet-visible (UV/VIS) spectroscopy, or tandem mass spectrometry (MS/MS) for quantification. Additionally, ultra-high-performance liquid chromatography (UHPLC), often coupled with quadrupole time-of-flight

mass spectrometry (QTOF-MS), is widely utilized for accurate mass determination and structural elucidation.

HPLC is a fundamental highly efficient technique for separation, identifying, and quantifying of anthocyanins. This technique possesses reversed-phase columns and gradient elution systems, facilitating the resolution of complexed anthocyanin mixtures (Lianza and Antognoni, 2024). HPLC coupled with electrospray ionization-quadruple time of flight (HPLC-ESI-QTOF) and matrix assisted laser desorption/ionization (MALDI-TOF) technique has been investigated and authors reported that the combination of these techniques can be utilized for both extractable and non-extractable phenolic compounds, respectively (Perez-Ramirez *et al.*, 2018; Di Lorenzo *et al.*, 2019). Other authors reported synergistic effects between high-performance thin-layer chromatography (HPTLC) and HPLC-DAD in investigation of grape pomace phenolic complex, where HPTLC was useful in detection of phenolic acids, flavonoids and anthocyanins, while HPLC-DAD identified only anthocyanins (Bernardi *et al.*, 2019).

As an advanced variant of HPLC, UHPLC operates at higher pressures, and with an improved resolution, offering faster analysis times, and reduced solvent consumption. Coupled with Q-TOF MS/MS, UHPLC provides enhanced detection sensitivity and mass accuracy, enabling precise molecular characterization of anthocyanin species and their derivatives. The Q-TOF MS/MS system facilitates structural elucidation through tandem mass spectrometry, identifying unique fragmentation patterns indicating specific anthocyanins and anthocyanins derivatives. Comparative investigation of micro-liquid (μLC) and ultra-high-performance liquid chromatography (UHPLC) coupled with hybrid tandem mass spectrometry (QqTOF MS) in red wine analysis showed differences regarding techniques. Papouskova *et al.* (2011) stated that UHPLC technique coupled with Q-TOF MS provides results with lower limit of detection (LOD) and limit of quantification (LOQ), enhancing productivity and precision of retention parameters and peak areas. Compared to UHPLC, the micro-LC technique demonstrated significant advantages, including reduced consumption of the mobile phase, decreased contamination of the mass spectrometer ion source, and an enhanced electrospray nebulization process. Although UHPLC coupled with mass spectrometry ensures fast and sensitive determination of anthocyanins, wine is a complex matrix having different bio-compounds of same molecular weights which complicates the analysis. Therefore, for the separation of isobaric compounds and more detailed anthocyanin analysis Alberts *et al.* (2012) suggests combination of reversed phase liquid chromatography (RP-LC). Their approach was applied in the investigation of red wine anthocyanins and their derivatives and involved two series of analysis, first neutral loss scanning to selectively detect anthocyanin glucosides, diglucosides, acylated anthocyanins and compounds formed during wine aging. Neutral loss scanning also provides information on molecular weight and mass of the attached sugar moiety, additionally helping the investigation. The second set of analysis included characterization of aglycone cation, allowing detection and identification of 121 red wine anthocyanins and their derivatives (pyranoanthocyanins and flavanol-anthocyanins).

Using these techniques, the identification of compounds such as anthocyanins within the matrix is achieved by comparing their characteristics to pre-established reference standards, which is often costly. Li *et al.* (2022) while investigating quantification of grape anthocyanins by UHPLC-QTOF MS, combined it with quantitative analysis of multiple-components by single marker (QAMS) using only peonidin 3-O-glucoside. QAMS method enables the possibility of simultaneous detection of content of multicomponents in the sample utilizing only one reference standard (Ning *et al.*, 2016). The results of the research showed that QAMS method, in comparison with standard external quantification method can indeed determine anthocyanins in grapes with the high precision. With this knowledge, identification and quantification of anthocyanins might be more environmentally safe, cost-effective and with lower operational complexity.

Liquid chromatography electrospray-ionization mass spectrometry (LC-ESI-MS) coupled with quadruple-time-of-flight mass spectrometry (Q-TOF MS/MS) can also be utilized in investigations of anthocyanins, the kinetics of their degradation and co-pigmentation to predict the occurrence of different compounds during wine aging (Mohammadi *et al.*, 2023). Similarly to this, Pinasseau *et al.* (2017) developed a targeted metabolomic based method for investigating the grape skin phenolics among

different cultivars and their response to drought. Utilizing UHPLC coupled with triple quadruple mass spectrometry (QqQ-MS), they developed method for rapid and sensitive identification and quantification of grape skin phenolic compounds, including anthocyanins.

2.1.7. Influence of anthocyanins and procyanidins on sensory characteristics of red wines

Red wine sensorics, the study of its sensory attributes, integrates chemistry (Hufnagel and Hofmann, 2008), physiology (Malfeito-Ferreira, 2021), and psychology (Parr, 2019) to evaluate its aromatic, gustatory, and textural characteristics, with particular emphasis on astringency perception. Astringency, a key tactile sensation and quality attribute of red wine, arises primarily from tannins, which interact with salivary proteins to produce a drying or puckering effect in the mouth (Breslin *et al.*, 1993). This sensation is influenced by the concentration, size, and structure of tannins, as well as their interactions with acids, ethanol, and anthocyanins (Cala *et al.*, 2011; Garcia-Estevez *et al.*, 2017). Anthocyanins, responsible for red wine's color, also contribute to sensory outcomes by stabilizing tannins through copigmentation and forming tannin-anthocyanin complexes (Boulton, 2001). Since copigmentation interactions are lowering the content of free phenolic compounds, they can soften astringency and modify the wine's mouthfeel by reducing the harshness of isolated tannins. Textural descriptors, such as dryness, roughness, and roundness, are shaped into the mouthfeel wheel, a terminology guide for communicating the sensorial gustatory characteristics of red wine (Gawel *et al.*, 2000). In addition to this, advanced techniques, high-performance liquid chromatography, gel electrophoresis, and sensory panels combined, provide critical sensometabolomic insights into the complex interplay between anthocyanins, tannins, and other wine components, advancing understanding of red wine's sensory qualities and consumer preferences (Ferrero-del-Teso *et al.*, 2024). Red wine evaluation involves various sensory analysis techniques, each targeting specific attributes of the wine. These techniques include visual assessment, olfactory analysis, gustatory evaluation, and tactile examination (Fairbairn *et al.*, 2024; Ortega-Heras *et al.*, 2024). Visual assessment focuses on parameters such as color, clarity, and viscosity (Hensel *et al.*, 2024). Olfactory analysis identifies aromatic compounds and their intensities, which define the wine's bouquet (Horberg *et al.*, 2025; Carreiras *et al.*, 2022). Gustatory evaluation examines taste elements, including sweetness, acidity, bitterness, and astringency (Paissoni *et al.*, 2023). Tactile examination assesses mouthfeel and body (de-la-Fuente-Blanco *et al.*, 2024). Together, these sensory analyses provide a detailed and holistic characterization of the wines' sensory property (Pires *et al.*, 2020). For investigating sensory consequences and implications of different phenolic comp1ounds on the red wine mouthfeel and astringency perceptions, recent studies are using multiple rapid sensory profiling techniques such as triangle test, check-all-that-apply (CATA), sorting and polarized sensory positioning (PSP), as alternatives to descriptive sensory analysis (Fleming *et al.*, 2016). Additionally, recent studies have provided proof of influence of saliva protein composition on wine preferences among consumers, stating that statistically significant variations in concentration of salivary proteins have been noticed for proline-rich proteins and lipocalin-1 (Luo *et al.*, 2023). These findings should be further investigated since the affinity of phenolic compounds towards salivary proteins differs regarding their molecular weight and polarity.

Among scientists, there is a lack of consensus regarding whether anthocyanins directly contribute to and cause the development of astringency. However, the majority agrees that anthocyanins play a role in influencing the sensory perception of wine, modifying the sensory perception of condensed tannins (proanthocyanidins). Investigating taste and mouthfeel properties of different classes of phenolic compounds of red wine, Vidal *et al.* (2004a) stated that purified grape anthocyanins (monoglucosides and monoglucoside coumarates) "do not contribute astringency nor bitterness to wine." Results of a similar study in which the influence of key wine components on mouthfeel perception was investigated, showed that anthocyanin fraction contributed to fullness and coarseness of red wine (Vidal *et al.*, 2004b). They also reported that, in the context of interactions between phenolic compounds, the presence of anthocyanins did not have a direct impact on medium surface smoothness. However, their presence was observed to reduce the influence of procyanidin (tannin) concentration on this parameter. Contrary to these results, other groupof researchers reported that the anthocyanins may contribute to the in-mouth sensorial consequences, connecting them to the descriptors such as astringency and bitterness (Paissoni

et al., 2018). Investigating the affinity of the different anthocyanins' fractions (glucoside, acetylated and cinnamoylated) towards salivary proteins and bovin serum albumin (BSA), they also found that anthocyanins reacted differently regarding the fractions. Results showed that cynnamoylated anthocyanins were the most reactive towards salivary proteins. The same groupof researchers investigated the effects of anthocyanins on in-mouth sensory perceptions and their ability to modify condensed tannins. The anthocyanin acylation groups studied included glucoside, acetylglucoside, and p-coumaroylglucosides, extracted from grape skins of *Vitis vinifera* L. cultivars 'Nebbiolo' and 'Barbera'. The sensory evaluation methods employed in the study were the triangle test, Check-All-That-Apply (CATA), and descriptive analysis. The findings indicated that pure anthocyanin fractions exerted minimal sensory impact. However, for total anthocyanins and glucoside fractions at a concentration of 400 mg/L, anthocyanins were found to influence the perception of astringency, particularly in the subqualities described as "velvety" and "chalky" (Paissoni *et al.*, 2020). Nevertheless, it is still unknown whether anthocyanins really have a mild taste, as it has been reported by Singelton and Noble (1976). Further, regarding anthocyanins' taste, it's been reported that anthocyanins activate bitterness receptors (Soares *et al.*, 2013). Same group of authors investigated the impact of co-pigmentation between malvidin-3-*O*-glucoside and epicatechin on the interaction of flavonols with proline-rich proteins (PRPs) using saturation-transfer difference nuclear magnetic resonance (STD-NMR) and isothermal titration calorimetry (ITC). Their findings indicated that the mixture of epicatechin and malvidin-3-*O*-glucoside exhibited similar binding affinities to PRPs as the individual compounds (Soares *et al.*, 2019). The study highlighted distinct interaction mechanisms: epicatechin engaged in both hydrophobic and hydrophilic interactions, while malvidin-3-*O*-glucoside primarily demonstrated electrostatic interactions. Building on these findings, Torres-Rochera *et al.* (2023) explored the role of anthocyanins in mediating interactions between salivary mucins and wine astringent compounds. Their results revealed that malvidin-3-*O*-glucoside, when isolated, exhibited the strongest binding affinity to salivary mucins compared to catechin, epicatechin, and quercetin-3-β-glucopyranoside. Additionally, the study suggested that co-pigmentation could play a broader role in modifying the intensity and nature of interactions between mucins and other phenolic compounds. Similarly, Mao *et al.* (2024) examined the interactions between oral mucins and cyanidin-3-*O*-glucoside, with a focus on the influence of oxidized quinones. Their research demonstrated that the oxidation of anthocyanins into quinones facilitated covalent binding with mucins in the oral cavity, forming tighter cross-linkages and intensifying oral astringency. Advancing the understanding of phenolic interactions in the oral environment, Soares *et al.* (2020) developed in vitro models representing buccal mucosa, tongue, human saliva, and mucosal pellicles to investigate the interaction of anthocyanin-rich red wine extract and green tea flavanol extract with oral epithelia. Their findings showed that anthocyanins preferentially interacted with oral cells. Notably, anthocyanins such as delphinidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside exhibited comparable interaction capabilities. These studies collectively highlight the complexity of phenolic compound interactions within the oral cavity, suggesting that various oral constituents are functioning distinctly across different phases of phenolic intake. This knowledge presents promising avenues for future research into the contribution of anthocyanins to the astringency of red wine.

2.2. Salivary proteins

2.2.1. Sensory perception of red wine and salivary proteins composition

Saliva ("whole saliva," "mixed saliva," "oral fluid") is an important body fluid of the oral cavity responsible for various functions (Schipper *et al.*, 2007). Saliva has major roles in speech, lubrication, digestion, maintaining a healthy oral cavity, enamel and teeth protection, antimicrobial action, and health in general (Bongaerts *et al.*, 2007; Slomiany *et al.*,1996; Gibbins and Carpenter, 2013). Examining saliva composition may discover the presence of systemic disease in an individual, and exposure to harmful substances. Saliva is an exocrine fluid secretion of major (parotid, submandibular, and sublingual glands) and minor (glands in the lower lip, tongue, palate, cheeks, and pharynx) salivary glands (Contreras-Aguilar and Gomez-Garcia, 2020; Tvarijonaviciute *et al.*, 2020). Although mainly composed of water (99.5%),

saliva has different viscosity due to proteins, inorganic and trace substances. Salivary proteins are glycoproteins, enzymes, immunoglobulins, and peptides such as cystatins, statherin, histatins, and proline-rich proteins. Each protein has its function, although some are not completely understood or described. Inorganic parts of the salivary fluid are usually electrolytes (sodium, potassium, chloride, and bicarbonate), while whole saliva also consists of blood, oral tissues, microorganisms, and food remains. Saliva controls sensory perception in the oral cavity, controlling the transport, adsorption, and metabolism of the flavor molecules, and the friction of the oral cavity (Canon *et al.*, 2018). Additionally, saliva influences aroma release and perception, through different mechanisms, affecting food acceptance (Ployon *et al.*, 2017). Saliva constitution, oral health, lubrication, and saliva secretion differ among individuals, based on their circadian rhythm, diet, drugs, age, gender, blood type, physiological status, and type and size of salivary glands. Consequently, sensory perceptions of food intake are individually predefined.

2.2.2. Astringency development of red wine and mouthfeel during wine tasting

The influence of salivary composition, mainly salivary proteins, on the perception of red wine astringency, has been largely investigated. Astringency is a tactile sensation, associated with the shrinking, drawing, or puckering of epithelium in the oral cavity, due to exposure to alums or tannins (Brossaud *et al.*, 2008; ASTM 1989, Yao *et al.*, 2010). The mechanism of the development of red wine astringency has not been fully understood (Garcia-Estevez *et al.*, 2018), but it is presumed it occurs as precipitation of salivary proteins by astringent molecules (phenolic compounds) (Bate-Smith, 1954). Precipitation reduces saliva viscosity, enhancing friction (Green, 1993; Smith and Noble, 1998). It is believed that salivary proteins, such as proline-rich proteins protect against the antinutritional effects of dietary tannins. Polyphenols such as tannins can bind the salivary proteins, forming insoluble tannin-protein precipitates, causing a decrease in lubrication and increasing friction (Baxter *et al.*, 1997). This mechanism of binding proteins and phenolic compounds operates based on the fixed number of binding sites available on proteins for tannin attachment and the similarly fixed binding sites on polyphenols. Maximum precipitation and the most extensive network occur when the total binding sites of polyphenols and proteins are equivalent. Variations in the ratio of protein to tannin lead to the formation of distinct protein-polyphenol complexes (Siebert *et al.*, 1996). Protein-polyphenol precipitates can be soluble or insoluble, depending on the presence and structure of different types of peptides and phenolic compounds. The class of salivary proteins that demonstrates the highest affinity and reactivity toward tannins comprises proline-rich proteins (PRPs). Salivary proline-rich proteins belong to intrinsically disordered proteins (IDPs), with particular sequences. IDPs are unstructured, without well-structured 3D folds, and stable tertiary structure (Uversky, 2002; Ward *et al.*, 2004; Receveur-Brechot *et al.*, 2006). The characteristic sequence signature of unfolded proteins, or unfolded regions within proteins, is defined by the following features: (1) an enrichment in polar and charged amino acids, such as glutamine (Gln), serine (Ser), proline (Pro), glutamic acid (Glu), and lysine (Lys); (2) a depletion of bulky hydrophobic residues, including valine (Val), leucine (Leu), methionine (Met), phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr); and (3) in some instances, low sequence complexity, characterized by repetitive short amino acid motifs. Boze *et al.* (2010) investigated the interactions of plant tannins with salivary proteins and reported that salivary PRPs have extended conformations. PRPs represent approximately two-thirds of the proteins secreted by human parotid glands. These proteins are characterized by a high content of repetitive amino acid sequences, predominantly proline (Pro), glycine (Gly), glutamine (Gln), and glutamic acid (Glu) residues (Tompa, 2003). Salivary PRPs are a family of salivary proteins divided into glycosylated (gPRPs), acidic (aPRPs), and basic (bPRPs) types, and regarding being structurally similar, each group possesses different functional roles (Chan and Bennick, 2001). Due to the high content of Pro in their structure, which makes them favorable for interactions with complex polyphenols, the function of basic PRPs is to bind plant polyphenols (tannins) and protect against their anti-nutritional effects (Lu and Bennick, 1998; Mehansho *et al.*, 1987). Baxter *et al.* (1997) demonstrated that hydrophobic stacking of the polyphenol aromatic ring against the pro-S face of the proline residue represents the predominant mode of interaction with proline-rich proteins (PRPs). They also stated that more complex polyphenols have stronger interactions than

the smaller ones, through self-association or stack when bound. On the contrary, investigating binding sites of different tannins (epigallocatechin gallate, procyanidin dimers B2, and B2 3'O-gallate) on human salivary PRPs (IB5), Canon *et al.* (2010) reported that the structure of the tannins (degree of polymerization and galoylation) does not influence the modifications on binding sites on the bPRP used in the research. This interaction model postulates that the clusters within the protein's binding site constitute rigid regions, serving as anchoring points to facilitate efficient tannin binding. The second group of PRPs, also partially responsible for the binding of phenolic compounds are glycosylated proline-rich proteins, responsible for lubrication and oral bacteria binding. Lu and Bennick (1998) proposed that deglycosylation of gPRPs enhances tannin binding capacity, as the carbohydrate side chains inhibit tannin binding to the protein. This contrasts with the findings of other authors who suggest that proline-rich glycoproteins exhibit an enhanced affinity for condensed tannins due to the presence of oligosaccharides in their structure (Asquith *et al.*, 1987). Ramos-Pineda *et al.* (2019) examined the synergistic effect of a mixture of acidic proline-rich proteins (aPRPs) and basic proline-rich proteins (bPRPs) on their interaction with wine flavonols. Their findings indicated an enhanced interaction between (epi)catechin and PRPs when both protein types were combined, suggesting that the formation of medium-sized aggregates between flavanols and bPRPs may facilitate the interaction with aPRPs. Beyond proline-rich proteins (PRPs), certain studies have reported that mucins, statherin, and histatin also exhibit binding interactions with phenolic compounds found in wine (Soares *et al.*, 2011). Naurato *et al.* (1999) reported that histatins 1, 3, and 5 are capable of precipitating condensed tannins, specifically epigallocatechin gallate (EGCG) and pentagalloyl glucose (PGG), with notable differences in their interactions. Their findings further demonstrated that the precipitated complexes were insoluble under conditions analogous to those in the stomach and small intestine, suggesting functional similarities to basic proline-rich proteins.

Nevertheless, recent studies have proposed theories of synergistic interactions among various phenolic compounds in red wine, particularly anthocyanins. These interactions indicate that the binding affinity of proteins is modulated by the specific qualitative composition of phenolic compounds and their associated molecular constituents (Paissoni *et al.*, 2020).

2.2.3. Potential mechanism of grape anthocyanin-salivary proteins interactions

As previously discussed, anthocyanins are reactive phenolic compounds that contribute to the diverse range of hues observed in red wine. Having the flavylium nucleus with positively charged oxygen and conjugated double bonds, the positive charge is delocalized over the entire cycle. This occurrence is stabilized by resonance (Ribereau-Gayon *et al.*, 2006). To better understand the mechanism of astringency development, some authors investigated the extent to which wine anthocyanins influence the precipitation of salivary proteins and their potential contribution to the perception of astringency. First evidence of interactions between anthocyanins extracted from grape and proteins from human whole saliva were reported by Yao *et al.* (2011). They investigated the affinity interactions with whole saliva between natural pigments theaflavin, curcumin and cyanidin, from black tea, turmeric and *Vitis vinifera* L., respectively. Although the other pigments showed greater affinity towards salivary proteins, this was the first time that interactions between anthocyanin and salivary proteins were characterized. Recent studies are showing positive results regarding the interactions between the grapevine/red wine anthocyanins and different classes of salivary proteins. Regardless of that salivary proline-rich proteins are the main class in the development of astringency, Torres-Rochera *et al.* (2023) reported that interaction between anthocyanins and mucins is stronger than that of catechin and epicatechin. The lack of interaction between mucin and other compounds underscores the pivotal role of anthocyanins in the development of astringency and the precipitation of higher molecular weight proteins. They also reported that the binding of malvidin-3-*O*-glucoside to mucins was through hydrogen (H) bonds, which is in accordance with the results of the previous authors. Paissoni *et al.* (2018) reported that anthocyanins react with anthocyanins, to different extent depending on the anthocyanins and anthocyanins' derivatives. According to them, cinnamoylated anthocyanins showed the highest reactivity towards salivary proteins.

During winemaking and aging, anthocyanins undergo co-pigmentation reactions, leading to a gradual decrease in free anthocyanins in aged wine over time due to the formation of novel complex anthocyanins-derived pigments, pyranoanthocyanins (Delić *et al.*, 2023). Pyranoanthocyanins are formed via cycloaddition reactions between anthocyanins and acetaldehyde, pyruvic acid, and vinyl phenols, which are yeast by-products (Brouillard and Dubois, 1977; Oliveira *et al.*, 2017). The following complex compounds are classified as pyranoanthocyanins: 1) vitisins (Romero and Bakker, 1999; Morata *et al.*, 2003); 2) pinotins (Marquez *et al.*, 2013); and 3) flavanyl-pyranoanthocyanins (Rentzsch *et al.*, 2007). Furthermore, pyranoanthocyanins, due to their electrophilic properties, may react with other compounds during wine aging, forming pyranoanthocyanin pigments such as oxovitisins, portisins, and pyranoanthocyanins dimers (He *et al.*, 2010). To gain deeper insights into the mechanisms underlying astringency development, these anthocyanin-derived pigments were also investigated. Garcia-Estevez *et al.* (2018) investigated the interaction between pyranoanthocyanins and aPRPs, using saturation transfer difference-NMR and MALDI-TOF. Their results showed that wine phenolic compounds other than tannins may also contribute to the astringency perception. Soares *et al.* (2019) investigated the possibility of effect of interaction of malvidin-3-*O*-glucoside and epicatechin on their ability to interact with acidic and basic PRPs. Consequently, the mechanism of co-pigmentation might contribute to the development of red wine astringency. The results showed that the mixture had a possible synergic effect toward the interactions with PRPs, with distinctions between interaction types. Interactions between malvidin-3-*O*-glucoside and PRPs were electrostatically driven, while the ones between tannin and PRPs were hydrophobic and hydrophilic. Ferrer-Gallego *et al.* (2015) reported that anthocyanins have the ability of interacting with salivary proteins, forming new soluble complexes. In summary, anthocyanins may influence the mechanism of astringency development both directly, through the formation of complexes with salivary proteins as free anthocyanins, and indirectly, via their interaction with salivary proteins in the form of anthocyanin-derived compounds.

Further research should focus on identifying the precise sites of interaction between anthocyanins and salivary proteins, elucidating the hierarchy of binding affinities, and determining which anthocyanins exhibit a preferential advantage in forming complexes with salivary proteins, complemented by an assessment of the sensory implications across varying anthocyanin ratios.

2.3. Analytical techniques for investigating grape/wine anthocyanin-salivary protein interactions

Monitoring and identifying interactions between grape anthocyanins and salivary proteins are crucial for understanding their role in sensory perception, particularly astringency and color stabilization. These interactions are primarily driven by non-covalent bonds such as hydrogen bonding, hydrophobic interactions, and van der Waals forces, influenced by the structural characteristics of both anthocyanins and salivary proteins. Spectroscopic techniques, including UV-Vis and fluorescence spectroscopy, are commonly used to monitor binding interactions, as they detect changes in anthocyanin absorption or emission properties upon complex formation. Circular dichroism (CD) spectroscopy provides insights into conformational changes in proteins induced by anthocyanin binding. To investigate how salivary proteins might influence the bioavailability of anthocyanins, Wiese *et al.* (2009) conducted research with cyanidin-3-*O*-glucoside and whole saliva proteins via intrinsic fluorescence quenching, at different levels of pH. They reported changes in protein structure and binding of cyanidin-3-*O*-glucoside extracted from blackberries, with the strongest affinity towards human serum albumin at pH 7. Further results suggest, based on the association constants for different salivary proteins, weak non-covalent interactions between cyanidin-3-*O*-glucoside and proteins. These findings are in accordance with previous reports (Bian *et al.*, 2004; Boulton *et al.*, 1998).

Advanced analytical methods like isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) are employed to quantify binding affinities and kinetics, offering detailed thermodynamic profiles of the interactions. Mass spectrometry (MS), particularly when coupled with chromatography (e.g., HPLC-MS/MS), allows for the identification of specific binding sites and the characterization of anthocyanin-protein complexes (Grassl *et al.*, 2016).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used analytical technique for monitoring and identifying interactions between anthocyanins and salivary proteins. This method separates proteins based on their molecular weight by denaturing them into linear forms, ensuring consistent charge-to-mass ratios. When studying anthocyanin-protein interactions, SDS-PAGE can be employed to detect changes in protein mobility or the formation of protein-anthocyanin complexes, which may appear as altered band intensities or shifts on the gel. Pre-staining proteins with anthocyanin-binding dyes or post-gel staining with anthocyanins can further highlight these interactions. Additionally, SDS-PAGE coupled with Western blotting allows for the specific identification of target proteins involved in binding. Future advancements in SDS-PAGE could include the incorporation of high-resolution gels or gradient gels to improve the separation of closely related proteins, particularly in complex salivary matrices. Combining SDS-PAGE with proteomics techniques, such as in-gel digestion followed by LC-MS/MS, offers the potential to precisely identify and characterize anthocyanin-bound proteins. Moreover, innovations in fluorescence labeling of anthocyanins could enable real-time visualization of their binding dynamics on gels. Recent investigations are focusing on developing different oral cell-based models, resulting in valuable insights regarding the astringency perception, anthocyanin-salivary proteins reactions and mucosal pellicle (Soares *et al.*, 2020). These developments would enhance the sensitivity, specificity, and functional understanding of anthocyanin-protein interactions, supporting applications in food science, nutrition, and oral health research.

3. Materials and Methods

3.1. Wine, skin and seed samples for UHPLC Q-ToF MS characterization

3.1.1. Preparation of 'Prokupac' and 'Kadarka' grape skin and seed samples for UHPLC Q-ToF MS analysis

Skin and seed from two indigenous grape varieties ('Kadarka' and 'Prokupac'), were manually separated, frozen and lyophilised. After that, skin and seed samples (1g) were finely ground, and extracted with 80% acidified methanol (1:10 w/v), for 1h, on a mechanical shaker (Milinčić, *et al*., 2021b; Pešić, *et al*., 2019). After that, samples were centrifuged at 4000g, for 10 min, and supernatants were collected. The same procedure was repeated three times, and combined supernatants of all individual skin and seed samples were evaporated to dryness (Heidolph, Laborota 4000, Schwabach, Germany), by rotary evaporator under reduced pressure at 40°C. The residues after evaporation were reconstituted in 10 mL milliQ water, and freeze-dried.

Before chromatographic analysis, skin and seed water extracts were passed through the SPE cartridge, with aims to remove sugars and other polar constituents from prepared extracts. The SPE cartridge was conditioned by washing with 5 mL of acidified methanol and milliQ water, respectively. After that, samples were passed through the cartridge and washed with 5 mL of milliQ water. Then, adsorbed phenolics were eluted with acidified methanol (0.1% methanol), filtered through 0.22 μm syringe filters and analyzed by UHPLC Q-ToF MS.

3.1.2. Preparation of purified 'Cabernet Sauvignon'/ 'Merlot' anthocyanins

Protocol for preparation and purification of 'Cabernet' / 'Merlot' anthocyanins comprise following steps:

(a) Grape berries

The black grapes of the 'Cabernet Sauvignon' and 'Merlot' varieties were harvested at maturity, then stored at -20°C before the beginning of the course.

(b) Sampling

2.57 kg of 'Cabernet Sauvignon' and 'Merlot' grapes were used to obtain anthocyanins. First, the berries were peeled, washed and dried to obtain fresh skins. In a second step, the 652 g of fresh skins obtained were freeze-dried, then ground with a blender (Waring commercial blender) before being weighed.

(c) Extraction of anthocyanins by acidified methanol maceration

A methanolic extraction of anthocyanins by dynamic maceration, was performed on the dry film powder obtained following the protocol described by Paissoni *et al.* (2018). Briefly, 111 g of powders were extracted by 4L of acidified methanol (0.1% TFA) under stirring. The extraction was performed in two 2-h cycles with 2L of solvents at room temperature. At the end of the extraction, the organic solvent was evaporated using a rotary evaporator under vacuum. The residue was then taken up in aqueous medium to allow freeze-drying of the extract.

(f) Pre-purification of anthocyanin extracts with Amberlite XAD-16 resin

To remove sugars, salts and amino acids, the obtained crude extract was pre-purified on Amberlite XAD-16 resin (Sigma-Aldrich) packed in an open column. Purification was performed according to the method described by Sadilova *et al.* (2006), with some modifications. Before the extracts were applied, the resin was conditioned and equilibrated by rinsing with 2 L of milli-Q water (acidified with TFA (0.1%) to remove salts (sodium chloride and sodium carbonate) and other impurities. In this work, 6 series of purifications were performed to purify the 68.6 g of crude extract obtained, i.e. approximately 10 g of purified crude extract per series. For each run, 10g of crude extract was dissolved in 30 mL of Milli-Q water (0.1% TFA), before being progressively loaded onto the column. Then, 2 L of acidified water was progressively poured into the column to remove polar compounds. The pigment fraction was then eluted with 2 L of acidified methanol (0.1% TFA) until the column was colorless. The resulting anthocyanin-rich extracts were concentrated using rotary vacuum evaporation at a temperature not exceeding 35°C, and rediluted in Milli-Q water before freeze drying.

3.1.3. Young and aged wines analysed by UHPLC Q-ToF MS analysis

In this study, young and aged wines from different grape varieties ('Prokupac', 'Kadarka', 'Merlot' and 'Cabernet Sauvignon') were analysed by UHPLC Q.ToF MS. Wines from the 2024 vintage, collected immediately after fermentation and separation from pomace, were marked as young 'Prokupac' (YP), 'Merlot' (YM) and 'Cabernet Sauvignon' (YC) wine. By contrast, bottled 'Prokupac' (2021 vintage)-AP, 'Kadarka' (2017 vintage)-AK, 'Merlot' (2021 vintage)-AM and 'Cabernet Sauvignon' (2019 vintage)-AC wine, with minimally undergone 18 or 24 months of barrique maturation were labeled as aged wines. Wines were filtered through 0.22 μm syringe filters and analyzed by UHPLC Q-ToF MS. Young and aged 'Prokupac' wine were selected and used to evaluate phenolics/salivary protein interactions.

3.1.4. LC/MS quantification of anthocyanins and proanthocyanidins in various indigenous and international wines

A total of 54 red wine samples employed in this research, generously provided by 18 Serbian wineries, obtaining a selection of autochtonous Serbian varietal ('Prokupac', 'Kadarka', and 'Black Tamjanika'), as well as the international wines produced in Serbia ('Cabernet Sauvignon', 'Merlot' and 'Cabernet Sauvignon' / 'Merlot' blends). Detailed characteristics of red wine samples are provided in Supplementary Table S1. The specific identities of the contributing wineries are maintained in confidentiality to ensure an unbiased representation of data. All analyses of technological characteristics and phenolic composition of Serbian red wines were performed in triplicate to ensure reproducibility and account for any potential variation between measurements.

3.1.4.1. HPLC analysis of anthocyanins of aged red wines

The experimental procedure was based on the OIV method MA-AS315-11. Wine samples were filtered using a 0.45 μm syringe filter prior to HPLC injection. 20 μL were then injected into a Thermo Scientific Accela (Thermo Fisher Scientific, Waltham, MA, USA) HPLC with an Accela 600 pumpmodule and a UV-Visible diode array detector and Xcalibur Software. The HPLC column was a reversed-phase C18 Nucleosil (250 x 4.6 mm, 5 μm). Mobile phase A consisted of water/formic acid (95:5, v/v) and mobile phase B was acetonitrile/formic acid (95:5, v/v). The flow rate was 1 mL/minute, and the gradient was as follows: 10 % to 35 % B in 25 minutes, 100 % B at 35 minutes, 100 % B from 35 to 40 minutes, 10 % B at 41 minutes, and then 10 % B for 4 minutes before the next injection. Detection was carried out at 520 nm. Anthocyanin 3-O-monoglucosides (delphinidin, cyanidin, petunidin, peonidin, and malvidin), along with the acetylated and p-coumaroylated forms of peonidin and malvidin, were identified by comparison of retention times to injected standards and previous results (Chira *et al.*, 2009).

3.1.4.2. HPLC analysis of proanthocyanidin monomers and dimers

The experimental procedure was based on González-Centeno *et al.* (2017). Wine samples were filtered through a 0.45 μm syringe filter prior to HPLC injection. 10 μL were then injected into a Vanquish HPLC system (ThermoFischer Scientific, Waltham, MA, USA) with a Thermo-Finnigan UV-Visible detector (UV-vis 200), a Vanquish autosampler and a Vanquish ternary pumpcoupled to a Chromeleon data system software. Separation was performed on a reverse-phase Lichrosphere 100-RP18 (250 mm x 2 mm, 5 μm; Merck, France) column. The mobile phases were 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B) at a flow rate of 1 mL/minute. The gradient was: 5 % to 18% B in 30 minutes, 100 % B for 1 minute, 100 % B for 7 minutes, from 100 to 5% B in 1 minute, 5 % B for 3 minutes. Eluting peaks were monitored by a UV-detector at 280 nm and a fluorescence detector (λ excitation = 280 nm, λ emission = 320 nm). Catechin and epicatechin monomers, as well as B1, B2, B3 and B4 dimers, were identified by comparison to external standards and previous results (Chira *et al.*, 2009). Quantification used a catechin equilibration curve with results expressed as mg of catechin equivalents per liter of wine.

3.2. Preparation of saliva sample

The saliva of 10 volunteers (5 men and 5 women, aged 24 to 47 years) was collected in the morning between 11 am to 12 noon, following the circadian rhythm. Saliva collection was performed in accordance with ethical permission using the method previously described by Paissoni *et al.* (2018). Briefly, the study and saliva collection were approved by the ethical committee of the Laboratory Research Unit USC 1366 of the Institute of Viticulture and Enology of the University of Bordeaux (ISVV). All participating volunteers signed a consent form with information about the type of the research, voluntary participation, and the spitting protocol. Participants were asked not to eat or drink for at least one hour before samples were taken. The saliva was then collected in Eppendorf tubes (15 mL), pooled, frozen at −20 ◦C, and freeze-dried.
Prior to the saliva test, freeze-dried saliva (10 mg/mL) was reconstituted in phosphate buffered solution at pH 6.8, vortexed, and stored in a refrigerator at 4 ◦C for 1 h. The reconstituted solution was then centrifuged at 4000× g for 10 min to obtain the salivary protein solution (SP), which was used for mixing wine and skin/seed extract samples.

3.3. Saliva test

The binding test of wine, grape skin and seed phenolics with salivary proteins was performed according to the methodology previously described by Ma, Waffo-Teguo, Jourdes, Li, and Teissedre (2016) and Paissoni, *et al.* (2018), with a slight modification of the protocol. The freeze-dried seed and skin extracts ('Prokupac' and 'Kadarka') and purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins were prepared at a concentration of 1 mg/mL in the model wine solution (12% ethanol, 4 g/L tartaric acid, pH 3.5), mixed intensively for 1 h, and centrifuged at 3000× g, for 5 min to remove any insoluble particles in the solution. Then, the skin solutions, seed solutions and purified anthocyanins solution (4 mL) were mixed with 1 mL of salivary protein solution and incubated at 37°C, for 5 min. After incubation, the mixtures were centrifuged at 17,000× g, for 5 min. The collected supernatants were filtered through 0.22 μm nylon syringe filters (samples labeled as „filtrates") and used for electrophoretic analysis and untargeted UHPLC Q-ToF MS analysis. „Mixture controls" were taken after incubation of salivary protein - skin, seed and purified anthocyanins mixtures, at 37 ◦C, for 5 min. Control salivary proteins were prepared by mixing 1 mL of salivary protein solution with 4 mL of model wine solution.
For saliva test were used following wine: Young 'Prokupac' wine-YP; young 'Merlot' wine-YM; young 'Cabernet Sauvignon' wine-YC; as well as Young and Aged 'Prokupac' wine (YPW and APW). In brief, 4 mL of wine samples was mixed with 1 mL of salivary protein solution or 1 mL of phosphate-buffered solution (pH 6.8) and incubated at 37 ◦C, for 5 min. After incubation, the mixtures were centrifuged at 17,000× g for 5 min. The collected supernatants were filtered through 0.22 μm nylon syringe filters („filtrates") and analyzed electrophoresis, and for selected anthocyanins and procyanidins via targeted UHPLC-QTOF-MS. Control wine samples (Control young 'Prokupac' wine-CYPW; Control young 'Merlot' wine-CYMW; Control young 'Cabernet Sauvignon' wine-CYCW; Control aged 'Prokupac' wine-CAPW) were were prepared by mixing 1 mL of phosphate buffered solution at pH 6.8, with 4 mL of wine, and filtration through 0.22 μm nylon syringe filters.

3.4. Untargeted and Targeted UHPLC Q-ToF MS Analysis

The analyses of phenolic compounds (identification, separation and quantification) were carried out on Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system coupled with a quadrupole time-of-flight mass spectrometry (6530C Q-ToF MS) from Agilent Technologies, Inc., CA, USA, using previously in detail described method Kostić *et al.* (2023). The chromatographic separation was performed at 40°C on a Zorbax C18 column (2.1 × 50 mm, 1.8 μm) from Agilent Technologies, Inc., CA, USA. The mobile phase mixtures comprised: (A) ultrapure water and (B) acetonitrile (MS grade), both A and B containing 0.1% HCOOH (MS grade). The flow rate was constant and set to 0.3 mL min−1, while the injection volume was 5 μL. The gradient elution program started with 2% solvent

B for the twice minute, which then reached 98% B over the next 17 minutes, and for the next 5 minutes the gradient was returned to initial conditions (2% B) to re-equilibrate the column to initial conditions.
The QToF-MS system was equipped with a Dual Agilent Jet Stream electrospray ionization (ESI) source, operating in both positive (ESI+) and negative (ESI-) ionization modes. Anthocyanins were analyzed in positive ionization mode, while flavan-3-ols, procyanidins, and other phenolics were monitored in negative ionization mode. The operation parameters for ESI were set as follows: nebulizer pressure of 45 psi, a drying gas temperature of 225°C and a flow rate of 8 L/min, sheath gas temperature of 300°C and sheath gas flow 10L/min, capillary voltage of 2500 V, fragmentor energy of 175 V, skimmer voltage of 65 V, octopole RF Peak at 750 V. The QToF-MS system was recorded spectra over the m/z range 100–1700, with a scan rate of 2 Hz. Data dependent acquisition (DDA) was employed for suspect screening, using the Auto MS/MS acquisition mode with collision energy at 30 eV. The parameters for the auto MS/MS mode were as follows: mass range (100–1700 m/z), acquisition rate (1 spectra/s), and acquisition time (1000 ms/spectrum). Agilent MassHunter software was used for data evaluation and analysis. The grape skin and seed ('Kadarka' and 'Prokupac'), young (YP, YM, YC) and aged (AP, AM, AC, and AK) wines were analyzed in auto MS/MS acquisition mode (untargeted analysis) to gain more detailed insight into their phenolic profiles and evaluate differences/similarities between wines.
Phenolics were identified based on their monoisotopic mass, MS fragmentation and date from literature (Milinčić, *et al.*, 2021a; Milinčić, *et al.*, 2021b; Pantelić, *et al.*, 2016; Pešić, *et al.*, 2019; Šuković, *et al.*, 2020). Accurate masses of components were calculated by using ChemDraw software (version 12.0, CambridgeSoft, Cambridge, MA, USA). Quantification was performed for selected phenolic compounds from young and aged wines, for which standards were available, and the content of each compound was expressed as mg/L wine. In addition, semi-quantification of skin and seed phenolics was performed, using available standards, while content of phenolics were expressed in equivalents of specific standards (mg/kg lyophilised seed or skin). Phenolic standards were purchased from Chem Faces, with purity >98% (Wuhan, Hubei, China). The equation parameters, correlation coefficient (R2), limit of quantification (LOQ), and limit of detection (LOD) of the applied phenolic standards for quantification are shown in Supplementary Table X.
Binding affinities (%) of salivary protein for grape seed flavan-3-ols/procyanidins (BAKSe and BAPSe) and grape skin anthocyanins (BAKSk, BAPSk and PCM) were monitored by untargeted analysis and calculated as ratio of areas of each individually identified compounds in control seed/skin/purified 'Cabernet Sauvignon' / 'Merlot' samples and filtrates.
After the applied saliva-wine test, typical anthocyanins (malvidin derivatives), flavan-3-ols (epicatechin), and procyanidins (procyanidin dimer to pentamer) were selected and monitored via targeted UHPLC-QTOF-MS analysis to obtain more information about the chemical affinity of these compounds for salivary proteins. Targeted analysis is more sensitive than untargeted analysis and can be applied to detect predominant or trace compounds in the sample. The percentage of each individual anthocyanins and procyanidins bound to salivary proteins was calculated as the ratio of the areas of target compounds in the filtrate and control wine samples.

3.5. Electrophoretic analysis

In this study, sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions (SDS-R-PAGE) was performed to analyse the salivary proteins before and after interaction with wine, skin, and seed phenolics. For this analysis, separating gels (12.5% w/v; pH 8.85) and stacking gels (5% w/v; pH 6.8) as well as Tris-Glycine running buffer [0.05 M Tris, (pH 8.5), 0.19 M glycine, 0.1% w/v SDS] were prepared as previously described in detail by Pešić *et al.* (2012).
The following salivary protein solution/seed extracts and salivary protein solution/skin extracts were used for electrophoretic analysis:
(a) Salivary protein solution/ 'Kadarka' seed extract after incubation (37 ◦C, 5 min)—SP/KSe-I;
(b) Salivary protein solution/ 'Prokupac' seed extract after incubation (37 ◦C, 5 min)—SP/PSe-I;
(c) Salivary protein solution/ 'Kadarka' skin extract after incubation (37 ◦C, 5 min)—SP/KSk-I;
(d) Salivary protein solution/ 'Prokupac' skin extract after incubation (37 ◦C, 5 min)—SP/PSk-I;

(e) Salivary protein solution/purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins after incubation (37 ◦C, 5 min)—SP/PCM-I;
(f) Salivary protein solution/ 'Kadarka' seed extract filtrate (after filtration through 0.22 μm filter)—SP/KSe-F;
(g) Salivary protein solution/ 'Prokupac' seed extract filtrate (after filtration through 0.22 μm filter)—SP/PSe-F;
(h) Salivary protein solution/ 'Kadarka' skin extract filtrate (after filtration through 0.22 μm filter)—SP/KSk-F;
(i) Salivary protein solution/ 'Prokupac' skin extract filtrate (after filtration through 0.22 μm filter)—SP/KSk-F;
(j) Salivary protein solution//purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins filtrate (after filtration through 0.22 μm filter)—SP/PCM-F;
(k) Control salivary proteins—CSP;
The following salivary protein solution/wine samples were used for electrophoretic analysis:
(l) Control salivary proteins—CSP;
(m) Salivary protein solution/young or aged wine filtrate (after filtration through 0.22 μm filter)—SP/YP-F ('Prokupac' young wine); SP/YM-F ('Merlot' young wine); SP/YC-F ('Cabernet Sauvignon' young wine); SP/AP-F ('Prokupac' aged wine);
(n) Salivary protein solution/young or aged wine precipitate (after centrifugation)—SP/YP-P ('Prokupac' young wine); SP/YM-P ('Merlot' young wine); SP/YC-P ('Cabernet Sauvignon' young wine); SP/AP-P ('Prokupac' aged wine);
(o) Control young or aged wine—CYPW ('Prokupac' young wine); CYMW ('Merlot' young wine); CYCW ('Cabernet Sauvignon' young wine); CAPW ('Prokupac' aged wine).
Prior to electrophoretic analysis, the samples were dissolved in sample buffer [0.055 M Tris-HCl (pH = 6.8), 2% (w/v) SDS, 7% (v/v) glycerol, 0.0025% (w/v) bromophenol blue and 5% β-mercaptoethanol]. All samples were mixed with sample buffer in a 1:1 (v/v) ratio, except for the precipitates (n). The precipitates, obtained after centrifugation of the salivary protein solution/wine mixtures and removal of the supernatant, were reconstituted in 500 μL of sample buffer, stirred with a mechanical shaker for 1 h, and centrifuged before loading into the wells. For all samples, 75 μL was loaded into the wells. Upon completion of analysis, the gels were stained with Coomassie blue dye for 45 min, then destained, scanned, and analyzed using SigmaGel software (SigmaGel software version 1.1, Jandal Scientific, San Rafael, CA, USA).

3.6. Sensorial analysis of young and aged 'Prokupac', 'Cabernet Sauvignon' and 'Merlot' wines

This study involved sensory evaluation of wine samples conducted by twelve trained adult panelists (6 men and 6 women) from the Faculty of Agriculture, Belgrade, Serbia. The sensory evaluation included tastings of the investigated young and aged 'Prokupac', 'Cabernet Sauvignon' and 'Merlot' wines. The panelists were selected based on their interest, availability, and experience in sensory analysis. The evaluation did not involve any invasive procedures, collection of sensitive personal data, or commercial interests. All participants were fully informed about the nature of the study and voluntarily provided their written consent prior to participation, with the right to withdraw at any time. In accordance with the Code of Professional Ethics of the University of Belgrade, adopted by the Senate of the University of Belgrade and published in the Official Gazette of the Republic of Serbia, No. 189/16, p. 16, and considering the non-invasive nature of the sensory analysis, this study was exempt from ethical committee approval.
Sensory analysis took place in a thermo-regulated room at 20 °C and controlled humidity, according to ISO 8589:2007 standards, in individual booths. For each test, 15 mL of wine was presented in a colored glass, according to ISO 3591:1977, coded with a three-digit number. The panelists rated the intensity of various descriptors on a 10-point scale (0–9), with 0 being the lowest and 9 being the highest intensity. The de-scriptors rated included the following mouthfeel attributes: acidity, bitterness, astringency, and tannin quality. In addition, the sensory evaluation of wines was also performed using Boxbaum's model

of positive rating with a maximum of 20 points on the following four sensory characteristics: color, clearness, aroma, and taste (Kovačević Ganić *et al.*, 2003).

3.7. Statistical analysis

All data were statistically analyzed using Microsoft Excel (Microsoft 365 version). For normally distributed data with homogeneous variances, Tukey's post hoc parametric test was applied to determine the presence and degree of significant differences. One-way ANOVA was used for statistical analysis of the sensory analysis data. The statistical significance was set at a p-value < 0.05.

4. Results and Discussion

4.1. Characterization and semi-quantification of phenolic compounds of 'Prokupac' and 'Kadarka' grape skin and seed

To identify, characterize and semi-quantify the non-anthocyanin phenolic compounds of skin and seed samples of indigenous grape varieties 'Prokupac' and 'Kadarka' the UHPLC Q-ToF MS analysis was conducted. The results are presented in Table 4.1. The total of 87 targeted phenolic compounds were identified, and semi-quantified. To facilitate the comparison of differences and similarities in phenolic composition between the skin and seeds of the two grape varieties analyzed, all identified phenolic compounds were categorized into the following groups: (I) hydroxybenzoic acid and derivatives (23 compounds); (II) hydroxycinnamic acid derivatives (5 compounds); (III) flavan-3-ols and derivatives (14 compounds); (IV) procyanidins and proanthocyanidins (19 compounds); (V) flavonols and derivatives (16 compounds); (VI) stilbenoids and derivatives (7 compounds); (VII) other detected phenolic compounds (3 compounds).

The highest total content of non-phenolic compounds was detected in 'Prokupac' and 'Kadarka' seeds with 310.3 and 269.6 mg/kg LM, respectively. The skin samples had significantly lower content of 178.4 mg/kg LM in 'Kadarka', and 163.3 mg/kg LM in 'Prokupac'. These results are expected, considering the significantly higher concentration of phenolic compounds in grape seeds relative to skin. This has been previously stated by Milinčić *et al.*, 2021. The differences between total content among varieties could be due to their intrinsic differences. It is known that 'Prokupac' has a specific phenolic profile, distinct from other varieties, characterized by a high antioxidant potential.

Among hydroxybenzoic acid and derivatives a few compounds were notably present in the skin and seed samples of both varieties. Hydroxybenzoic acid was detected in the skin and seed samples of both 'Kadarka' and 'Prokupac', with higher concentrations in seed samples (7.83 and 8.34 mg/kg LM, respectively).

Gallic acid was detected in both 'Kadarka' and 'Prokupac' seeds, and 'Kadarka' skin. Hexosides detected above limit of quantification were isomer I dihydroxybenzoic acid hexoside and syringic acid hexosides detected in skin of both varieties, while gallic acid hexoside was detected in 'Prokupac' skin and seeds of both varieties investigated. Galloylshikimic acid was detected only in seeds, with the content higher in 'Prokupac' (16.27 mg/kg LM).

Hydroxycinnamic acid derivatives were detected mainly in skin of both varieties. Ferulic, coutaric, fertaric, and caffeic acid hexoside were detected only in the skins of both 'Kadarka' and 'Prokupac', with selectively different concentrations, probably variety dependent. Caftaric acid was only compound detected in both skin and seed samples, with the content twice as higher in skin than in seed for both varieties.

Flavan-3-ols and derivatives were, as expected, detected primarily in seeds, only catechin was detected in both skin and seeds of both varieties, at comparable concentrations. The concentration of catechin was almost ten times higher in seed than in skin, which is in accordance with previous results (Milinčić *et al.*, 2021). Epigallocatechin was detected only in skin of both varieties. Epicatechin gallate, chalcan-flavan-3-ol dimer isomer II and chalcan-flavan-3-ol isomer I are detected in seeds of both varieties, which can be attributed to the high abundance and diversity of flavan-3-ols and their derivatives in grape seeds.

Procyanidins and proanthocyanidins were detected mainly in seeds of both varieties, at similar concentrations. The only procyanidin detected both in skin and seeds in both varieties investigated was procyanidin dimer B type isomer I, but the concentrations were notably higher in seeds, which is expected. Since procyanidins and proanthocyanidins represent flavan-3-ol polymers, known as condensed tannins, they are predominantly found in grape seeds. Procyanidins found in grape seeds of both varieties above limit of quantification are procyanidin B-type isomer II, B type procyanidin dimer gallate isomer I, B type procyanidin dimer isomer II, B type procyanidin trimer isomer I, B type procyanidin trimer isomer II, and B type procyanidin trimer isomer III.

Table 4.1. Identification, characterisation and semi-quantification of non-anthocyanin phenolic compounds of indigenious grape ('Prokupac' - P and 'Kadarka' – K) skin and seed samples, using UHPLC Q-ToF MS. Target compounds, mean expected retention times (RT), molecular formula, calculated mass, m/z exact mass, mean mass accuracy (mDa), and MS fragments are presented.

No.	RT	Tentative identified compounds*	Formula	Calculated mass	*m/z* exact mass	mDa	MS fragments (% of base peaks)	Samples (mg/kg LM)			
								Skin		Seed	
								Kad.	Prok.	Kad.	Prok.
					Hydroxybenzoic acid and derivatives						
1	4.92	**Hydroxybenzoic acid is. I**[b]	$C_7H_5O_3^-$	137.02390	137.02574	-1.84	**108.02213(100)**, 109.02733(12)	4.57	4.26	7.83	8.34
2	5.32	**Dihydroxybenzoic acid is. II**[b]	$C_7H_5O_4^-$	153.01880	153.02053	-1.73	**107.01611(100)**, 108.02055(22)	<LOQ	<LOQ	<LOQ	<LOQ
3	2.85	**Dihydroxybenzoic acid is. I**[b]	$C_7H_5O_4^-$	153.01880	153.02149	-2.69	**108.02201(100)**, 109.03004(84)	6.85	6.42	<LOQ	—
4	1.41	**Gallic acid**[a]	$C_7H_5O_5^-$	169.01370	169.01559	-1.89	**125.02466(100)**, 124.01705(81)	3.03	<LOQ	14.15	17.91
5	5.98	**Methyl gallate**[b]	$C_8H_7O_5^-$	183.02930	183.03266	-3.36	**124.0177(100)**, 125.02109(8)	17.99	<LOQ	<LOQ	<LOQ
6	7.48	**Ethyl gallate**[b]	$C_9H_9O_5^-$	197.04500	197.04934	-4.34	**124.01747(100)**, 125.02386(31), 169.01416(2)	—	—	—	<LOQ
7	3.30	**Glyceryl gallate**[b]	$C_{10}H_{11}O_7^-$	243.05050	243.05291	-2.41	**124.01673(100)**, 125.02369(27), 169.01497(8)	<LOQ	—	<LOQ	<LOQ
8	3.77	**Hidroxybenzoic acid hexoside**[b]	$C_{13}H_{15}O_8^-$	299.07670	299.07905	-2.35	**137.02579(100)**, 138.02767(12)	<LOQ	<LOQ	<LOQ	<LOQ

9	7.82	**Ellagic acid**[a]	$C_{14}H_5O_8^-$	300.99840	301.00142	-3.02	**301.00142(100)**, 145.03032(9), 173.02539(10), 185.02559(11), 229.01581(20), 245.01103(11), 257.01066(8), 283.99839(17)	—	—	<LOQ	2.06
10	2.83	**Dihydroxybenzoic acid hexoside is. I**[b]	$C_{13}H_{15}O_9^-$	315.07160	315.07706	-5.46	**108.02243(100)**, 109.02959(38), 152.0126(60), 153.01945(16)	3.16	10.02	<LOQ	<LOQ
11	5.85	**Dihydroxybenzoic acid hexoside is. II**[b]	$C_{13}H_{15}O_9^-$	315.07160	315.07872	-7.12	**153.02053(100)**, 152.01055(3)	<LOQ	<LOQ	<LOQ	—
12	3.44	**Galloylshikimic acid**[b]	$C_{14}H_{13}O_9^-$	325.05600	325.06009	-4.09	**125.0251(100)**, 111.04656(9), 124.01691(44), 155.03748(2), 168.00717(6), 169.01523(65)	—	—	6.73	16.27
13	3.77	**Vanillic acid hexoside is. I**[b]	$C_{14}H_{17}O_9^-$	329.08730	329.09141	-4.11	**108.02248(100)**, 123.04567(41), 152.01254(73), 167.03619(40)	<LOQ	5.31	<LOQ	<LOQ
14	5.31	**Vanillic acid hexoside is. II**[b]	$C_{14}H_{17}O_9^-$	329.08730	329.09191	-4.61	**123.04567(100)**, 124.0491(10), 167.03691(6)	<LOQ	<LOQ	<LOQ	<LOQ
15	2.62	**Gallic acid hexoside**[b]	$C_{13}H_{15}O_{10}^-$	331.06650	331.07192	-5.42	**125.02515(100)**, 169.01533(96)	<LOQ	48.84	35.77	41.32
16	4.85	**Methyl gallate hexoside**[b]	$C_{14}H_{17}O_{10}^-$	345.08220	345.08697	-4.77	**168.00751(100)**, 124.01726(52), 125.02035(5), 169.01022(10), 183.03132(83)	<LOQ	<LOQ	—	<LOQ
17	6.87	**Syringic acid hexoside**[b]	$C_{15}H_{19}O_{10}^-$	359.09780	359.09837	-0.57	**197.04704(100)**	4.49	12.11	<LOQ	<LOQ
18	7.58	**Ellagic acid pentoside**[c]	$C_{19}H_{13}O_{12}^-$	433.04070	433.04662	-5.92	299.99268(100), 301.0002(71), 433.04707(8)	—	—	—	<LOQ
19	5.93	**Digalloyl hexoside is. II**[b]	$C_{20}H_{19}O_{14}^-$	483.07750	483.08282	-5.32	**169.01558(100)**, 125.02495(17), 211.02656(42), 271.0479(48), 313.0594(11), 331.06844(7)	—	—	<LOQ	4.15
20	2.35	**Digalloyl hexoside is. I**[b]	$C_{20}H_{19}O_{14}^-$	483.07750	483.08001	-2.51	**169.01462(100)**, 125.02454(18), 331.07191(13)	<LOQ	—	—	—

21	1.55	**Gallic acid dihexoside is. I[b]**	$C_{19}H_{25}O_{15}$—	493.11930	493.12539	-6.09	**169.0152(100)**, 331.06968(63)	125.02521(10),	313.06049(18),	<LOQ	<LOQ	<LOQ	3.92
22	2.42	**Gallic acid dihexoside is. II[b]**	$C_{19}H_{25}O_{15}$—	493.11930	493.12485	-5.55	**313.05941(100)**, 331.07122(11)	125.02492(14),	169.01498(91),	—	—	<LOQ	8.08
23	6.54	**Galloyl-HHDP-hexose[b]**	$C_{27}H_{21}O_{18}$—	633.07280	633.07895	-6.15	**301.00132(100)**, 633.07895(45)	169.01364(5),	463.04904(11),	—	—	<LOQ	<LOQ
				∑						**40.09**	**86.95**	**64.48**	**102.0**
					Hydroxycinnamic acid derivatives								
24	6.60	**Ferulic acid[a]**	$C_{10}H_9O_4$—	193.05010	193.05228	-2.18	**134.03893(100)**, 117.03595(17), 133.02944(51)			6.04	3.09	<LOQ	<LOQ
25	6.06	**Coumaroyl tartaric acid (Coutaric acid)[d]**	$C_{13}H_{11}O_8$—	295.04540	295.04986	-4.46	**119.05083(100)**, 163.04135(25)			10.11	6.24	<LOQ	3.49
26	4.65	**Caffeoyl tartaric acid (Caftaric acid)[d]**	$C_{13}H_{11}O_9$—	311.04030	311.04546	-5.16	**135.04593(100)**, 179.03595(18)			37.15	13.43	14.70	7.40
27	6.66	**Feruloyl tartaric acid (Fertaric acid)[d]**	$C_{14}H_{13}O_9$—	325.05600	325.06452	-8.52	**134.03845(100)**, 135.04218(11), 178.02915(4), 193.05309(14)	149.06191(4),		59.36	34.94	<LOQ	<LOQ
28	6.06	**Caffeic acid hexoside[d]**	$C_{15}H_{17}O_9$—	341.08730	341.09036	-3.06	**161.02601(100)**, 135.04627(85), 179.03685(59)			6.84	4.97	—	—
				∑						**119.5**	**62.68**	**14.70**	**10.89**
					Flavan-3-ols and derivatives								
29	6.40	**Catechin[a]**	$C_{15}H_{13}O_6$—	289.07120	289.07539	-4.19	**123.04621(100)**, 109.0306(92), 137.02585(27), 151.04115(31), 203.0728(21), 221.0842(13)	125.02564(43),	161.06055(14),	4.53	2.51	31.67	32.19

30	7.06	**Epicatechin**[a]	$C_{15}H_{13}O_6^-$	289.07120	289.07590	-4.70	**123.04585(100)**, 109.03027(98), 125.02516(41), 137.02502(28), 149.02552(17), 151.04101(31), 159.04629(12), 161.06023(14), 187.04211(12), 203.07274(20), 221.08344(14)	<LOQ	<LOQ	11.24	12.94
31	3.37	**Epigallocatechin**[e]	$C_{15}H_{13}O_7^-$	305.06610	305.07082	-4.72	**125.02553(100)**, 109.03026(13), 137.02527(33), 139.04116(27), 165.01968(18), 167.03705(20), 219.06779(10)	1.38	2.66	<LOQ	<LOQ
32	9.37	**(Epi)catechin 3-*O*-coumarate**[e]	$C_{24}H_{19}O_8^-$	435.10800	435.11633	-1.91	**145.03033(100)**, 109.03074(189, 125.0255(45), 137.02573(22), 165.01879(29), 179.03654(15), 203.07383(20), 205.05297(15), 245.08379(47), 287.02046(15), 289.07459(45)	—	—	<LOQ	<LOQ
33	8.62	**Amurensisin**[e]	$C_{22}H_{15}O_{10}^-$	439.06650	439.07184	-5.34	**287.02276(100)**, 243.03184(2), 259.02687(3), 274.01418(10), 288.02737(29), 439.07184(27)	—	—	<LOQ	<LOQ
34	7.92	**(-)-Epicatechin gallate**[a]	$C_{22}H_{17}O_{10}^-$	441.08220	441.08791	-5.71	**169.0158(100)**, 125.02527(46), 245.08349(15), 289.07419(23)	<LOQ	—	5.76	5.85
35	5.93	**(-)-Epicatechin 3-*O*-hexoside**[e]	$C_{21}H_{23}O_{11}^-$	451.12400	451.12816	-4.16	**289.07494(100)**, 290.07818(20), 203.07266(26), 245.08423(65)	—	—	<LOQ	<LOQ
36	5.46	**Catechin 3-*O*-hexoside**[e]	$C_{21}H_{23}O_{11}^-$	451.12400	451.12946	-5.46	**289.07396(100)**, 290.07732(20), 137.02492(17), 203.07212(14), 245.08384(43)	—	—	<LOQ	4.98
37	8.08	**(Epi)gallocatechin 3-*O*-gallate methyl ether**[e]	$C_{23}H_{19}O_{11}^-$	471.09270	471.10141	-8.71	**125.02479(100)**, 169.01514(30), 269.04694(10), 287.05518(9), 303.05186(3), 313.03636(3)	—	—	<LOQ	<LOQ
38	7.01	**Chalcan-flavan-3-ol dimer is. II**[e]	$C_{30}H_{27}O_{12}^-$	579.15030	579.15422	-3.92	**289.07433(100)**, 125.0252(8), 137.02517(6), 179.03609(8), 205.05235(9), 245.08445(33)	—	—	8.71	11.30
39	6.33	**Chalcan-flavan-3-ol dimer is. I**[e]	$C_{30}H_{27}O_{12}^-$	579.15030	579.15519	-4.89	**289.07449(100)**, 290.07826(20), 109.03023(6), 125.02525(9), 137.02556(7), 179.03632(8), 205.05283(9), 245.08441(35)	—	—	23.22	24.77

40	8.76	**Ethyl (epi)catechin-(epi)catechin (Epicatechin ethyl dimer) is. I[e]**	$C_{32}H_{29}O_{12}^-$	605.16590	605.17139	-5.49	**289.07469(100)**, 290.07831(16), 245.08447(15), 271.09736(6), 315.09086(87)			—	—	<LOQ	<LOQ
41	9.77	**Ethyl (epi)catechin-(epi)catechin (Epicatechin ethyl dimer) is. II[e]**	$C_{32}H_{29}O_{12}^-$	605.16590	605.17171	-5.81	**315.0905(100)**, 316.09157(26), 137.02496(12), 163.04255(15), 205.05341(11), 245.08353(18), 289.07381(99)			—	—	<LOQ	<LOQ
42	6.74	**(Epi)catechin-methyl-(epi)gallocatechin[e]**	$C_{31}H_{27}O_{13}^-$	607.14520	607.14815	-2.95	**125.02406(100)**, 161.02595(19), 243.03512(24), 287.05793(41), 405.07159(9), 424.0777(6), 452.08265(6)			<LOQ	<LOQ	<LOQ	<LOQ
				∑						**5.91**	**5.16**	**80.61**	**92.04**
		Procyanidins and proanthocyanidins											
43	8.56	**Procyanidin dimer A type[f]**	$C_{30}H_{23}O_{12}^-$	575.11900	575.12194	-2.94	**125.02677(100)**, 137.02627(47), 161.02593(32), 243.03276(38), 271.02889(54), 287.05802(37), 289.07332(45), 394.0712(88), 407.08035(57), 449.09018(33), 575.12194(61)			—	—	<LOQ	<LOQ
44	6.80	**Procyanidin dimer B type is. II[f]**	$C_{30}H_{25}O_{12}^-$	577.13460	577.14255	-7.95	**289.07435(100)**, 109.03032(5), 205.05228(8), 245.08446(33)			<LOQ	<LOQ	16.74	19.86
45	6.20	**Procyanidin dimer B type is. I[f]**	$C_{30}H_{25}O_{12}^-$	577.13460	577.14353	-8.93	**289.07472(100)**, 125.0255(8), 137.02542(6), 179.03673(8), 205.05255(9), 245.08471(33)			9.24	4.20	36.34	36.77
46	8.62	**Proanthocyanidin A[f]**	$C_{31}H_{27}O_{12}^-$	591.15030	591.15375	-3.45	**289.07424(100)**, 109.0305(6), 125.02537(9), 137.02439(11), 215.07428(20), 245.08533(14), 257.08277(8), 301.07548(62), 439.10872(5)			—	—	<LOQ	<LOQ
47	6.27	**Procyanidin A4[f]**	$C_{30}H_{23}O_{13}^-$	591.11390	591.12283	-8.93	**259.02698(100)**, 260.03015(19), 125.02455(17), 137.02464(14), 139.00439(60), 151.00582(23), 161.02515(23), 271.02747(24), 289.07099(37), 423.07534(39)			<LOQ	—	<LOQ	<LOQ

48	8.69	**Coumaroyl procyanidin dimer B type[f]**	$C_{39}H_{31}O_{14}^-$	723.17140	723.18385	-12.45	**407.08041(100)**, 125.0256(69), 287.05626(14), 289.07244(73), 425.09557(14), 451.11186(16), 577.14613(24)	—	—	<LOQ	<LOQ
49	7.41	**B type procyanidin dimer gallate is. I[f]**	$C_{37}H_{29}O_{16}^-$	729.14566	729.15485	-9.20	**407.08217(100)**, 125.02538(36), 169.01573(19), 271.06413(15), 289.07469(71), 425.09292(9), 441.08779(11), 451.10971(21), 577.13756(16)	<LOQ	-	8.62	8.33
50	8.42	**B type procyanidin dimer gallate is. II[f]**	$C_{37}H_{29}O_{16}^-$	729.14566	729.15466	-9.00	**407.08151(100)**, 125.02470(35), 169.01534(22), 245.05482(8), 271.06474(12), 289.07385(65), 441.08746(10), 451.10855(22), 577.13504(12)	—	—	1.60	1.75
51	8.76	**Feruloyl procyanidin dimer B type[f]**	$C_{40}H_{33}O_{15}^-$	753.18190	753.19340	-11.50	**407.08182(100)**, 125.02531(60), 137.02521(14), 289.07542(63), 425.08997(17), 451.11037(27), 577.14084(20), 601.14425(20)	—	—	<LOQ	<LOQ
52	9.97	**B type procyanidin dimer dimethylgallate[f]**	$C_{39}H_{33}O_{16}^-$	757.17698	757.18240	-5.42	**315.09069(100)**, 169.01507(19), 289.07453(81), 441.08787(17), 467.10542(13), 605.15055(4)	—	—	<LOQ	<LOQ
53	3.64	**B type procyanidin trimer is. I[f]**	$C_{45}H_{37}O_{18}^-$	865.19802	865.20736	-9.34	**287.05936(100)**, 289.07388(61), 125.02529(77), 407.08227(56), 425.09211(42), 451.10883(31), 577.14140(55), 695.14857(57), 713.15927(31)	<LOQ	<LOQ	26.59	21.90
54	6.60	**B type procyanidin trimer is. II[f]**	$C_{45}H_{37}O_{18}^-$	865.19802	865.20997	-11.95	**287.05924(100)**, 289.07468(72), 125.02566(82), 407.08219(69), 425.09226(54), 575.12678(37), 577.14171(65), 695.14954(41), 713.15910(25)	1.69	<LOQ	14.50	11.49
55	7.27	**B type procyanidin trimer is. III[f]**	$C_{45}H_{37}O_{18}^-$	865.19802	865.21018	-12.15	**287.05935(100)**, 289.07498(73), 125.02520(83), 407.08198(74), 413.09254(33), 425.09242(50), 451.10667(38), 575.12602(40), 577.14135(59), 695.14719(42), 713.15949(28)	<LOQ	<LOQ	5.48	5.25
56	9.84	**B type procyanidin trimer derivative[f]**	$C_{46}H_{35}O_{18}^-$	875.18231	875.19410	-11.79	**407.07787(100)**, 125.02514(64), 169.01525(52), 287.05863(49), 289.07489(33), 451.11018(30), 559.12861(79), 577.13658(25), 723.17459(83)	—	—	<LOQ	<LOQ

57	8.76	**Procyanidin dimer B type digallate is. II**[f]	$C_{44}H_{33}O_{20}$—	881.15650	881.17110	-14.60	**407.0831(100)**, 125.02441(41), 269.04858(22), 287.05833(32), 289.07614(28), 559.12367(52), 577.12648(27), 711.14477(25), 729.15397(91)	—	—	<LOQ	<LOQ
58	7.82	**Procyanidin dimer B type digallate is. I**[f]	$C_{44}H_{33}O_{20}$—	881.15650	881.17145	-14.95	**407.081(100)**, 125.02543(27), 169.01524(21), 287.06033(27), 289.07356(34), 559.12691(67), 560.13294(27), 577.13232(31), 729.15439(81)	—	—	<LOQ	<LOQ
59	8.63	**Ethyl (epi)catechin-procyanidin dimer B type**[f]	$C_{47}H_{41}O_{18}$—	893.22930	893.22688	2.42	**451.1105(100)**, 125.02511(42), 289.07182(89), 315.09033(56), 407.08124(34), 425.08806(41), 433.09141(49), 452.10782(37), 577.14324(71), 603.15842(91)	—	—	<LOQ	<LOQ
60	8.35	**Procyanidin trimer B type gallate**[f]	$C_{52}H_{41}O_{22}$—	1017.20890	1017.21788	-8.98	**1017.21788(100)**, 1018.22113(69), 125.02449(28), 287.06002(45), 289.07288(22), 407.07832(31), 575.12659(34), 577.12377(22), 729.15415(67), 847.16829(29), 865.18078(20)	—	—	—	<LOQ
61	7.41	**Procyanidin tetramer B type**[f]	$C_{60}H_{49}O_{24}$—	1153.26140	1153.27427	-12.87	**1153.27486(100)**, 1154.27696(88), 125.02419(28), 287.0598(58), 413.09356(23), 425.09254(27), 575.12729(71), 576.12993(34), 577.14026(72), 865.20233(55)	—	—	—	<LOQ
			∑					**10.94**	**4.20**	**109.8**	**105.3**
							Flavonols and derivatives				
62	10.37	**Kaempferol**[a]	$C_{15}H_9O_6$—	285.03990	285.04274	-2.84	**285.04274(100)**, 143.04999(7), 159.04592(9), 185.06198(13), 187.04158(10), 211.04135(9), 229.05202(11), 239.03597(10)	<LOQ	<LOQ	<LOQ	<LOQ
63	9.53	**Quercetin**[g]	$C_{15}H_9O_7$—	301.03480	301.03633	-1.53	**151.00448(100)**, 107.01472(44), 121.03012(46), 178.99899(13), 187.0427(3), 245.04613(4)	<LOQ	<LOQ	<LOQ	—
64	10.16	**Rhamnetin**[g]	$C_{16}H_{11}O_7$—	315.05050	315.05534	-4.84	**165.02089(100)**, 121.03074(10), 137.0254(48), 166.02367(10), 167.02547(1), 256.04252(4)	—	<LOQ	—	—

65	10.51	**Isorhamnetin**[g]	$C_{16}H_{11}O_7^-$	315.05050	315.05567	-5.17	**300.03045(100)**, 301.03325(23), 151.00533(54), 164.01262(13), 227.03581(7), 243.03159(6), 255.0317(7), 271.02701(12), 283.02774(9)	<LOQ	<LOQ	—	<LOQ
66	10.38	**Syringetin**[g]	$C_{17}H_{13}O_8^-$	345.06100	345.06582	-4.82	**315.01847(100)**, 316.02146(21), 259.02754(13), 287.02279(38), 329.03284(2), 330.0418(20), 331.04529(4)	<LOQ	<LOQ	—	—
67	7.95	**Quercetin 3-*O*-hexoside**[g]	$C_{21}H_{19}O_{12}^-$	463.08770	463.09266	-4.96	**300.03134(100)**, 301.03752(50), 151.00507(4), 179.00037(3), 255.03217(6), 271.02829(11)	<LOQ	<LOQ	<LOQ	<LOQ
68	7.88	**Quercetin 3-*O*-hexuronide**[g]	$C_{21}H_{17}O_{13}^-$	477.06690	477.07151	-4.61	**301.03947(100)**, 302.04242(20), 151.00495(17), 179.00017(12)	2.00	<LOQ	<LOQ	<LOQ
69	8.36	**Isorhamnetin 3-*O*-hexoside**[g]	$C_{22}H_{21}O_{12}^-$	477.10330	477.10548	-2.18	**314.04709(100)**, 315.05177(31), 151.00591(3), 243.03266(14), 300.03025(4)	<LOQ	<LOQ	<LOQ	—
70	7.48	**Myricetin 3-*O*-hexoside**[g]	$C_{21}H_{19}O_{13}^-$	479.08260	479.08902	-6.42	**316.02656(100)**, 317.03146(31)	<LOQ	<LOQ	—	—
71	8.01	**Laricitrin 3-*O*-hexoside**[g]	$C_{22}H_{21}O_{13}^-$	493.09820	493.10659	-8.39	**287.0594(100)**, 288.06393(19), 151.00538(19), 272.03663(32), 285.04239(18), 315.02107(14), 330.04267(37), 331.04979(49)	<LOQ	<LOQ	—	—
72	8.36	**Syringetin 3-*O*-hexoside**[g]	$C_{23}H_{23}O_{13}^-$	507.11390	507.11950	-5.60	**301.07534(100)**, 286.05184(36), 327.05555(21), 328.05754(5), 344.05713(13), 345.06544(22)	<LOQ	<LOQ	—	<LOQ
73	7.75	**Quercetin 3-*O*-(6"-rhamnosyl)hexoside**[g]	$C_{27}H_{29}O_{16}^-$	609.14560	609.15069	-5.09	**300.03104(100)**, 151.00572(3), 179.00037(3), 301.03768(73), 609.15401(81)	<LOQ	<LOQ	<LOQ	—
74	10.24	**Isorhamnetin 3-*O*-(6"-coumaroyl) hexoside**[g]	$C_{31}H_{27}O_{14}^-$	623.14010	623.14864	-8.54	**271.06405(100)**, 272.06776(17), 297.04394(21), 298.04758(5), 315.05525(80)	<LOQ	<LOQ	—	<LOQ
75	9.36	**Myricetin 3-*O*-(6"-coumaroyl)hexoside**[g]	$C_{30}H_{25}O_{15}^-$	625.11930	625.13385	-14.55	**317.03389(100)**, 243.03255(5), 255.03142(9), 273.04413(66), 299.02298(62), 300.026(13)	<LOQ	<LOQ	—	—

76	9.84	**Laricitrin 3-*O*-(6"-coumaroyl)hexoside**[g]	$C_{31}H_{27}O_{15}$—	639.13500	639.14960	-14.60	**331.05013(100)**, 287.05948(93), 313.03913(39)	<LOQ	<LOQ	—	—
77	9.64	**Syringetin 3-*O*-(6"-coumaroyl)hexoside**[g]	$C_{32}H_{29}O_{15}$—	653.15060	653.16065	-10.05	**301.07733(100)**, 315.05466(11), 327.05434(19), 329.07091(14), 345.06646(99)	<LOQ	<LOQ	—	—
					∑			**2.00**	**—**	**—**	**—**
					Stilbenoids and derivatives						
78	9.64	**Resveratrol**[a]	$C_{14}H_{11}O_3$—	227.07080	227.07508	-4.28	**143.05134(100)**, 157.06731(6), 181.06663(9), 185.06183(24)	<LOQ	—	—	—
79	8.48	**Resveratrol 3-*O*-glucoside**[h]	$C_{20}H_{21}O_8$—	389.12360	389.12959	-5.99	**227.07289(100)**, 185.06145(8), 228.07513(20)	—	<LOQ	—	—
80	10.17	**Resveratrol dimer**[h]	$C_{28}H_{21}O_6$—	453.13380	453.13749	-3.69	**225.05814(100)**, 226.06482(71), 227.06963(18), 197.06286(47), 333.08098(40), 346.08748(24), 347.09447(47), 435.12718(23), 453.13969(39)	<LOQ	<LOQ	<LOQ	<LOQ
81	10.47	**Resveratrol dimer (like Gneafricanin A)**[h]	$C_{29}H_{23}O_8$—	499.13930	499.14716	-7.86	**453.13745(100)**, 454.14064(46), 225.05476(15), 226.06708(13), 347.09784(32), 359.09519(24), 411.12258(19), 435.12883(15),	<LOQ	<LOQ	<LOQ	<LOQ
82	10.31	**Resveratrol trimer**[h]	$C_{42}H_{31}O_9$—	679.19680	679.20289	-6.09	**679.20492(100)**, 335.094(11), 345.08415(17), 451.12471(16), 573.16477(22), 585.16367(18)	<LOQ	4.29	<LOQ	—
83	9.84	**Resveratrol tetramer is. I**[h]	$C_{56}H_{41}O_{12}$—	905.25980	905.26054	-0.74	**905.27264(100)**, 906.27425(85), 357.0811(23), 358.0873(44), 359.09749(64), 451.12485(27), 717.18325(21), 811.22758(51), 812.23069(33)	<LOQ	<LOQ	<LOQ	—
84	11.19	**Resveratrol tetramer is. II**[h]	$C_{56}H_{41}O_{12}$—	905.25980	905.27112	-11.32	**905.27074(100)**, 906.27682(70), 359.09768(11), 799.23293(17), 887.26176(3)	<LOQ	—	<LOQ	<LOQ
					∑			**—**	**4.29**	**—**	**—**

							Other detected phenolic compounds					
85	9.50	**Luteolin**[a]	$C_{15}H_9O_6^-$	285.03990	285.04358	-3.68	**133.02986(100)**, 151.00508(22), 285.04065(46)	134.03372(15), 215.04099(7),	107.01387(23), 268.97616(6),	—	—	<LOQ	—
86	9.30	**Eriodictyol 7,3'-dimethyl ether**[i]	$C_{17}H_{15}O_6^-$	315.08690	315.08861	-1.71	**109.02966(100)**, 163.03787(16), 203.06644(11)	123.04609(76), 177.05295(12),	151.04034(51), 187.04248(13),	—	—	<LOQ	<LOQ
87	7.61	**Morelloflavone acetyl-hexoside**[i]	$C_{38}H_{31}O_{17}^-$	759.15610	759.15919	-3.09	**287.05816(100)**, 269.04843(26), 437.08823(32), 575.12526(41)	288.06355(19), 303.05236(22),	125.02462(84), 405.06511(53),	<LOQ	<LOQ	<LOQ	<LOQ
					∑					—	—	—	—
					∑∑					**178.4**	**163.3**	**269.6**	**310.3**

Abbreviations: „—„ non-identified compounds. *Compound quantities expressed using available standards a; Compound content expressed as gallic acid equivalent b; Compound content expressed as ellagic acid equivalent c; Compound content expressed as ferulic acid equivalent d; Compound content expressed as catechin equivalent e; Compound content expressed as procyanidin B2 equivalent f; Compound content expressed as kaempferol equivalent g; Compound content expressed as resveratrol equivalent h; Compound content expressed as luteolin equivalent i; <LOQ-less of limit of quantification.

Among the identified flavonols and their derivatives, the only compound quantified above limit of quantification was quercetin 3-*O*-hexuronide, detected in 'Kadarka' skin, with the concentration of 2 mg/kg lyophilized material. This indicates a limited but specific accumulation of this glycosylated flavonol in the epidermal tissue of 'Kadarka' variety.

Resveratrol was the sole stilbenoid quantified in the analyzed samples, detected exclusively in the seeds of the 'Prokupac' variety at a concentration of 4.29 mg/kg lyophilized material. This selective presence may be due to the cultivar-specific biosynthesis of this compound.

Apart from the compounds above-mentioned, other quantified non-anthocyanin phenolic compounds were either present in trace amounts (<LOQ) or completely absent from the samples analyzed.

To characterize the anthocyanins' content in 'Prokupac' and 'Kadarka' skin samples, UHPLC Q-ToF MS analysis was conducted. The results are presented in the Table 4.2. Different classes and anthocyanin derivatives were detected and semi-quantified, namely malvidin, peonidin, petunidin, delphinidin, and cyanidin derivatives.

The total content of anthocyanins was 738.13 mg/kg lyophilized matter in 'Kadarka' skin, and 544.89 mg/kg lyophilized matter in 'Prokupac'.

The most abundant anthocyanin in both skin samples was malvidin-3-*O*-glucoside, at the concentrations of 167.98 mg/kg lyophilized material in 'Kadarka', that is 150.82 mg/kg lyophilized material in 'Prokupac' skin sample. Malvidin hexosides detected both in 'Kadarka' and 'Prokupac' skin were, malvidin 3-*O*-(6''-acetyl)hexoside, malvidin 3-*O*-(6''-*p*-coumaroyl)hexoside, and malvidin 3-*O*-(6''-caffeoyl)hexoside. In contrast, Vitisin A, a pyranoanthocyanin typically formed during wine aging, was present at levels below the limit of quantification, suggesting limited formation or accumulation in the fresh grape skin matrix.

Significantly higher content of peonidin-3-*O*-glucoside and its derivatives was detected in 'Kadarka' skin sample, suggesting a cultivar-specific accumulation pattern of methoxylated anthocyanins. This may be attributed to the enhanced expression or activity of *O*-methyltransferase enzymes in 'Kadarka', which catalyze the methylation of cyanidins derivatives into peonidin forms, influencing the anthocyanins' profile and color stability.

Petunidin derivatives quantified were petunidin-3-*O*-hexoside, petunidin 3-*O*-(6''-acetyl)hexoside, and petunidin 3-*O*-(6''-*p*-coumaroyl)hexoside, detected in skin sample of both investigated varieties.

The second most abundant anthocyanin detected in 'Kadarka' skin was delphinidin-3-*O*-hexoside, quantified at the concentration of 136.23 mg/kg lyophilized matter. The content of the same anthocyanin was only 34.16 mg/kg lyophilized matter in 'Prokupac' skin, suggesting significant differences among varieties regarding anthocyanin profile. Delphinidin hexosides were, in contrast, detected only in 'Prokupac' skin, in marginal concentrations.

Cyanidin-3-*O*-hexoside was the solely anthocyanin detected from cyanidin derivatives in both 'Kadarka' and 'Prokupac' sample, with the concentrations of 16.37, and 5.44 mg/kg lyophilized matter, respectively.

Table 4.2. Identification, characterisation and semi-quantification of anthocyanins of indigenious grape (Prokupac - P and Kadarka – K) skin samples, using UHPLC Q-ToF MS. Target compounds, mean expected retention times (RT), molecular formula, calculated mass, m/z exact mass, mean mass accuracy (mDa), base peak and MS fragments are presented.

RT	Compound name*	Formula	Calculated mass	*m/z* exact mass	mDa	Base peak	MS fragments (% of base peak)	Samples (mg/kg LM)	
								Skin	
								Kad.	Prok.
				Malvidin derivatives					
7.08	**Malvidin 3-*O*-glucoside**[a]	$C_{23}H_{25}O_{12}^+$	493.1346	493.1357	1.1	331.0819	**331(100)**, 332, 333, 315, 316, 287, 270	167.98	150.82
7.85	**Malvidin 3-*O*-(6"-acetyl)hexoside**[b]	$C_{25}H_{27}O_{13}^+$	535.1452	535.1460	0.83	331.0815	**331(100)**, 332, 333, 315, 316	102.46	85.47
7.47	**Malvidin 3-*O*-hexoside-pyruvate (Vitisin A)**[b]	$C_{26}H_{25}O_{14}^+$	561.1244	561.1252	0.77	399.0713	**399(100)**, 400, 401, 383, 384	<LOQ	—
8.73	**Malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside**[b]	$C_{32}H_{31}O_{14}^+$	639.1714	639.17235	0.97	331.08165	**331(100)**, 332, 333	72.48	93.85
8.14	**Malvidin 3-*O*-(6"-caffeoyl)hexoside**[b]	$C_{32}H_{31}O_{15}^+$	655.1663	655.16715	0.85	331.08145	**331(100)**, 332, 333	23.26	24.35
				∑Malvidin derivatives				**366.19**	**354.49**
				Peonidin derivatives					
6.96	**Peonidin 3-*O*-glucoside**[b]	$C_{22}H_{23}O_{11}^+$	463.124	463.1248	0.76	301.0709	**301(100)**, 302, 286, 303	53.40	12.43
7.84	**Peonidin 3-*O*-(6"-acetyl)hexoside**[b]	$C_{24}H_{25}O_{12}^+$	505.1346	505.13535	0.75	301.0709	**301(100)**, 302, 286, 303	34.13	5.22
8.51	**Peonidin 3-*O*-(6"-*p*-coumaroyl)hexoside**[b]	$C_{31}H_{29}O_{13}^+$	609.1608	609.16155	0.73	301.0707	**301(100)**, 302, 303, 286	30.47	9.79
				Petunidin derivatives					
6.58	**Petunidin 3-*O*-hexoside**[b]	$C_{22}H_{23}O_{12}^+$	479.119	479.11965	0.7	317.06575	**317(100)**, 318, 319. 302	57.64	61.27

7.58	**Petunidin 3-*O*-(6"-acetyl)hexoside**[b]	$C_{24}H_{25}O_{13}^+$	521.1295	521.1303	0.78	317.06585	**317(100)**, 318, 319, 302	18.85	15.86
8.24	**Petunidin 3-*O*-(6"-*p*-coumaroyl)hexoside**[b]	$C_{31}H_{29}O_{14}^+$	625.1557	625.15645	0.72	317.06575	**317(100)**, 318, 319, 302	20.03	32.18
				Delphinidin derivatives					
6.46	**Delphinidin 3-*O*-hexoside**[b]	$C_{21}H_{21}O_{12}^+$	465.1033	465.1039	0.6	303.05	**303(100)**, 304, 305	136.23	34.16
7.31	**Delphinidin 3-*O*-(6"-acetyl)-hexoside**[b]	$C_{23}H_{23}O_{13}^+$	507.1139	507.115	1.13	303.05045	**303(100)**, 304, 305	4.83	4.29
7.94	**Delphinidin 3-*O*-hexuronide**[b]	$C_{21}H_{19}O_{13}^+$	479.0826	479.0831	0.53	303.0501	**303(100)**, 304, 113, 305, 141	—	5.29
8.07	**Delphinidin 3-*O*-(6"-*p*-coumaroyl)-hexoside**[b]	$C_{30}H_{27}O_{14}^+$	611.1401	611.1412	1.12	303.05	**303(100)**, 304, 305	—	4.48
				Cyanidin derivatives					
6.62	**Cyanidin 3-*O*-hexoside**[b]	$C_{21}H_{21}O_{11}^+$	449.1084	449.10905	0.66	287.0553	**287(100)**, 288, 289	16.37	5.44
8.28	**Cyanidin 3-*O*-(6"-*p*-coumaroyl)-hexoside**[b]	$C_{30}H_{27}O_{13}^+$	595.1452	595.1469	1.73	287.0554	**287(100)**, 288, 289, 205, 217	<LOQ	
				∑∑				**738.13**	**544.89**

4.2. Binding affinity of salivary proteins for selected anthocyanins and flavan-3-ols/procyanidins derived from grape skin and seed

To investigate the binding affinity of salivary proteins for grape anthocyanins a targeted UHPLC-Q-ToF-MS analysis of selected anthocyanins before and after interaction with salivary proteins was conducted. The skin samples of 'Prokupac', 'Kadarka', and pre-purified 'Cabernet Sauvignon'/'Merlot' are used as reference sources of anthocyanins' extracts for further comparative analysis. The objective of this analysis was to investigate the potential impact of anthocyanins on sensory attributes, with a particular focus on their contribution to astringency perception. The relative content of main anthocyanins in control skin extracts is presented in the Supplementary Table S2.
The results of the analysis are presented in Table 4.3.

Table 4.3. Binding affinities (%) of salivary proteins for selected anthocyanins derived from grape skin analyzed by UHPLC-Q-ToF-MS.

Anthocyanins	Samples			Range (%)
	BAKSk	BAPSk	BAPCMA	
Malvidin derivatives	Binding affinity (%)			
Malvidin 3-*O*-hexoside	60.41	52.05	52.62	52.05-60.41
Malvidin 3-*O*-(6''-acetyl)hexoside	69.05	61.84	58.04	58.04-69.05
Malvidin 3-*O*-(6''-*p*-coumaroyl)hexoside	55.73	52.38	54.04	52.38-55.73
Peonidin derivatives				
Peonidin 3-*O*-glucoside	47.22	44.53	44.92	44.53-47.22
Peonidin 3-*O*-(6''-acetyl)hexoside	67.48	64.30	35.82	35.82-67.48
Peonidin 3-*O*-(6''-*p*-coumaroyl)hexoside	64.17	58.31	47.74	47.74-64.17
Delphinidin derivatives				
Delphinidin 3-*O*-glucoside	94.87	95.01	67.63	67.63-95.01
Delphinidin 3-*O*-(6''-acetyl)hexoside	100.00	100.00	68.39	68.39-100
Delphinidin 3-*O*-(6''-*p*-coumaroyl)hexoside	34.30	79.00	64.09	34.30-79.00
Petunidin derivatives				
Petunidin 3-*O*-hexoside	97.87	98.20	64.58	64.58-98.20
Petunidin 3-*O*-(6''-acetyl)hexoside	98.43	98.36	55.51	55.51-98.43
Petunidin 3-*O*-(6''-*p*-coumaroyl)hexoside	94.47	83.07	64.25	64.25-94.47
∑Glucosylated anthocyanin derivatives (%)	**64.15**	**53.90**	**55.21**	53.90-64.15
∑Acetylated anthocyanin derivatives (%)	**71.80**	**64.86**	**49.27**	49.27-71.80
∑Coumaroylated anthocyanin derivatives (%)	**62.21**	**56.87**	**54.68**	54.68-62.21

Abbreviations: BAKSk-Binding affinity (%) of individual 'Kadarka' skin anthocyanins; BAPSk-Binding affinity (%) of individual 'Prokupac' skin anthocyanins; BAPCMA-Binding affinity (%) of purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins.

According to the results presented, anthocyanins showed different chemical affinities to salivary proteins, dependent on the anthocyanins composition, variety and the relative content of the compounds investigated.
Being the most abundant anthocyanin in all the grape skin samples investigated, malvidin-3-*O*-hexoside and its acetylated derivatives showed moderate binding affinity across all the samples. As can be seen, delphinidin derivatives exhibited the highest binding potential with delphinidin 3-*O*-(6''-acetyl) hexoside reaching 100% affinity across both 'Kadarka' (BAKSk) and 'Prokupac' (BAPSk) skin samples. Petunidin derivatives also demonstrated strong interactions, with binding affinities above 90% in most samples. In contrast, peonidin derivatives, particularly peonidin 3-*O*-(6''-acetyl) hexoside from BAPCMA, showed the lowest binding affinity with the value of 35.82%, indicating weaker interactions with salivary proteins. Regarding substitution patterns, acylated anthocyanins exhibited the highest binding affinity, depending on the variety investigated (from 49.27 for BAPCMA to 71.80 for BAKSk). Coumaroylated (54.68-62.21%) and glycosylated (53.90-64.15%) derivatives showed lower binding affinity, suggesting that anthocyanin structural features, particularly acylation and hydroxylation patterns significantly influence the affinity for salivary proteins.

With respect to relative content of major anthocyanins identified in the analyzed grape skin samples, the results suggest that certain anthocyanins exhibited high binding affinity despite their low concentrations (Supplementary Table 2). This inverse relationship may be particularly for delphinidin and petunidin derivatives, whose low relative content in skin samples was accompanied by the highest observed binding affinities.
Nevertheless, the crude anthocyanins extracts used as controlled, pure matrixes, exhibited relatively high binding affinities, indicating a moderate potential for these compounds to influence the sensory properties of red wines through their interaction with salivary proteins. This is in accordance with previously stated by Paissoni *et al.*, 2020 and Ferrer-Gallego *et al.*, 2015.

To compare the binding affinities of skin and seed samples as controlled matrixes, a targeted UHPLC-Q-ToF-MS analysis of selected flavan-3-ols, procyanidins and their derivatives before and after interaction with salivary proteins was conducted. The analysis was conducted for 'Kadarka' and 'Prokupac' seed samples. The relative content of targeted flavan-3-ols/procyanidins in control seed samples, their identification, and characterization is presented in Supplementary Table S3.
The results are presented in the Table 4.4.

Table 4.4. Binding affinities (%) of salivary proteins for selected flavan-3-ols/procyanidins derived from grape seed, analyzed by UHPLC-QTOF-MS.

Compound name	Extracts	
	BAKSe	**BAPSe**
Flavan-3-ols and derivatives		
Catechin	0	0
Epicatechin	0	0
Epicatechin gallate	0	0
Epicatechin-3-O-hexoside	0	0
Procyanidins and derivatives		
Procyanidin dimer B type is. I	16.45	30.72
Procyanidin dimer B type is. II	30.79	56.23
Procyanidin dimer B type gallate is. I	23.94	45.61
Procyanidin dimer B type gallate is. II	25.45	21.40
Procyanidin trimer B type is. III	16.79	—
Procyanidin trimer B type is. I	50.83	64.21
Procyanidin trimer B type is. II	21.73	51.82
Procyanidin trimer B type gallate is. I	24.25	9.97
Procyanidin trimer B type gallate is. II	100	52.84

Abbreviations: BAKSe- Binding affinity of individual flavan-3-ols/procyanidins derived by 'Kadarka' seed; **BAPSe**- Binding affinity of individual flavan-3-ols/procyanidins derived by 'Prokupac' seed. „—„ nonidentified phenolic compounds.

The results showed no measurable binding affinity (0%) for all monomeric flavan-3-ols (catechin, epicatechin, epicatechin gallate and epicatechin-3-*O*-hexoside) in both varieties analyzed, suggesting limited or negligible direct interaction with salivary proteins in their monomeric forms.

In contrast, significant binding was observed among oligomeric procyanidins, particularly procyanidin dimers and trimers, indicating that increased polymerization and galloylation are associated with enhanced affinity for salivary proteins, consistent with established models of tannin-protein interactions. In regards to variety differences, 'Prokupac' seeds generally demonstrated higher binding affinity than 'Kadarka' sample, proposing a potentially greater contribution to the astringency perception of wines made from this variety.

The absence of binding observed for monomeric flavan-3-ols underscores the significance of oligomeric structures and functional substitutions in mediating interactions with salivary proteins. This finding further highlights the importance of biochemical transformations occurring during wine aging in enhancing phenolic complexity and shaping the sensory perception, particularly astringency, of red wine.

To understand the binding affinity and mechanism of interactions between phenolic compounds from skin and seeds and salivary proteins, and the tendency to form soluble and insoluble complexes between them, SDS-R-PAGE analysis of the salivary proteins was performed before and after mixing with skin and seed extracts (Figure 4.1).

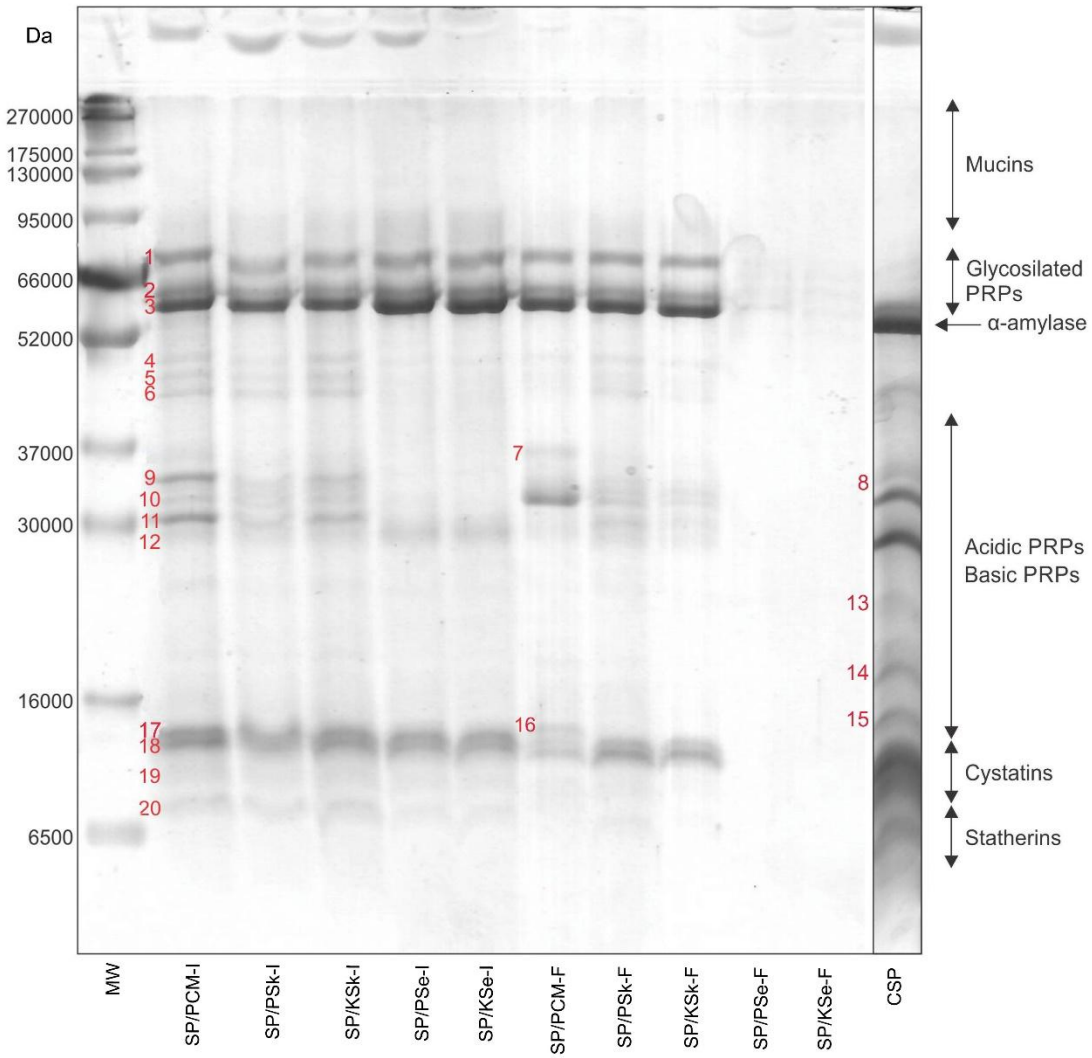

Figure 4.1. Electrophoretic patterns of salivary proteins before and after interaction (after incubation and filtration through 0.22μm filter), with phenolic compounds from grape seed extracts ('Kadarka' and 'Prokupac'); grape skin extracts ('Kadarka' and 'Prokupac'); and purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins, analyzed by SDS-PAGE under reducing conditions (SDS-R-PAGE). Abbreviations are explained in Section 3.5; MW—molecular weight standard; PRPs—proline-rich proteins. Red numbers on the electrophoretic patterns mark bands of salivary proteins and complexes formed after interaction with phenolics.

The salivary proteins were identified based on the literature data (Rinaldi *et al.*, 2014; Ramos-Pineda *et al.*, 2020; Soares *et al.*, 2011), with several predominant and/or diffuse bands corresponding to mucins (<95 kDa), amylase (~62 kDa), proline-rich proteins (PRPs), cystatins (10–14 kDa), and statherins (6.5–10 kDa) (Figure1, lines CSP). The polypeptide composition (%) of the salivary proteins is shown in Supplementary Table S6. Proline-rich proteins (acidic and basic PRPs), representing the main fraction of salivary proteins, had a share of 50.52% (Table S3). Bands of proline-rich proteins can be observed in two regions with the following MW ranges: (a) 14 to 37 kDa (acidic and basic PRPs) and (b) 66–95 kDa (weakly glycosylated PRPs). Previous studies have shown that proline-rich proteins and statherins are the most prone to reacting with phenolics, especially with acidic PRPs (Ramos-Pineda *et al.*, 2020).
The analysis aimed to assess the affinity of phenolic compounds for salivary proteins and their capacity to form soluble or insoluble complexes, particularly those that persist after filtration through a 0.22 μm filter. The electrophoretic profiles reveal notable changes in protein band intensity and migration patterns following interactions with phenolic extracts, indicating those interactions and potential aggregation.
In samples with 'Prokupac' seed extracts (SP/PKSe-I and SP/PKSe-F) several high molecular weight bands, from 1 to 6 (glycosylated PRPs, α-amylase, acidic and basic PRPs) showed reduced intensity or disappearance, indicating strong interactions with phenolics and formation of insoluble complexes removed by filtration. In contrast, skin extracts and anthocyanin fractions of all varieties showed less depletion of protein bands, suggesting lower binding affinity or weaker aggregation tendency due to formation of soluble complexes.
The difference between samples after incubation and filtrate highlight the extent of complex solubility. Also, the intensity of bands 7, 13, 14, and 15 (acidic and basic PRPs) was notably reduced or absent in filtrates of samples treated with seed extracts, confirming strong and mostly insoluble complex formation. These results confirm previously aforementioned analysis and binding affinity of salivary proteins towards anthocyanins, flavan-3-ols and procyanidins, suggesting that the highest binding affinity is, in fact, of polymerized flavan-3-ols, or condensed tannins.

To provide additional clarification and representation of soluble and insoluble complexes formed, electrophoregrams of salivary proteins and skin and seed extracts are presented in Figure 4.2. It is evident that salivary proteins/skin anthocyanins interactions form only soluble aggregates, both after incubation and filtration, while seed procyanidins interact with salivary proteins (acidic and basic PRPs) resulting in insoluble complexes.

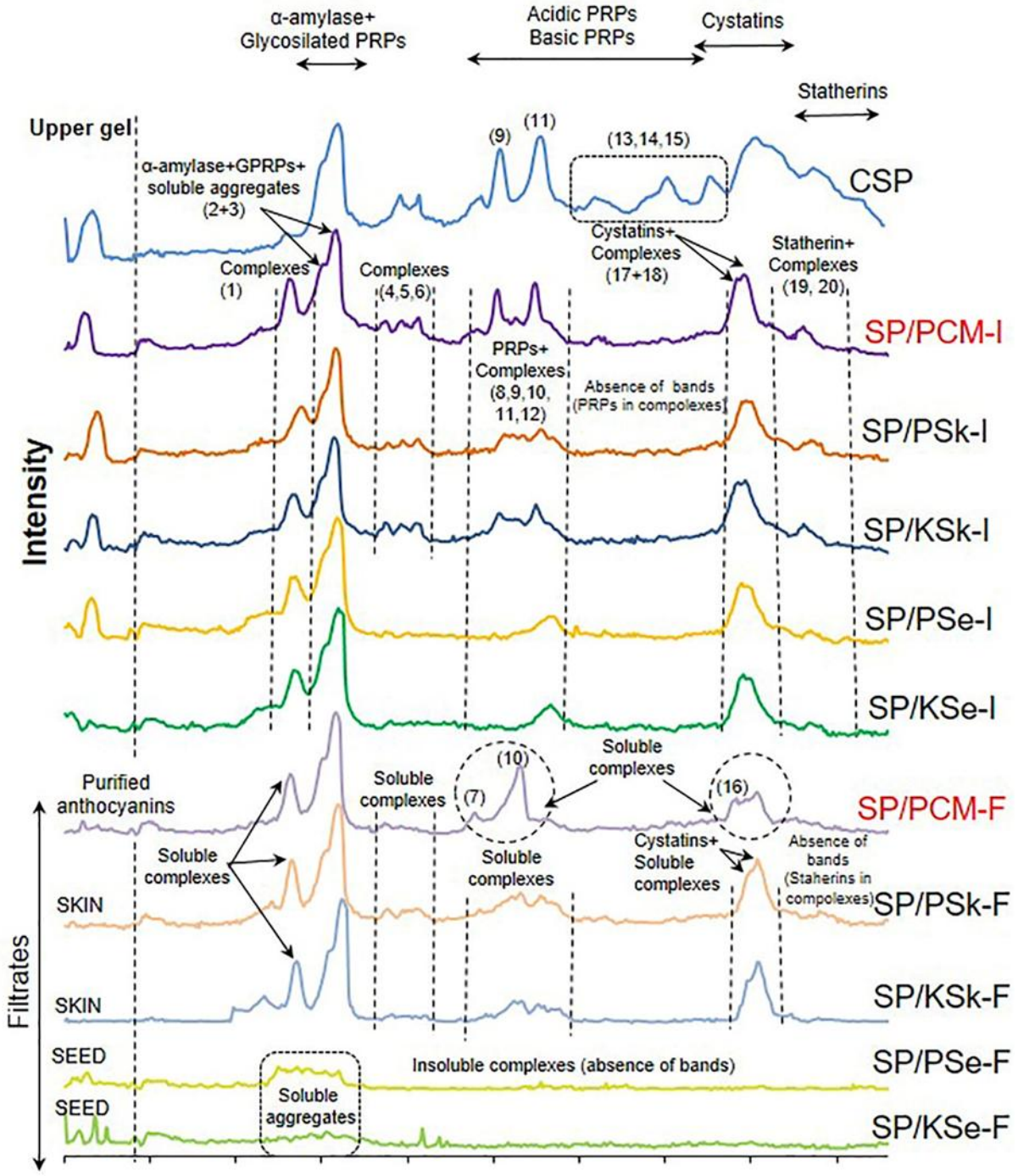

Figure 4.2. Electrophoregrams of salivary proteins (CSP); and CSP/seed phenolics, and CSP/skin phenolics and CSP/purified 'Cabernet Sauvignon' / 'Merlot' anthocyanins after incubation (SP/KSe-I; SP/PSe-I; SP/KSk-I; SP/PSk-I; SP/PCM-I), and their filtrates (SP/KSe-F; SP/PSe-F; SP/KSk-F; SP/PSk-F; SP/PCM-F). Abbreviations are also explained in Section 1.5; PRPs—proline-rich proteins. The numbers mark peaks (Figure Xa) in accordance with the numbers marked in Figure Xa.

4.3. LC/MS quantification of anthocyanins and proanthocyanidins in various indigenous and international wines

The results presented in Table 4.5. showed malvidin-3-*O*-glucoside as a predominant anthocyanin in most samples, which is in accordance with previous investigations of *Vitis vinifera* L. wines (Garcia-Beneytez *et al.*, 2003). The anthocyanin profiles of wine samples analyzed reveal significant varietal differences. Malvidin derivatives are primarily contributors to color intensity and stability in *Vitis vinifera* L. varieties. The highest concentration of mal-3-glc are found in 'Prokupac' (12 PK), with 78.65 ± 3.26 mg/L, and 'Cabernet Sauvignon' (10CS) with 64.73 ± 0.13 mg/L), suggesting a significant influence of variety and potentially vintage. These values are in accordance with previously reported by Menković *et al.* 2014. 'Prokupac' samples show a wide range of anthocyanin concentrations, with higher variability in individual samples compared to 'Cabernet Sauvignon', 'Merlot', 'Black Tamjanika' and blends. Among 'Prokupac' samples, substantial variability was observed in mal-3-glc, ranging from 1.78 mg/L (5PK) to 78.65 mg/L (12PK). Banc *et al.* (2020) stated that the anthocyanin profiles and composition of the wines depends on the vineyard location even in the wines from the same grape variety which could be the reason for big variations among samples in this case. The concentrations of derivatives such as mal-acet and mal-coum showed similar trends, suggesting that winemaking / wine aging may influence these values, since they are later developed. However, mal-3-glc is dominantly represented in 'Prokupac' wines which confirmes previously cited Menković *et al.* (2014). 'Cabernet Sauvignon' samples showed consistency in high levels of anthocyanins, particularly in mal-3-glc, which is already reported by Kropek *et al.* (2023). Anthocyanins' concentration and composition in 'Merlot' samples demonstrated a broad distribution of values, showing high concentrations (e.g. 7ME, and 9ME). 'Merlot' samples demonstrate intermediate anthocyanin concentrations compared to 'Prokupac' and 'Cabernet Sauvignon', with considerable levels of derivatives such as mal-acet and peo-coum, possibly influencing specific sensory attributes. 'Black Tamjanika' samples display the lowest anthocyanin concentrations, often below levels of detection for some compounds, due to its varietal properties. In comparison to glucosides, acetylated and *p*-coumaroylated anthocyanins are present in lower concentrations, though they greatly increase color stability over aging (Boulton, 2001). Samples having higher concentrations of acetylated anthocyanins are 10CS and 12PK, showing greater potential for aging (Razungles, 2022). Anthocyanins, such as delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside are present in low concentrations, almost traces, which is in accordance with previously reported results of anthocyanin content of Croatian red wines given by Kropek *et al.* (2023).

Table 4.5. Mean values with standard deviations of the concentration of molecular anthocyanins (mg/L mal-3-glc)

Samples	del-3-glc	cyan-3-glc	pet-3-glc	peo-3-glc	mal-3-glc	peo-acet	mal-acet	peo-coum	mal-coum
1PK	0.81 ± 0.03	0.18 ± 0.02	1.34 ± 0.07	0.83 ± 0.10	20.23 ± 0.26	0.30 ± 0.04	3.55 ± 0.09	0.83 ± 0.01	2.64 ± 0.32
2PK	1.34 ± 0.18	0.37 ± 0.04	2.85 ± 0.04	3.89 ± 0.17	34.43 ± 0.14	0.93 ± 0.01	6.10 ± 0.15	0.68 ± 0.03	2.80 ± 0.14
3PK	1.36 ± 0.07	0.21 ± 0.05	2.87 ± 0.22	3.34 ± 0.56	48.77 ± 1.14	0.91 ± 0.17	7.43 ± 0.14	0.65 ± 0.02	3.54 ± 0.19
4PK	2.93 ± 0.15	0.69 ± 0.04	3.55 ± 0.18	4.35 ± 0.17	17.98 ± 0.96	0.46 ± 0.28	1.98 ± 0.38	0.42 ± 0.05	1.56 ± 0.09
5PK	0.06 ± 0.01	0.03 ± 0.02	0.09 ± 0.03	0.14 ± 0.02	1.78 ± 0.05	0.12 ± 0.03	0.27 ± 0.04	0.14 ± 0.02	3.64 ± 0.03
6PK	0.18 ± 0.07	0.06 ± 0.01	0.09 ± 0.04	0.33 ± 0.06	3.56 ± 0.22	0.08 ± 0.03	0.57 ± 0.03	0.12 ± 0.03	1.42 ± 0.06
7PK	0.55 ± 0.17	0.16 ± 0.00	0.46 ± 0.05	0.26 ± 0.05	4.37 ± 0.42	0.10 ± 0.01	0.70 ± 0.08	0.19 ± 0.03	1.01 ± 0.11
8PK	1.25 ± 0.05	0.24 ± 0.03	1.42 ± 0.15	0.65 ± 0.06	11.54 ± 0.53	0.18 ± 0.02	1.80 ± 0.02	0.25 ± 0.05	0.86 ± 0.07
9PK	1.84 ± 0.14	0.22 ± 0.03	2.65 ± 0.16	2.66 ± 0.24	39.62 ± 0.40	0.61 ± 0.05	5.17 ± 0.08	0.70 ± 0.05	3.48 ± 0.18
10PK	0.69 ± 0.05	0.17 ± 0.03	1.12 ± 0.31	0.91 ± 0.16	20.91 ± 0.58	0.19 ± 0.05	3.40 ± 0.19	0.27 ± 0.04	1.93 ± 0.15
11PK	0.78 ± 0.07	0.22 ± 0.01	1.39 ± 0.20	1.00 ± 0.16	24.27 ± 3.10	0.35 ± 0.05	3.98 ± 0.51	0.43 ± 0.06	2.26 ± 0.48
12PK	2.85 ± 0.16	0.39 ± 0.12	4.81 ± 0.10	4.16 ± 0.25	78.65 ± 3.26	1.55 ± 0.19	9.74 ± 0.58	2.42 ± 0.20	9.82 ± 0.72
13PK	0.35 ± 0.11	0.06 ± 0.00	0.19 ± 0.03	0.30 ± 0.03	3.45 ± 0.03	0.05 ± 0.03	0.40 ± 0.05	0.06 ± 0.02	1.28 ± 0.11
14PK	0.71 ± 0.02	0.13 ± 0.03	0.76 ± 0.05	0.59 ± 0.08	15.00 ± 0.49	0.13 ± 0.06	1.73 ± 0.15	0.21 ± 0.01	1.82 ± 0.08
15PK	0.66 ± 0.13	0.18 ± 0.03	1.05 ± 0.04	0.84 ± 0.07	16.38 ± 0.27	0.34 ± 0.05	3.20 ± 0.11	0.35 ± 0.01	1.71 ± 0.02
16PK	0.75 ± 0.02	0.21 ± 0.06	0.82 ± 0.12	0.61 ± 0.04	17.24 ± 0.08	0.41 ± 0.01	1.92 ± 0.15	0.41 ± 0.02	1.66 ± 0.04
1CS	0.13 ± 0.02	0.05 ± 0.01	0.46 ± 0.09	0.14 ± 0.03	2.16 ± 0.19	0.07 ± 0.04	0.52 ± 0.05	0.10 ± 0.04	1.20 ± 0.10
2CS	1.12 ± 0.05	0.38 ± 0.05	0.77 ± 0.03	0.50 ± 0.08	5.34 ± 0.11	0.11 ± 0.02	1.65 ± 0.06	0.23 ± 0.01	0.67 ± 0.06
3CS	0.56 ± 0.03	0.31 ± 0.02	0.55 ± 0.01	0.50 ± 0.05	4.71 ± 0.24	0.09 ± 0.03	1.39 ± 0.05	0.25 ± 0.04	0.89 ± 0.08
4CS	1.08 ± 0.04	0.36 ± 0.03	0.63 ± 0.08	0.52 ± 0.13	5.07 ± 0.46	0.07 ± 0.03	1.16 ± 0.16	0.21 ± 0.02	1.03 ± 0.19
5CS	2.13 ± 0.04	0.32 ± 0.00	1.50 ± 0.02	0.47 ± 0.03	13.97 ± 0.10	0.10 ± 0.05	2.95 ± 0.13	0.24 ± 0.02	1.10 ± 0.15
6CS	2.64 ± 0.04	0.22 ± 0.02	2.21 ± 0.07	1.71 ± 0.14	20.19 ± 0.42	0.42 ± 0.12	6.04 ± 0.15	0.45 ± 0.02	1.37 ± 0.04
7CS	0.87 ± 0.17	0.52 ± 0.02	0.72 ± 0.04	0.72 ± 0.07	11.00 ± 0.08	0.17 ± 0.07	4.78 ± 0.22	0.55 ± 0.04	1.45 ± 0.08
8CS	3.91 ± 0.09	0.76 ± 0.08	3.38 ± 0.13	1.07 ± 0.16	30.11 ± 0.58	0.40 ± 0.02	9.71 ± 0.30	0.60 ± 0.05	1.08 ± 0.06
9CS	0.05 ± 0.02	0.07 ± 0.02	0.06 ± 0.03	0.09 ± 0.01	0.46 ± 0.03	0.08 ± 0.03	0.38 ± 0.10	0.06 ± 0.02	1.01 ± 0.03
10CS	5.58 ± 0.40	0.26 ± 0.02	6.27 ± 0.18	4.26 ± 0.52	64.73 ± 0.13	0.70 ± 0.07	16.02 ± 0.23	0.66 ± 0.10	7.21 ± 0.32
1ME	1.61 ± 0.02	0.29 ± 0.02	1.31 ± 0.12	0.85 ± 0.06	6.97 ± 0.09	0.16 ± 0.04	1.16 ± 0.05	0.52 ± 0.03	2.65 ± 0.02
2ME	0.60 ± 0.05	0.18 ± 0.07	0.38 ± 0.07	0.69 ± 0.06	1.79 ± 0.06	0.10 ± 0.05	0.26 ± 0.06	0.22 ± 0.05	0.94 ± 0.06
3ME	2.99 ± 0.09	0.67 ± 0.08	2.93 ± 0.04	3.23 ± 0.16	19.15 ± 0.16	0.18 ± 0.07	1.79 ± 0.07	0.62 ± 0.03	1.93 ± 0.05

4ME	2.47 ± 0.10	0.92 ± 0.01	2.33 ± 0.04	3.52 ± 0.11	11.56 ± 0.23	0.13 ± 0.05	0.84 ± 0.06	0.56 ± 0.07	0.67 ± 0.06
5ME	0.81 ± 0.03	0.21 ± 0.03	0.52 ± 0.05	0.47 ± 0.07	3.06 ± 0.10	0.08 ± 0.02	0.56 ± 0.01	0.21 ± 0.04	0.53 ± 0.04
6ME	1.29 ± 0.08	0.22 ± 0.01	0.96 ± 0.04	0.58 ± 0.09	7.50 ± 0.12	0.05 ± 0.02	1.12 ± 0.03	0.29 ± 0.08	1.01 ± 0.01
7ME	4.06 ± 0.12	0.65 ± 0.04	3.93 ± 0.07	3.95 ± 0.13	24.08 ± 0.41	0.64 ± 0.04	5.53 ± 0.07	0.99 ± 0.10	3.79 ± 0.29
8ME	0.62 ± 0.04	0.39 ± 0.04	0.60 ± 0.03	0.72 ± 0.08	4.65 ± 0.12	0.24 ± 0.07	1.95 ± 0.18	0.40 ± 0.02	1.17 ± 0.06
9ME	3.36 ± 0.14	0.61 ± 0.10	4.05 ± 0.08	3.69 ± 0.07	25.28 ± 0.48	1.25 ± 0.10	6.45 ± 0.25	0.60 ± 0.02	1.49 ± 0.02
10ME	0.43 ± 0.01	0.10 ± 0.00	0.19 ± 0.02	0.29 ± 0.04	0.93 ± 0.03	0.10 ± 0.03	0.35 ± 0.05	0.11 ± 0.04	0.97 ± 0.08
11ME	1.22 ± 0.11	0.22 ± 0.02	0.84 ± 0.07	0.63 ± 0.08	5.87 ± 0.09	0.05 ± 0.02	1.07 ± 0.06	0.27 ± 0.03	1.13 ± 0.07
12ME	0.41 ± 0.07	0.07 ± 0.01	0.19 ± 0.03	0.22 ± 0.04	1.36 ± 0.06	0.05 ± 0.02	0.39 ± 0.03	0.14 ± 0.01	0.87 ± 0.03
1TM	0.36 ± 0.05	0.11 ± 0.02	0.40 ± 0.08	1.44 ± 0.20	3.06 ± 0.03	0.07 ± 0.06	0.36 ± 0.06	0.19 ± 0.07	0.35 ± 0.01
2TM	0.53 ± 0.04	0.21 ± 0.01	1.00 ± 0.02	3.28 ± 0.20	12.75 ± 0.05	0.27 ± 0.01	1.41 ± 0.09	0.57 ± 0.03	1.35 ± 0.04
3TM	-	-	-	0.27 ± 0.01	0.43 ± 0.03	-	-	-	-
4TM	-	-	0.14 ± 0.01	0.55 ± 0.04	3.14 ± 0.03	-	-	-	-
5TM	0.11 ± 0.03	-	0.09 ± 0.01	0.31 ± 0.02	1.61 ± 0.05	6.21 ± 0.16	-	-	0.34 ± 0.05
1CU	0.34 ± 0.09	0.19 ± 0.03	0.29 ± 0.03	0.30 ± 0.02	1.35 ± 0.03	0.05 ± 0.02	0.35 ± 0.10	0.10 ± 0.01	0.49 ± 0.08
2CU	1.62 ± 0.02	0.22 ± 0.03	1.91 ± 0.01	1.41 ± 0.06	18.23 ± 0.17	0.31 ± 0.03	3.85 ± 0.18	0.31 ± 0.02	1.31 ± 0.08
3CU	3.59 ± 0.18	0.32 ± 0.03	4.07 ± 0.21	4.14 ± 0.57	23.17 ± 2.03	0.75 ± 0.02	5.09 ± 0.12	0.86 ± 0.03	3.43 ± 0.32
4CU	6.51 ± 0.10	0.47 ± 0.04	7.27 ± 0.24	4.73 ± 0.42	45.26 ± 0.58	1.14 ± 0.13	11.93 ± 0.19	0.92 ± 0.05	5.61 ± 0.34
5CU	0.54 ± 0.17	1.92 ± 0.12	1.83 ± 2.25	1.06 ± 0.09	4.57 ± 0.23	0.09 ± 0.02	0.57 ± 0.05	0.14 ± 0.09	0.88 ± 0.10
6CU	0.65 ± 0.04	7.72 ± 0.11	17.22 ± 0.12	0.56 ± 0.20	3.12 ± 0.06	0.13 ± 0.00	0.75 ± 0.14	0.20 ± 0.10	0.84 ± 0.06
7CU	0.12 ± 0.02	0.03 ± 0.00	0.05 ± 0.01	0.09 ± 0.02	1.80 ± 0.06	0.03 ± 0.01	0.30 ± 0.03	0.03 ± 0.00	0.25 ± 0.04
8CU	0.68 ± 0.53	0.22 ± 0.13	2.85 ± 0.16	2.77 ± 0.10	47.81 ± 0.57	1.42 ± 0.12	12.44 ± 0.22	0.90 ± 0.08	4.27 ± 0.16
9CU	0.16 ± 0.01	0.05 ± 0.01	0.15 ± 0.02	0.17 ± 0.05	2.33 ± 0.06	0.08 ± 0.05	0.46 ± 0.04	0.06 ± 0.03	0.24 ± 0.02
10CU	0.96 ± 0.06	0.45 ± 0.02	0.67 ± 0.02	0.79 ± 0.07	4.65 ± 0.02	0.07 ± 0.02	1.62 ± 0.08	0.16 ± 0.04	0.52 ± 0.30
1KA	0.81 ± 0.06	1.36 ± 0.15	1.46 ± 0.02	1.41 ± 0.04	7.74 ± 0.02	0.38 ± 0.05	1.26 ± 0.11	1.01 ± 0.04	0.96 ± 0.02

Phenolic compounds such as monomeric flavan-3-ols units of catechins and polymeric and oligomeric proanthocyanidins (condensed tannins), are largely responsible for gustatory sensations of red wine. The most investigated sensorial characteristic of red wine regarding tannins are astringency and bitterness. Since astringency occurs as a response to salivary proteins precipitation induced by condensed tannins, hence the importance of their qualitative and quantitative analysis. It has been reported that monomers are more likely to influence bitter taste, while the astringency was correlated with higher molecular weight derivatives (Chira *et al.*, 2015).

Presented mean values in Table 4.6. are showing differences between concentration and content of proanthocyanidins. 'Prokupac' samples show moderate to high levels of B3, and CAT compared to other varieties, with some variability among samples. Levels of epicatechin are consistently moderate, indicating potential contributions to the astringency profile. Procyanidins B1 and B2 concentrations are moderate across samples, with variations suggesting the influence of processing or aging. Samples of 'Prokupac' show moderate variability in procyanidin B3 levels, with the highest in sample 1PK (17.69 mg/L) and the lowest in sample 7PK (3.62 mg/L). It is known that procyanidins contribute to bitterness and astringency, often perceived as a dry, puckering sensation. Higher procyanidin concentrations in young wine may enhance perceived bitterness, which can be smooth out with aging. Concentrations of procyanidin B1 are relatively consistent, with the highest observed in 1PK (3.28 mg/L). Lower concentrations in some samples may lead to softer mouthfeel. Levels of catechin are moderate, indicating that this variety may give structured, moderately astringent red wines. Higher levels may inflict interactions with salivary proteins, increasing sensations of drying. Concentration of procyanidin B4 is low in 'Prokupac' samples, highest in sample 1PK (1.81 mg/L), while procyanidin B2 is present in moderate levels, being highest in 1PK at 15.47 mg/L. Epicatechin levels vary from 25.64 mg/L in 1PK to 9.02 mg/L in 11PK. Higher levels of epicatechin are usually related to more persistent astringency perception.

'Cabernet Sauvignon' samples generally show high variability, particularly in catechin and epicatechin, which would contribute to pronounced sensory properties such as astringency and bitterness. Having generally higher proanthocyanidin concentrations than 'Prokupac' and other varieties compared, 'Cabernet Sauvignon' have higher potential for robust flavour profiles. Significantly higher concentrations of B3, B1, B2, catechin and epicatechin are noted in 10CS, 30.39, 4.12, 18.60, 69.75, and 31.37 mg/L, respectively. 'Cabernet Sauvignon' is a variety known for its characteristic full-bodied wines, which is correlated with the concentration of proanthocyanidins. Concentration of procyanidin B4 varies across samples, with lower concentrations in 4CS (0.31 mg/L) which may result in softer astringency.

'Merlot' wine samples have generally a high concentration of proanthocyanidins, with samples 1ME and 7ME standing out. In comparison with other varieties, 'Merlot' exhibited higher concentrations of epicatechin than 'Prokupac' and 'Cabernet Sauvignon', indicating that wines may have potentially smoother texture, which is in accordance with previous results of Rinaldi *et al.*, (2014). Notably high levels of procyanidin B3 and epicatechin in 1PK are insinuating phenolic complexity, with 16.57 and 35.23 mgl/L, respectively. Results showed that 'Black Tamjanika' have the lowest concentrations of most proanthocyanidins, with only catechin peaking. This indicates a variety of a lighter phenolic profile with sensorial characteristics softer compared to other varieties investigated. The low concentrations of epicatechin and procyanidin B3 are among the lowest. Low concentrations of procyanidin B3 (9.19 mg/L), B4 (0.74 mg/L), epicatechin (14.46 mg/L), and moderate concentrations of procyanidin B1 (2.55 mg/L) in sample 1TM indicates softer phenolic profile, and low astringency and bitterness levels. Wine samples of blends of varieties 'Cabernet Sauvignon' and 'Merlot' displayed a wide range of concentrations, combining characteristics of both varieties, with high levels of procyanidin B3 and catechin in samples 4CU and 7CU. Wine samples showed great similarities towards phenolic profile of 'Cabernet Sauvignon', indicating that this variety has stronger phenolic complex. These wines may have pronounced astringency and body, and enhanced bitterness, due to substantial variability in concentrations of procyanidin B3 (21.59 mg/L), B1 (5.15 mg/L), B2 (13.10 mg/L) and epicatechin (20.83 mg/L), highest in sample 3CU.

Table 4.6. Mean values with standard deviations of the concentration of proanthocyanidin monomers and dimers (mg/L gallic acid)

Sample No.	B3	B1	CAT	B4	B2	EPICAT
1PK	17.69 ± 0.17	3.28 ± 0.15	43.85 ± 0.34	1.81 ± 0.07	15.47 ± 0.13	25.64 ± 0.06
2PK	13.23 ± 0.06	2.65 ± 0.05	34.25 ± 0.23	0.76 ± 0.03	9.74 ± 0.22	17.23 ± 0.23
3PK	14.06 ± 0.05	2.50 ± 0.04	31.74 ± 0.80	1.04 ± 0.02	9.05 ± 0.06	15.90 ± 0.02
4PK	7.38 ± 0.40	1.51 ± 0.04	26.12 ± 0.02	1.02 ± 0.18	7.88 ± 0.23	16.79 ± 0.18
5PK	5.35 ± 0.02	1.14 ± 0.01	22.40 ± 0.05	1.51 ± 0.08	3.69 ± 0.06	10.84 ± 0.12
6PK	6.98 ± 0.13	1.15 ± 0.01	31.33 ± 0.13	1.63 ± 0.08	4.28 ± 0.02	14.01 ± 0.02
7PK	3.62 ± 0.18	1.80 ± 0.05	16.92 ± 0.53	1.16 ± 0.44	3.05 ± 0.22	9.83 ± 0.35
8PK	7.72 ± 0.05	0.93 ± 0.04	26.08 ± 0.08	0.99 ± 0.05	4.79 ± 0.05	9.86 ± 0.20
9PK	9.29 ± 0.01	0.70 ± 0.02	27.37 ± 0.11	0.99 ± 0.06	5.68 ± 0.11	10.93 ± 0.26
10PK	11.15 ± 0.11	0.77 ± 0.05	35.25 ± 0.14	1.36 ± 0.07	8.33 ± 0.07	17.82 ± 0.21
11PK	8.71 ± 0.22	0.53 ± 0.04	23.65 ± 0.12	0.49 ± 0.01	5.26 ± 0.14	9.02 ± 0.03
12PK	9.99 ± 0.10	0.91 ± 0.03	26.51 ± 0.03	0.84 ± 0.02	4.86 ± 0.02	8.62 ± 0.05
13PK	11.40 ± 0.07	1.55 ± 0.06	33.25 ± 0.05	0.82 ± 0.02	3.99 ± 0.13	9.87 ± 0.11
14PK	9.28 ± 0.10	3.37 ± 0.11	38.07 ± 0.07	2.52 ± 0.18	6.23 ± 0.03	18.71 ± 0.04
15PK	9.99 ± 0.05	0.97 ± 0.01	33.50 ± 0.06	0.63 ± 0.03	8.69 ± 0.08	17.50 ± 0.07
16PK	8.90 ± 0.05	1.12 ± 0.03	26.80 ± 0.08	0.58 ± 0.03	6.71 ± 0.11	13.94 ± 0.23
1CS	4.40 ± 0.09	0.79 ± 0.03	14.61 ± 0.07	3.19 ± 0.09	6.19 ± 0.13	10.25 ± 0.04
2CS	11.34 ± 0.05	1.34 ± 0.05	30.01 ± 0.48	0.57 ± 0.02	10.08 ± 0.09	15.04 ± 0.27
3CS	10.59 ± 0.23	1.29 ± 0.03	18.88 ± 0.06	0.97 ± 0.31	10.63 ± 0.38	21.77 ± 0.22
4CS	4.95 ± 0.07	1.94 ± 0.11	18.68 ± 0.11	0.31 ± 0.02	4.49 ± 0.09	7.04 ± 0.18
5CS	8.23 ± 0.04	2.12 ± 0.08	24.91 ± 0.67	0.49 ± 0.01	7.49 ± 0.21	9.63 ± 0.15
6CS	11.28 ± 0.04	2.03 ± 0.04	34.96 ± 0.15	1.00 ± 0.16	11.29 ± 0.26	16.66 ± 0.23
7CS	10.52 ± 0.35	2.29 ± 0.06	33.39 ± 0.19	2.85 ± 0.57	10.34 ± 0.62	15.03 ± 0.41
8CS	9.45 ± 0.13	1.98 ± 0.23	33.16 ± 0.75	1.76 ± 0.42	7.91 ± 0.02	13.21 ± 0.13
9CS	4.47 ± 0.07	0.15 ± 0.01	12.00 ± 0.04	0.79 ± 0.02	1.25 ± 0.11	1.27 ± 0.09
10CS	30.93 ± 0.16	4.12 ± 0.29	69.76 ± 0.34	2.65 ± 0.07	18.60 ± 0.14	31.37 ± 0.12
1ME	16.57 ± 0.43	3.52 ± 0.33	59.63 ± 2.79	3.25 ± 0.20	22.47 ± 1.14	35.25 ± 1.62
2ME	3.62 ± 0.06	4.38 ± 0.06	21.48 ± 0.14	1.81 ± 0.03	7.28 ± 0.39	10.40 ± 0.33

3ME	12.04 ± 0.08	3.54 ± 0.09	40.04 ± 0.24	2.78 ± 0.06	12.24 ± 0.15	19.23 ± 0.14
4ME	7.26 ± 0.03	3.35 ± 0.06	31.52 ± 0.09	3.13 ± 0.26	8.89 ± 0.37	17.91 ± 0.19
5ME	8.65 ± 0.06	1.94 ± 0.07	30.24 ± 0.04	1.29 ± 0.04	7.71 ± 0.08	14.17 ± 0.19
6ME	12.78 ± 0.07	2.66 ± 0.06	41.53 ± 0.01	2.07 ± 0.07	12.68 ± 0.12	19.47 ± 0.26
7ME	16.06 ± 0.11	2.79 ± 0.08	43.85 ± 0.13	2.84 ± 0.12	14.61 ± 0.09	20.53 ± 0.13
8ME	11.25 ± 0.25	4.27 ± 0.17	31.82 ± 0.98	3.27 ± 0.18	11.83 ± 0.63	16.90 ± 0.05
9ME	11.59 ± 0.04	2.42 ± 0.03	31.53 ± 0.33	1.84 ± 0.12	8.92 ± 0.22	17.55 ± 0.10
10ME	3.67 ± 0.14	3.62 ± 0.11	22.00 ± 0.36	1.06 ± 0.09	6.17 ± 0.03	9.74 ± 0.13
11ME	13.06 ± 0.15	2.07 ± 0.05	43.39 ± 0.51	1.84 ± 0.06	11.98 ± 0.19	18.66 ± 0.19
12ME	5.57 ± 0.02	2.52 ± 0.05	27.54 ± 0.20	0.90 ± 0.04	5.88 ± 0.09	11.15 ± 0.03
1TM	9.19 ± 0.30	2.55 ± 0.03	34.11 ± 0.06	0.74 ± 0.03	6.25 ± 0.12	14.46 ± 0.09
2TM	6.36 ± 0.02	1.06 ± 0.04	21.00 ± 0.05	0.72 ± 0.05	4.54 ± 0.06	11.22 ± 0.93
3TM	1.61 ± 0.06	0.35 ± 0.01	15.02 ± 0.06	0.58 ± 0.04	1.10 ± 0.02	6.19 ± 0.06
4TM	2.22 ± 0.07	0.61 ± 0.01	9.47 ± 0.05	0.40 ± 0.01	0.62 ± 0.02	2.70 ± 0.05
5TM	1.85 ± 0.01	1.08 ± 0.04	23.56 ± 0.07	0.63 ± 0.02	2.47 ± 0.03	7.45 ± 0.02
1CU	3.83 ± 0.08	1.50 ± 0.05	14.70 ± 0.25	0.23 ± 0.01	3.45 ± 0.08	5.69 ± 0.11
2CU	10.16 ± 0.05	1.42 ± 0.03	28.39 ± 0.20	1.57 ± 0.42	8.03 ± 0.04	13.22 ± 0.23
3CU	13.88 ± 0.44	2.08 ± 0.10	36.73 ± 1.17	3.19 ± 0.06	13.10 ± 0.12	20.83 ± 0.46
4CU	15.23 ± 0.12	2.09 ± 0.07	33.92 ± 0.10	3.10 ± 0.10	13.45 ± 0.01	19.07 ± 0.16
5CU	4.46 ± 0.05	1.66 ± 0.04	19.16 ± 0.13	1.21 ± 0.02	6.17 ± 0.03	11.33 ± 0.14
6CU	4.07 ± 0.03	2.20 ± 0.09	15.06 ± 0.17	1.13 ± 0.02	5.74 ± 0.10	8.59 ± 0.07
7CU	21.59 ± 0.23	5.15 ± 0.05	1.43 ± 0.07	0.94 ± 0.03	2.15 ± 0.10	4.21 ± 0.02
8CU	17.19 ± 0.21	1.44 ± 0.03	19.66 ± 0.07	0.93 ± 0.31	8.09 ± 0.04	18.67 ± 0.08
9CU	1.84 ± 0.02	0.96 ± 0.04	7.88 ± 0.36	2.33 ± 0.09	12.65 ± 0.06	10.13 ± 0.04
10CU	6.65 ± 0.12	3.28 ± 0.09	27.02 ± 0.13	1.22 ± 0.07	8.54 ± 0.9	11.08 ± 0.10
1KA	18.12 ± 0.09	50.09 ± 0.05	3.28 ± 0.04	4.14 ± 0.03	11.76 ± 0.12	13.96 ± 0.00

4.4. Untargeted UHPLC Q-ToF MS profile of young and aged wines from indigenous and international grape varieties

The untargeted analysis of phenolic compounds in young and aged red wines from indigenous ('Prokupac' and 'Kadarka') and international ('Merlot' and 'Cabernet Sauvignon') grape varieties, yielded a total of 72 compounds, as shown in Table 7. All identified compounds were divided into several groups to gain a better insight into the differences/similarities between the analyzed wine samples: (I) phenolic acids and derivatives (14 compounds); (II) coumarins (2 compounds); (III) flavan-3-ols and procyanidins (13 compounds); (IV) flavonols and glycosides (13 compounds); (V) flavanonols, flavanons and chalcons (5 compounds); (VI) stilbenoids (4 compounds); and (VII) anthocyanins – malvidin derivatives (12 compounds), peonidin derivatives (3 compounds), petunidin derivatives (3 compounds), and other anthocyanins (3 compounds).

Phenolic acids were detected mainly in the form of aglycones, ethyl derivatives, or esters with tartaric acid. Gallic and vanillic acids were the only hydroxybenzoic acids confirmed. Gallic acid was detected in all the wines analyzed. However, vanillic acid was found only in young wines and its absence in AP, AK, AM, and AC was probably due to transformations (polymerization or oxidation) caused by the maturation of the wine. Coutaric acid, caftaric acid, and fertaric acid were confirmed in all wine samples and are typical hydroxycinnamoyltartaric acid derivatives identified in grapes and wine (Milinčić *et al.*, 2021; Šuković *et al.*, 2020). In contrast, coumaric and ferulic acids were selectively detected in the analyzed wines (YP and AC, AP and AK, respectively), while caffeic acid was found in all wine samples. The presence of hydroxycinnamic acid in aged wine samples may be due to the hydrolysis of hydroxycinnamoyltartaric acid derivatives and caffeoylquinic acid that occurs during aging (Gutiérrez *et al.*, 2005). But a prolonged aging period may also affect the loss or absence of phenolic acids in aged wine (Gutiérrez *et al.*, 2005), as in the case of coumaric acid. Ethyl gallate and ethyl caffeate were detected in all wine samples. Ethyl derivatives of phenolic acids are typical compounds in products undergoing alcoholic fermentation (Milinčić *et al.*, 2021). Ellagic acid was found in all wine samples. Although previous studies have shown a high content of ellagic acid in Prokupac grape seeds, apparently passing easily into the wine during fermentation, ellagic acid can also be extracted from oak barrels and is a characteristic marker for wine that has undergone barrique maturation (Jordão *et al.*, 2005; Matějíček *et al.*, 2005).

Coumarins were detected selectively, only in aged wines. Esculetin was detected in AC, while aesculin was detected in AP, AM, and AK. This may be attributed to the chemical transformations occurring during the wine aging process. Coumarins originate from the degradation of phenolic precursors such as hydroxycinnamic acids, undergoing enzymatic and non-enzymatic reactions over time. Additionally, as these compounds can be naturally found in oak wood, wine aging in oak barrels may contribute to the presence of coumarins. The prolonged contact with wood and oxidative conditions during barrel aging facilitate the release of coumarin compounds which explains their absence in young wines.

The main flavan-3-ols (catechin and epicatechin) were detected in both young and aged wines. On the other hand, epicatechin gallate was detected only in YM, while chalcan-flavan 3-ol dimer isomer II, and procyanidin dimer B-type gallate were confirmed in YP, YM and YC. Procyanidin dimer type II (like Procyanidin B1) was detected in YP and YM. The absence of these compounds in aged wines can be caused by oxidative flavanol (interflavan) polymerization and aldehyde-mediated polycondensation of anthocyanins and flavanols during wine aging (Gutiérrez *et al.*, 2005).

Table 4.7. Untargeted UHPLC Q-ToF MS phenolic profile of young and aged red wines from indigenous ('Prokupac' and 'Kadarka') and international ('Merlot' and 'Cabernet Sauvignon') grape varieties. Tentative identified phenolics, mean expected retention times (RT), molecular formula, calculated mass, m/z exact mass, mean mass accuracy (mDa), and MS fragments are presented.

No.	RT	Tentative identified compounds*	Formula	Calculated mass	*m/z* exact mass	mDa	MS fragments (% of base peaks)	Presence of identified phenolics in analysed young and aged wines
				Phenolic acid and derivatives				
1	6.38	**Coumaric acid**	$C_9H_7O_3^-$	163.0395	163.0401	0.58	**119.0497(100)**	**YP, AC**
2	7.38	**Vanillic acid**	$C_8H_7O_4^-$	167.0344	167.0356	1.17	**123.0439(100)**, 107.0133	**YP, YM, YC**
3	1.00	**Gallic acid**	$C_7H_5O_5^-$	169.0137	169.0148	1.10	**125.0239(100)**, 124.0163	**YP, AP, YM, AM, YC, AC, AK**
4	4.37	**Caffeic acid**	$C_9H_7O_4^-$	179.0344	179.0356	1.17	**135.0445(100)**, 134.0371, 107.0499	**YP, AP, YM, AM, YC, AC, AK**
5	3.92	**Ferulic acid**	$C_{10}H_9O_4^-$	193.0501	193.0503	0.22	**134.0365(100)**, 133.0283, 117.0342, 148.0133, 164.0119	**AP, AK**
6	6.59	**Ethyl gallate**	$C_9H_9O_5^-$	197.045	197.0465	1.50	**124.0162(100)**, 125.0227, 169.0144	**YP, AP, YM, AM, YC, AC, AK**
7	9.42	**Ethyl caffeic acid**	$C_{11}H_{11}O_4^-$	207.0657	207.0670	1.27	**133.0292(100)**, 135.0446, 134.036, 161.0244, 179.0343	**YP, AP, YM, AM, YC, AC, AK**
8	3.20	**Coutaric acid**	$C_{13}H_{11}O_8^-$	295.0454	295.0470	1.61	**119.0501(100)**, 163.0400	**YP, AP, YM, AM, YC, AC, AK**
9	7.52	**Ellagic acid**	$C_{14}H_5O_8^-$	300.9984	301.0001	1.66	**300.9992(100)**, 299.9913, 283.9966, 229.016, 201.0202, 151.0033, 245.0144, 185.0251, 173.0229, 257.0103	**YP, AP, YM, AM, YC, AC, AK**
10	1.54	**Caftaric acid**	$C_{13}H_{11}O_9^-$	311.0403	311.0421	1.79	**135.0447(100)**, 149.0089, 179.0352, 134.0372	**YP, AP, YM, AM, YC, AC, AK**
11	4.17	**Fertaric acid**	$C_{14}H_{13}O_9^-$	325.056	325.0600	4.04	**134.0368(100)**, 193.0506, 178.027, 149.0089	**YP, AP, YM, AM, YC, AC, AK**
12	4.79	**Coumaric acid hexoside**	$C_{15}H_{17}O_8^-$	325.0923	325.0936	1.26	**145.0292(100)**, 119.0496, 163.0401	**YM, AM, YC, AC, AK**
13	5.98	**Vanillic acid hexoside**	$C_{14}H_{17}O_9^-$	329.0873	329.0893	2.04	**167.0346(100)**, 123.0448	**YM**
14	3.84	**Caffeoylquinic acid (like Chlorogenic acid)**	$C_{16}H_{17}O_9^-$	353.0873	353.0887	1.44	**191.0559(100)**, 161.0239, 127.0395, 173.0451, 135.0449	**YP**
				Coumarins				
15	3.84	**Esculetin**	$C_9H_5O_4^-$	177.0188	177.0195	0.72	**105.0339(100)**, 133.0288, 121.0291, 149.0238, 107.0139, 177.0198	**AC**
16	7.81	**Aesculin**	$C_{15}H_{15}O_9^-$	339.0716	339.0734	1.79	**161.0241(100)**, 159.0295, 133.0285, 177.0398, 115.0392	**AP, AM, AK**
				Flavan-3-ols and procyanidins				
17	3.42	**Catechin**	$C_{15}H_{13}O_6^-$	289.0712	289.0727	1.49	123.045(100), 109.0294, 125.0244, 151.0398, 137.0244, 203.0712, 149.025, 221.0821, 187.0402, 245.0813	**YP, AP, YM, AM, YC, AC, AK**
18	6.13	**Epicatechin**	$C_{15}H_{13}O_6^-$	289.0712	289.0727	1.49	123.045(100), 109.0294, 125.0244, 151.0399, 137.0243, 203.0713, 149.0253, 221.0819, 187.0403, 245.0820	**YP, AP, YM, AM, YC, AC, AK**

19	7.46	**Epicatechin gallate**	$C_{22}H_{17}O_{10}^-$	441.0822	441.0842	2.03	**169.0136(100)**, 125.024, 289.0711, 245.0828, 271.0621, 203.0707, 151.0406, 137.0255, 109.0292	**YM**
20	2.48	**Procyanidin B type dimer is. I**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0724(100)**, 407.0780, 125.0243, 245.0805, 161.0248, 137.0242, 273.0408, 425.0884, 451.1036, 255.0339, 229.0511	**YP, AP, YM, AM, YC, AC, AK**
21	4.11	**Procyanidin B type dimer is. II (like Procyanidin B1)**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0718(100)**, 407.0776, 125.0241, 245.0798, 161.0249, 137.0239, 273.0404, 425.0885, 451.1047, 255.0377, 229.0512, 205.0485	**YP, YM**
22	5.38	**Procyanidin B type dimer is. III (like Procyanidin B3)**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0722(100)**, 407.0778, 125.0242, 245.0803, 161.0250, 137.0242, 273.0407, 425.0882, 451.1031, 255.0352, 229.0512, 205.0476, 109.0291	**YP, AP, YM, AM, YC, AC, AK**
23	3.41	**Chalcan-flavan 3-ol dimer is. I**	$C_{30}H_{27}O_{12}^-$	579.1503	579.1522	1.95	**289.0720(100)**, 245.0824, 271.0607, 179.0352, 205.0510, 165.0187, 151.0400, 137.0245, 125.0242, 109.0293	**YP, YM, YC, AK**
24	6.07	**Chalcan-flavan 3-ol dimer is. II**	$C_{30}H_{27}O_{12}^-$	579.1503	579.1522	1.95	**289.0719(100)**, 245.0824, 271.060719, 179.0352, 205.0510, 165.0188, 151.0397, 137.0241, 125.0241, 109.0293, 221.0825	**YP, YM, YC**
25	6.84	**Procyanidin dimer B type gallate**	$C_{37}H_{29}O_{16}^-$	729.1456	729.1481	2.54	**407.0772(100)**, 289.0716, 125.0239, 408.0807, 451.1023, 169.0141, 577.1319, 271.0612, 287.0567, 441.0825, 161.0246, 245.0591, 203.0206, 137.0237	**YP, YM, YC**
26	6.71	**Procyanidin derivative I (like Cinchonain)**	$C_{39}H_{31}O_{15}^-$	739.1663	739.1675	1.20	**289.0725(100)**, 339.0505, 177.0182, 587.1185, 739.1642, 287.0555, 451.1019, 407.0777, 569.1104, 125.0249, 449.0869	**AK**
27	7.14	**Procyanidin derivative II (like Cinchonain)**	$C_{39}H_{31}O_{15}^-$	739.1663	739.1675	1.20	**289.0711(100)**, 341.0662, 451.1011, 339.0518, 177.0186, 569.1088, 459.0729, 287.0561, 407.0813	**AK**
28	3.81	**Procyanidin B type trimer is. I**	$C_{45}H_{37}O_{18}^-$	865.198	865.1992	1.21	**287.0551(100)**, 125.0238, 289.0709, 407.0766, 577.1359, 695.141, 425.0874, 451.102, 243.0313	**YM, AM**
29	5.51	**Procyanidin B type trimer is. I (like Procyanidin C1)**	$C_{45}H_{37}O_{18}^-$	865.198	865.2003	2.31	**287.0554(100)**, 125.0239, 407.0783, 577.136, 289.0704, 695.1423, 865.1987, 425.0851, 451.1021, 243.0332	**YM, AK**
					Flavonols and glycosides			
30	10.10	**Kaempferol**	$C_{15}H_9O_6^-$	285.0399	285.0411	1.19	**285.0405(100)**, 185.0609, 229.0515, 239.035, 159.0447, 211.0396, 143.0497, 151.0038, 227.0347, 255.0301,	**YP, YM, YC**
31	9.30	**Quercetin**	$C_{15}H_9O_7^-$	301.0348	301.0368	1.97	**151.0036(100)**, 121.0292, 178.9984, 149.0237, 301.0334, 245.0456, 273.0400, 229.0500, 201.0549	**YP, AP, YM, AM, YC, AC, AK**

32	10.27	**Isorhamnetin**	$C_{16}H_{11}O_7^-$	315.0505	315.0516	1.12	**300.0276(100)**, 151.0033, 301.031, 107.0133, 271.0251, 283.0259, 255.0293, 227.0344, 243.0301, 179.0001	**YP, AP, YM, AM, YC, AC**
33	8.41	**Myricetin**	$C_{15}H_9O_8^-$	317.0297	317.0315	1.76	**151.0036(100)**, 137.0241, 107.0137, 178.9987, 165.0191, 227.0349, 243.0311, 271.0247, 317.0306	**YP, AP, YM, AM, YC, AC, AK**
34	9.27	**Laricitrin**	$C_{16}H_{11}O_8^-$	331.0454	331.0473	1.91	**151.0062(100)**, 316.0231, 178.9995, 271.0243, 317.0257, 287.0179, 259.0252, 243.0300, 107.0135	**AP, AK**
35	10.15	**Syringetin**	$C_{17}H_{13}O_8^-$	345.061	345.0634	2.36	**315.0144(100),** 163.0028, 287.0211, 330.0383, 316.019, 271.0243, 259.0244, 243.0282, 345.0607	**YP, YC, AC**
36	7.60	**Quercetin 3-*O*-hexuronide**	$C_{21}H_{17}O_{13}^-$	477.0669	477.0687	1.78	**301.0358(100),** 151.0034, 178.9984, 283.0251, 273.0403, 255.0301, 245.0451	**YP, AP, YM, AM, YC, AC, AK**
37	8.14	**Isorhamnetin 3-*O*-hexoside**	$C_{22}H_{21}O_{12}^-$	477.1033	477.1038	0.5	**314.0420(100),** 301.0350, 151.0031, 477.0992, 243.0305, 271.0251, 285.0398, 227.0672, 178.9985	**YM, YC**
38	7.13	**Myricetin 3-*O*-hexoside**	$C_{21}H_{19}O_{13}^-$	479.0826	479.0847	2.13	**316.0229(100),** 271.0245, 287.0194, 178.9982, 151.0035, 479.0832	**YP, AP, YM, AM, YC, AC**
39	7.05	**Myricetin 3-*O*-hexuronide**	$C_{21}H_{17}O_{14}^-$	493.0618	493.0647	2.87	**317.0304(100),** 318.0312, 178.9971, 151.0049, 137.0232, 271.0281, 299.0174	**AP, YM, AM, AC, AK**
40	7.65	**Laricitrin 3-*O*-hexoside**	$C_{22}H_{21}O_{13}^-$	493.0982	493.0988	0.58	**330.0382(100),** 331.0446, 315.0150, 316.0201, 287.02, 493.1013, 271.0245, 243.0285, 151.0055, 178.9975	**YP**
41	8.11	**Syringetin 3-*O*-hexoside**	$C_{23}H_{23}O_{13}^-$	507.1139	507.1156	1.73	**344.0541(100)**, 345.0591, 507.1147, 273.0405, 301.0369, 316.0588, 329.0321, 258.0160, 151.0034	**YP, AP, YM, AM, YC, AC, AK**
42	8.71	**Syringetin 3-*O*-(6"-acetyl)hexoside**	$C_{25}H_{25}O_{14}^-$	549.1244	549.1269	2.47	**344.0540(100)**, 549.1240, 329.0324, 316.0601, 301.0351, 273.0367, 387.0744	**YC**
				Flavanonols, Flavanons and Chalcons				
43	9.83	**Naringenin**	$C_{15}H_{11}O_5^-$	271.0606	271.0622	1.55	**119.0501(100)**, 151.0034, 107.0133, 177.0182, 161.0586, 145.0275, 229.0541	**YP, AP, YM, AM, YC, AC, AK**
44	7.39	**Taxifolin**	$C_{15}H_{11}O_7^-$	303.0505	303.0522	1.72	**125.0249(100)**, 151.0216, 174.0312, 199.0390, 137.0211, 193.0515, 243.0271	**AP, AC, AK**
45	5.05	**Dihydromyricetin**	$C_{15}H_{11}O_8^-$	319.0454	319.0469	1.51	**125.0242(100)**, 165.019, 151.0038, 167.0346, 137.0241, 175.0040, 193.0137, 205.0501, 233.0457	**YP, YM, YC**
46	7.69	**Taxifolin 3-*O*-rhamnoside**	$C_{21}H_{21}O_{11}^-$	449.1084	449.1108	2.41	**151.0038(100)**, 285.0402, 125.0240, 107.0134, 135.0444, 180.0064, 303.0509	**YM, AK**
47	8.40	**Phloretin-2'-*O*-hexoside (like Phlorizin)**	$C_{21}H_{23}O_{10}^-$	435.1291	435.1316	2.48	**167.0351(100)**, 273.0778, 125.0238, 274.0802, 179.0348, 123.0452, 168.0388	**YP, YC**
				Stilbenoids				
48	9.34	**Resveratrol**	$C_{14}H_{11}O_3^-$	227.0708	227.0721	1.28	**143.0501(100)**, 185.0593, 117.0347, 157.0655, 167.0535	**YP, AP, YM**

49	7.32	**Resveratrol hexoside is. I**	$C_{20}H_{21}O_8^-$	389.1236	389.1253	1.66	**227.0710(100)**, 185.0603, 143.0496, 159.0829, 157.0678	**YP, YM, AM, AC, AK**
50	8.22	**Resveratrol hexoside is. II (like Piceid)**	$C_{20}H_{21}O_8^-$	389.1236	389.1253	1.66	**227.0711(100)**, 185.0605, 143.0499, 159.0811	**YP, AP, YM, AM, YC**
51	9.48	**Resveratrol tetramer (like r-Viniferin)**	$C_{56}H_{41}O_{12}^-$	905.2598	905.2613	1.50	**905.2599(100)**, 359.2678, 811.2179, 717.1771, 451.1165, 265.0543, 579.1602,	**AK**
				Anthocyanins				
				Malvidin derivatives				
52	6.59	**Malvidin 3-*O*-glucoside**	$C_{23}H_{25}O_{12}^+$	493.1346	493.1375	2.9	**331.0831(100)**, 332.0854, 315.0508, 316.0578, 287.0555	**YP, AP, YM, AM, YC, AC, AK**
53	7.13	**Malvidin 3-*O*-hexoside-acetaldehyde (like Vitisin B)**	$C_{25}H_{25}O_{12}^+$	517.1346	517.1367	2.1	**355.0819(100)**, 356.0854, 317.0662	**YP, AP, YM, YC**
54	7.40	**Malvidin 3-*O*-(6"- acetyl)hexoside**	$C_{25}H_{27}O_{13}^+$	535.1452	535.1475	2.33	**331.0819(100)**, 332.085, 333.0878, 315.0505	**YP, YM, AM, YC, AC, AK**
55	7.45	**Malvidin-3-*O*-(6"- acetyl)hexoside-acetaldehyde**	$C_{27}H_{27}O_{13}^+$	559.1452	559.147	1.83	**355.0822(100)**, 356.0848, 397.0921	**YP, YM, YC**
56	7.12	**Malvidin 3-*O*-hexoside-pyruvate (Vitisin A)**	$C_{26}H_{25}O_{14}^+$	561.1244	561.1266	2.17	**399.0722(100)**, 400.0754	**YP, AP, AM, YC, AC, AK**
57	7.37	**Malvidin 3-*O*-(6"-acetyl)hexoside-pyruvate**	$C_{28}H_{27}O_{15}^+$	603.135	603.1367	1.7	**399.0720(100)**, 400.0755	**AM, AC**
58	8.64	**Malvidin 3-*O*-hexoside-4-vinylphenol**	$C_{31}H_{29}O_{13}^+$	609.1608	609.1626	1.78	**447.1079(100)**, 448.1112, 431.0755	**AP, AM, AC**
59	8.39	**Malvidin 3-*O*-hexoside-4-vinylcatechol (Pinotin A)**	$C_{31}H_{29}O_{14}^+$	625.1557	625.1577	1.97	**463.1026(100)**, 464.1059, 447.0745	**AP, AC, AK**
60	8.22	**Malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{32}H_{31}O_{14}^+$	639.1714	639.1739	2.52	**331.0819(100)**, 332.085, 333.0876	**YP, YM, AM, YC, AC, AK**
61	8.86	**Malvidin 3-*O*-(6"-acetyl)hexoside-4-vinylphenol**	$C_{33}H_{31}O_{14}^+$	651.1714	651.1736	2.22	**447.1082(100)**, 448.1112, 431.0746	**AC**
62	8.11	**Malvidin-3-*O*-(6"-*p*-coumaroyl) hexoside-acetaldehyde**	$C_{34}H_{31}O_{14}^+$	663.1714	663.1737	2.32	**355.0811(100)**, 356.0852, 357.087	**YP, YM, YC**
63	8.07	**Malvidin-3-*O*-(6"-*p*-coumaroyl) hexoside-pyruvate**	$C_{35}H_{31}O_{16}^+$	707.1612	707.1638	2.59	**399.0716(100)**, 400.0742, 401.0801	**AC**
				Peonidin derivatives				
64	6.48	**Peonidin 3-*O*-glucoside**	$C_{22}H_{23}O_{11}^+$	463.124	463.1261	2.06	**301.0711(100)**, 302.0741, 286.0475	**YM, AM, YC, AK**
65	7.59	**Peonidin 3-*O*-(6"-acetyl)hexoside**	$C_{24}H_{25}O_{12}^+$	505.1346	505.1362	1.6	**301.0704(100)**, 302.0746, 286.048	**YP, YM, YC**
66	8.30	**Peonidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{13}^+$	609.1608	609.1635	2.68	**301.0708(100)**, 302.0744, 303.076, 286.0477	**YP, YM, AM, YC**
				Petunidin derivatives				

67	6.06	**Petunidin 3-*O*-glucoside**	$C_{22}H_{23}O_{12}^+$	479.119	479.1205	1.55	**317.0657(100)**, 318.0698, 302.0423	**YP, YM, AM, YC, AC**
68	7.31	**Petunidin 3-*O*-(6"-acetyl)hexoside**	$C_{24}H_{25}O_{13}^+$	521.1295	521.1321	2.58	**317.0658(100)**, 318.0696, 302.042	**YM, YC**
69	8.06	**Petunidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{14}^+$	625.1557	625.1581	2.37	**317.0661(100)**, 318.0689, 302.0466	**YP, YM, YC**
				Other anthocyanins				
70	5.97	**Cyanidin-3-O-glucoside**	$C_{21}H_{21}O_{11}^+$	449.1084	449.1097	1.31	**287.0558(100)**, 288.0584, 289.0612	**YC**
71	5.19	**Delphinidin 3-O-glucoside**	$C_{21}H_{21}O_{12}^+$	465.1033	465.105	1.7	**303.05(100)**, 304.0539	**YM, AM, YC**
72	6.92	**Delphinidin 3-O-(6"-O-acetyl)hexoside**	$C_{23}H_{23}O_{13}^+$	507.1139	507.1161	2.23	**303.0503(100)**, 304.054	**YM, YC**

Abbreviations: is.-isomer; YP-Young 'Prokupac' wine; AP-Aged 'Prokupac' wine (Despotika 2021); YM-Young 'Merlot' wine; AM-Aged 'Merlot' wine (Despotika 2021); YC-Young 'Cabernet Sauvignon' wine; AC-Aged 'Cabernet Sauvignon' wine (Despotika 2019); AK-Aged 'Kadarka' wine (Tonković 2017).

Flavonols in wine usually originate from the grape skins (Šuković *et al.*, 2020; Pantelić *et al.*, 2016) and are present in the form of aglycones, hexuronide, or glycoside (hexoside). Of the flavonol aglycones, quercetin, and myricetin were detected in all wines analyzed, while isorhamnetin was excluded from the AK. Other aglycones (kaempferol and syringetin) were found selectively in YP, YC, YC and YP, YC, AC, respectively or AP and AK (laricitrin). Flavonol glycosides were selectively found in the young analyzed wines. Flavonol hexuronides were found in all wine samples; quercetin 3-*O*-hexuronide was found in all the wines analyzed, while myricetin 3-*O*-hexuronide was found only in AP, AM, AC, AK and YM. These results agreed with the previously reported by Gutierrez *et al.* (2005), who analyzed young and shortly aged red wines. The reduction in flavonol glycosides may be caused by hydrolysis of the glycosidic linkage and formation of aglycones (as in the case of laricitrin) or by oxidation to hexuronides. In addition, glucosides and aglycones can also be involved in other oxidative reactions and condensation during wine aging (Gutierrez *et al.*, 2005).

Of the other non-anthocyanin flavonoids, naringenin was detected in all wines analyzed, while other phenolics were found selectively in YP, YM, and YC (dihydromyricetin), YP, and YC (phlorizin) or AP, AC, and AK (taxifolin).

Stilbenoids are widely present in wine (Šuković *et al.*, 2020; Căpruciu *et al.*, 2025) and grape stems (Milinčić *et al.*, 2020), especially resveratrol, which is known for its health benefits (Căpruciu *et al.*, 2025). Resveratrol was confirmed in YP, AP and YM, while its hexoside (piceid) was detected in YP, AP, YM, AM, and YC.

Anthocyanin derivatives are responsible for the purple-red color of young wine, while the bricked color of aged wine comes from various pyranoanthocyanins (anthocyanin-derived pigments) formed during fermentation and aging (de Freitas *et al.*, 2011; Alcalde-Eon *et al.*, 2006). The presence or absence of anthocyanins and pyranoanthocyanins clearly showed differences between young and aged wines (Table 7). Malvidin and pyranomalvidin derivatives were predominant in the wines analyzed and were most frequently confirmed. Malvidin 3-*O*-glucoside was confirmed in all the wines analyzed, while vitisin A was found in YP, AP, AM, YC, AC and AK, and vitisin B was confirmed in YP, AP, YM, and YC. In addition, malvidin-3-*O*-(6"-acetyl)-hexoside, malvidin-3-*O*-(6"-*p*-coumaroyl)-hexoside, were not detected only in AP. These vitisin-like pyranoanthocyanins were probably formed during fermentation by the reaction of free anthocyanins and certain yeast byproducts, such as acetaldehyde and pyruvic acid (de Freitas *et al.*, 2011; Šuković *et al.*, 2020). Previous studies have shown that the content of these vitisin-like pyranoanthocyanins decreases during wine aging in the bottle, which is due to the formation of various condensation products, as well as the absence of air/oak compounds that favor their formation and protect them from degradation (Alcalde-Eon *et al.*, 2006). Other anthocyanins detected, such as peonidin and petunidin glucosides and acetyl and/or coumaroyl derivatives were confirmed selectively only mostly in young wines. Compounds recognized as malvidin-3-*O*-hexoside-4-vinylphenol (m/z 609, with major fragment at 447 m/z) and malvidin-3-*O*-hexoside-4-vinylcatechol (Pinotin A) (m/z 625, with major fragment at 463 m/z) were detected selectively only in some aged wines. These compounds were formed by different mechanisms during maturation in the barrel and during aging in the barrel and bottle and represent typical compounds in aged wine (Alcalde-Eon *et al.*, 2006)

The results of the quantification of phenolic compounds in young and aged red wines analyzed are shown in Table 4.8. The highest total content of all quantified phenolic compounds was detected in YC and YP (133.79 and 132.45 mg/L, respectively), while a slightly lower content was observed in YM (112.18 mg/L). The total content of all quantified phenolic compounds was significantly lower in all aged wines in comparison to young wines analyzed. The contents of individual phenolic compounds were also significantly higher in young than in aged wines. The lower contents of monomeric phenolic compounds in aged wines were likely due to oxidation, copigmentation, polymerization, and condensation of these compounds during aging of the wine.

Table 4.8. Content of phenolic compounds (mg/L), in young and aged red wines from indigenous ('Prokupac' and 'Kadarka') and international ('Merlot' and 'Cabernet Sauvignon') grape variety.

Compounds	Wine samples						
	YP	**AP**	**YM**	**AM**	**YC**	**AC**	**AK**
	mg/L wine						
Coumaric acid	3.07	—	—	—	—	<LOQ	—
Vanillic acid	5.95	—	3.04	—	4.20	—	—
Gallic acid	24.65	19.84	12.63	17.72	11.67	16.62	15.19
Caffeic acid	4.72	3.39	1.18	1.46	<LOQ	4.83	3.93
Ferulic acid	—	2.09	—	—	—	—	3.11
Ellagic acid	0.61	1.25	<LOQ	0.73	<LOQ	1.62	1.69
Catechin	16.32	11.40	16.88	11.25	17.70	8.49	16.54
Epicatechin	6.93	2.74	5.47	3.13	4.42	1.95	3.08
Epicatechin gallate	—	—	<LOQ	—	—	—	—
Procyanidin B type dimer is. I *	10.33	3.63	13.89	7.65	14.50	4.17	10.76
Procyanidin B type dimer is. II (like Procyanidin B1)*	2.02	—	3.76	—	—	—	—
Procyanidin B type dimer is. III (like Procyanidin B3)*	6.56	3.33	7.12	5.29	12.28	3.16	4.99
Procyanidin B type trimer is. I**	—	—	2.26	<LOQ	—	—	—
Procyanidin B type trimer is. II (like Procyanidin C1)**	—	—	<LOQ	—	—	—	<LOQ
Kaempferol	<LOQ	—	<LOQ	—	<LOQ	—	—
Myricetin	2.69	2.09	1.92	1.94	1.61	1.88	5.40
Naringenin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Resveratrol	1.38	<LOQ	<LOQ	—	—	—	—
Malvidin-3-*O*-glucoside	47.20	5.80	44.03	24.57	67.42	8.89	10.59
∑	**132.45**	**55.56**	**112.18**	**73.76**	**133.79**	**51.59**	**75.28**

* Expressed as Procyanidin B2 equivalent; ** Expressed as Procyanidin C1 equivalent. „–„ nonidentified and non-quantified compounds; <LOQ-less then limit of quantification.

Among the quantified phenolics, malvidin-3-*O*-glucoside was the most abundant in YC (67.42 mg/L), while in YP and YM slightly lower content was detected (47.20 and 44.03 mg/L, respectively). This phenomenon can be attributed to the intrinsic biological characteristics of the variety and differences among varieties, as Cabernet Sauvignon is known for its high anthocyanins content, particularly malvidin-3-*O*-glucoside and its derivatives. The content of gallic acid was highest in YP (24.65 mg/L), while a lower content was observed in YM and YC (12.63 and 11. 67 mg/L, respectively). The content of catechin was similar in all the young wines investigated. The contents of these compounds were significantly lower in all the aged wines, especially malvidin 3-*O*-glucoside, which was attributed to its sensitivity and tendency to form pyranoanthocyanins and complexes with other phenolics during maturation and aging. In all the aged wines, gallic acid and catechin were the predominant compounds. Apart from the compounds already mentioned, other quantified phenolic compounds were either less abundant (<6 mg/L), present in trace amounts (<LOQ), or completely absent from all wines.

4.5. Procyanidins and anthocyanins in young wine of different indigenous and international grape varieties: Evaluation of their reactivity toward salivary proteins.

To evaluate the biochemical interaction potential and complexation behavior between phenolic compounds of young red wines and salivary proteins, SDS-PAGE under reducing conditions was employed. The electrophoretic profiles (Figure 4.3.) provide a comparative visual representation of of the protein patterns before and after interaction with young red wine samples from 'Cabernet Sauvignon', 'Merlot', and 'Prokupac' varieties.

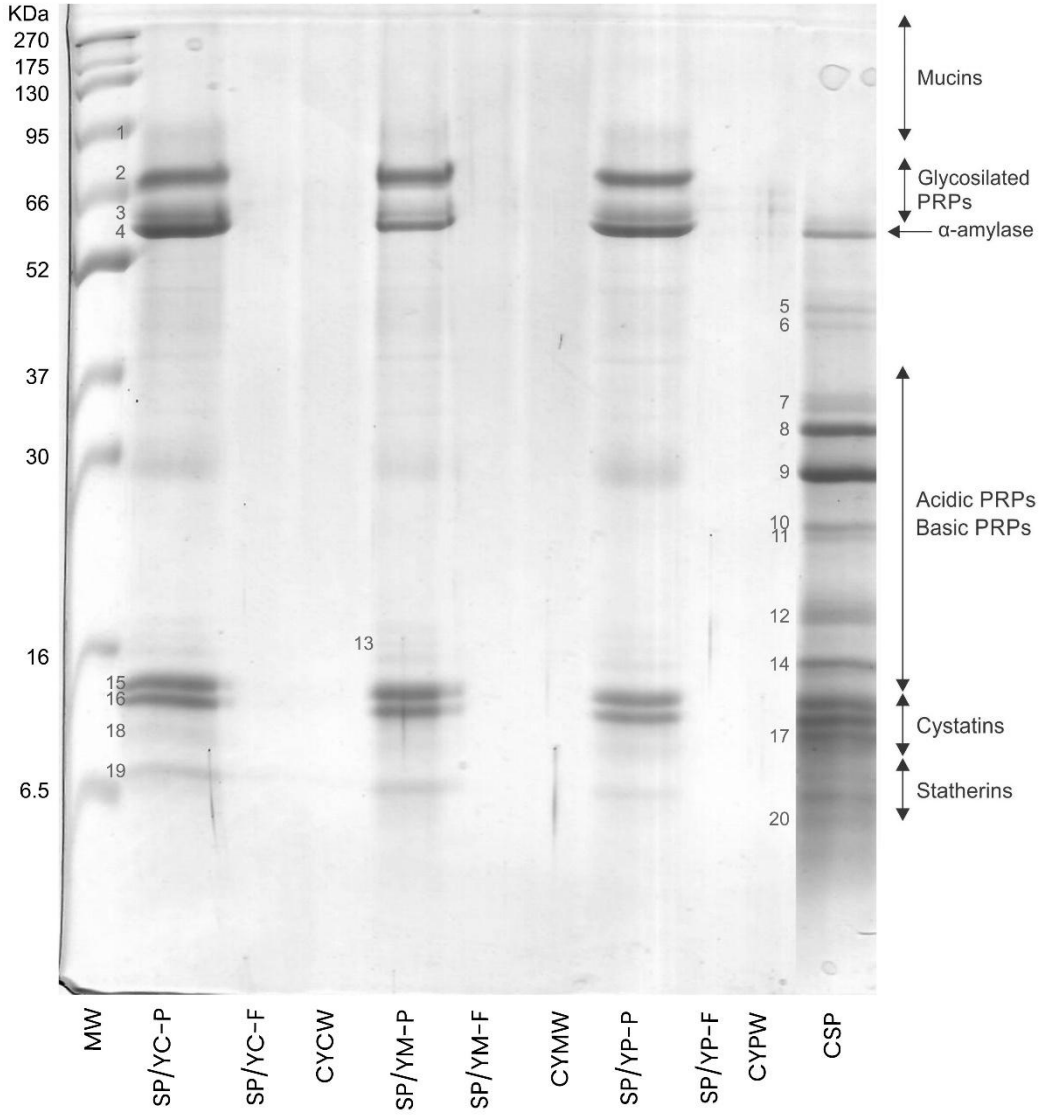

Figure 4.3. Electrophoretic patterns of salivary proteins before and after interaction with young wine produced from different grape varieties ('Cabernet Sauvignon', 'Merlot' and 'Prokupac'), analyzed by SDS-PAGE under reducing conditions (SDS-R-PAGE). Abbreviations are explained in Section 3.5; MW—molecular weight standard; PRPs—proline-rich proteins. Small numbers on the electrophoretic patterns of mark bands of salivary proteins and complexes formed after interaction with phenolics.

The salivary proteins were identified based on the aforementioned literature data (Rinaldi *et al.*, 2014; Ramos-Pineda *et al.*, 2020; Soares *et al.*, 2011), with several predominant and/or diffuse bands previously described in section 4.2 (Figure 1, lines CSP). The polypeptide composition (%) of the salivary proteins is shown in Supplementary Table S6.

The electrophoretic patterns reveal that the salivary proteins in interactions with young red wine samples lead to marked alterations in salivary protein profiles, particularly with wine samples within the molecular weight regions corresponding to proline-rich proteins (PRPs), cystatins, and statherins. A substantial reduction or disappearance of bands in the 10-30 kDa region (bands 7-14), characteristic of acidic and basic PRPs, is observed in lanes corresponding to precipitated fractions, especially in samples of 'Prokupac' and 'Cabernet Sauvignon' young wines. This indicates strong non-covalent interactions and subsequent precipitation of PRPs, which are known to play a central role in astringency perception. In filtrate lanes (-F), many of these bands are absent or greatly reduced, confirming that the resulting protein-phenolic complexes are largely insoluble and stayed on filters of 0.22 μm, after filtration.

Regarding high-molecular weight proteins such as mucins and glycosylated PRPs (>95 kDa; bands 1-4), they appear less affected, suggesting lower binding affinity or steric protection from interaction. The interaction strength appears to be variety-dependent, with 'Prokupac' and 'Cabernet Sauvignon' young wines exhibiting stronger binding capacity, likely due to concentrations and polymerization degrees of tannins and procyanidins, whereas 'Merlot' induced comparatively weaker depletion of protein bands.
This observation is consistent with the previously discussed findings of untargeted UHPLC-Q-ToF-MS analysis of young and aged wines (Table 4.7.), where the total phenolic content was the highest in young 'Prokupac' and 'Cabernet Sauvignon' wines, which explains the higher binding affinity in these varieties.

To ensure better visualization of the newly formed complexes and the decreasing/increasing band intensity of individual salivary proteins after interaction with wine phenolics, electrophoregrams of CSP and precipitates after interaction with young wines produced from different grape varieties (SP/YC-P; SP/YM-P; and SP/YP-P) are presented in Figure 4. As can be seen, the peaks of acidic and basic PRPs on SP/YM-P, SP/YC-P and SP/YP-P electrophoregrams were significantly reduced or absent compared to the same peaks on the CSP electrophoregram. This indicates that these fractions of salivary proteins were crucial for the formation of complexes with phenolic compounds.
Figure 4.4. exhibits the electrophoregram that highlights significant differences in binding between wine varieties, in the region of α-amylase and glycosylated PRPs (3+4 bands). The 'Cabernet Sauvignon' (SP/YC-P) variety exhibits the highest depletion, set to 100% of relative binding, followed by 'Prokupac' (SP/YP-P) with 80.8% and 'Merlot' (SP/YM-P) with 51.0%.
These data suggest that phenolic compounds in 'Cabernet Sauvignon' have the highest interaction potential with these salivary proteins, likely due to their higher procyanidin content and polymerization degree.
Figure 4.4. exhibits the noticeable alterations in cystatin region (15+16 bands), displaying the most pronounced intensity with SP/YC-P and SP/YM-P, while SP/YP-P showed moderate changes. The presence of smaller peaks in these lanes indicates that some cystatin proteins remained unbound or only partially interacted, possibly due to the lower reactivity of monomeric or less polymerized phenolics.

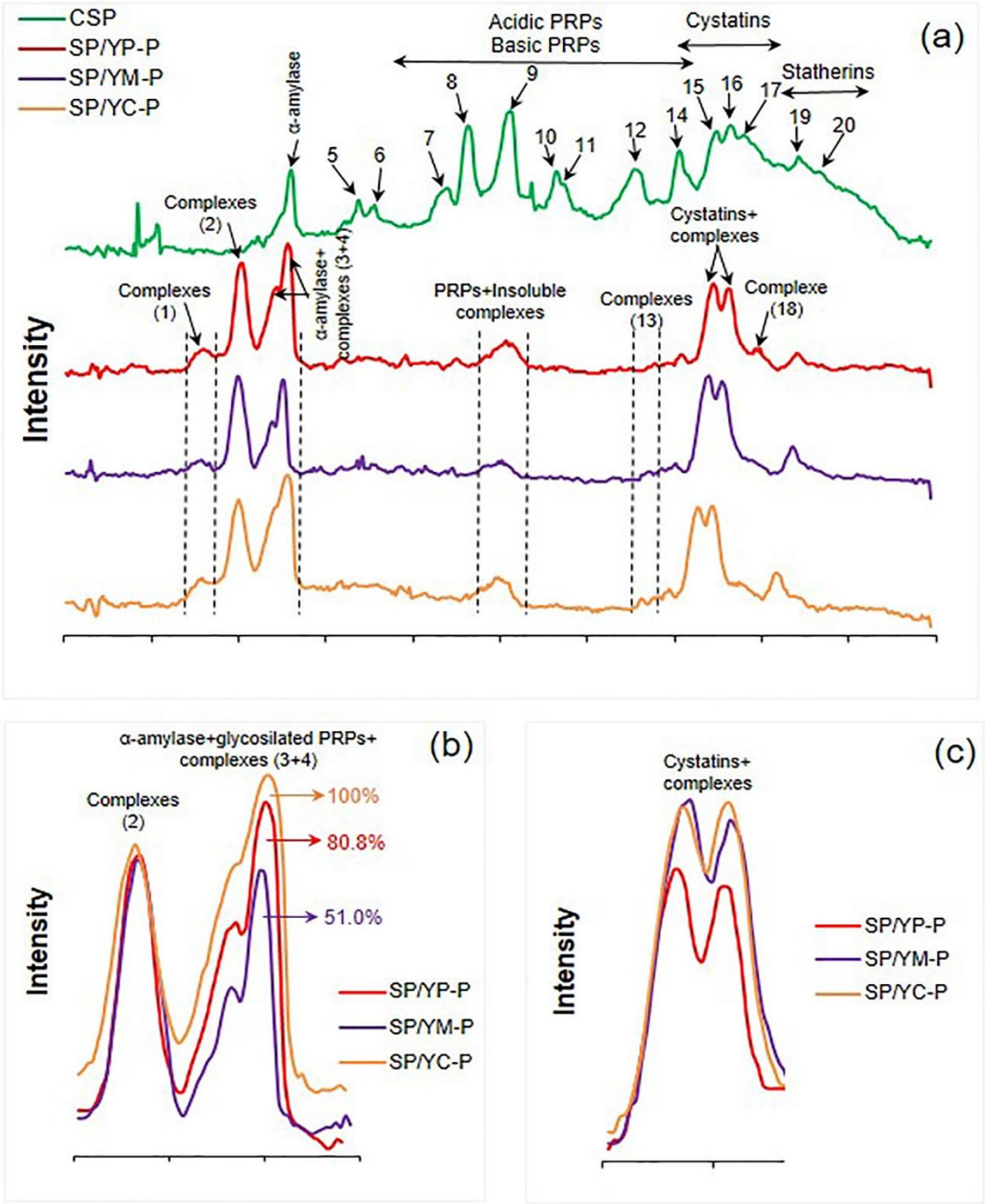

Figure 4.4. Electrophoregrams of: (a) salivary proteins (CSP) and precipitates after interaction with young wines produced from different grape varieties (SP/YC-P; SP/YM-P; and SP/YP-P); (b) Separated parts of electrophoregrams corresponding to bands of complexes (2); α-amylase+glycosylated PRPs+complexes (3+4); and (c) cystatins+complexes (15+16); with the aim of observed the difference in salivary protein/wine phenolics interaction depending on varieties. Abbreviations are also explained in Section 1.5; PRPs—proline-rich proteins. The numbers mark peaks (Figure Xd) in accordance with the numbers marked in Figure Xc.

The previous observations are also summarized in Table 4.9, which shows the changes (%) of individually salivary proteins content in control salivary solution (CSP) and after the interaction with phenolics from young wine produced from different grape varieties (SP/YP-P, SP/YC-P, and SP/YM-P), as all salivary proteins and complexes were retained in the precipitates after interaction. As can be seen, the previous observations agreed with the results of the densitometric analysis.

Table 4.9. The change (%) of individually salivary proteins content in control salivary solution (CSP), and after interaction with phenolics from young wine produced from different grape varieties (the same band confirmed on SP/YP-P, SP/YM-P and SP/YC-P patterns).

No. Polypeptide band	CSP (%)	SP/YP-P (%)	SP/YM-P (%)	SP/YC-P (%)	Characetrisation of identified bands (observations)
1	—	+	+	+	Complexes
2	—	+	+	+	Complexes
3+4	100	325.0	205.0	402.1	α-amylase+GPRPs+complexes
5	100	—	—	—	Salivary protein I
6	100	—	—	—	Salivary protein II
7	100	—	—	—	PRPs
8	100	—	—	—	PRPs
9	100	38.2	23.7	35.8	PRPs+insoluble complexes
10	100	—	—	—	PRPs
11	100	—	—	—	PRPs
12	100	—	—	—	PRPs
13	—	+	+	+	Complexes
14	100	14.9	14.2	13.5	PRPs+complexes
15	100	131.6	151.5	158.6	Cystatins+complexes
16	100	71.4	78.7	82.8	Cystatins+complexes
17	100	—	—	—	Cystatins
18	—	+	—	+	Complexes
19	100	22.5	45.9	53.9	Statherins
20	100	—	—	—	Statherins

Abbreviations: Content of each salivary proteins in CSP labeled as 100%. „—„ nonidentified polypeptides; „+“ identified polypeptide band only on SP/YP-P, SP/YM-P and SP/YC-P patterns (complexes).

The binding affinities of salivary proteins for selected anthocyanins, flavan-3-ols and procyanidins from young 'Cabernet Sauvignon', 'Merlot', and 'Prokupac' wines, quantified by targeted UHPLC Q-ToF-MS, reveal distinct trends based on phenolic profiles and grape variety (Table 4.10).

Procyanidins, particularly oligomeric forms (trimer, tetramer, and pentamer) showed the highest binding affinity, while monomeric flavan-3-ols and anthocyanins showed relatively lower interactions. Monomeric flavan-3-ol, (epi)catechin showed modest binding across samples, with the highest affinity in 'Cabernet Sauvignon' (11.36%), and lower percentage in 'Prokupac' (7.43%). No binding was detected in 'Merlot'. Binding of procyanidin dimers and trimers was significantly higher, with values reaching up to 42.7% (procyanidin trimer) for 'Cabernet Sauvginon' and 27.47% (procyanidin dimer) for 'Prokupac'. These results confirm that increased polymerization enhances protein affinity, due to the greater number of hydroxyl groups and binding sites available for hydrogen bonding and hydrophobic interactions with salivary proteins. Larger oligomers (tetramers and pentamers) were only detected in 'Cabernet Sauvignon' and 'Prokupac', showing high binding affinity (42.95% procyanidin pentamer in 'Cabernet Sauvignon'), further supporting the role of polymerization in interaction strength. Their absence in 'Merlot' young wine suggests a lower oligomeric procyanidin content.

The binding affinity of anthocyanins, particularly malvidin derivatives, was generally lower compared to procyanidins. Malvidin 3-*O*-glucoside exhibited similar low-to-moderate binding across all varieties (~5-7%), while the acylated derivative, malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside, showed slightly higher

binding in 'Cabernet Sauvignon' and 'Prokupac' (~8%), but significantly lower in 'Merlot' (3.72%). The acetylated derivative was not detected in any sample.

These results suggest that acylation or glycosylation of anthocyanins may influence their interaction potential, but to a lesser extent than degree of polymerization, and that anthocyanins are less involved in salivary protein precipitation compared to procyanidins.

When comparing binding affinities among varieties, 'Cabernet Sauvignon' young wine exhibited the strongest overall protein binding, due to the presence of trimeric and pentameric procyanidins; 'Prokupac' showed strong interaction especially for dimers and trimers, while 'Merlot' generally exhibited lower binding affinity, likely due to the differences in phenolic composition, suggesting lower oligomeric procyanidin content.

4.6. Procyanidins and anthocyanins in young and aged 'Prokupac' wines: Evaluation of their reactivity towards salivary proteins

To ensure a better interpretation and understanding of the interactions between phenolic compounds and salivary proteins, the structural formulas of representative flavan-3-ols, procyanidins, and anthocyanins, confirmed by untargeted analysis of young and aged Prokupac wines, are presented in Figure 4.5.

The untargeted UHPLC Q-ToF-MS phenolic profile of young and aging Prokupac red wine with the ration of each compound identified in young and aged wine is presented in the Table S5.

Table 4.10. Binding affinity (%) of salivary proteins for selected anthocyanins, flavan-3-ols and procyanidins from young 'Cabernet Sauvignon', 'Merlot' and 'Prokupac' wine, analyzed by targeted UHPLC Q-ToF MS.

RT	Target compounds	*m/z* exact mass	SP/YC	SP/YM	SP/YP
			Percentage of bound phenolics (%)		
	Monomeric flavan-3-ol and procyanidins (ESI-)				
6.37	**(Epi)catechin**	289.0712	11.36±0.69	0	7.43±0.75
6.20	**Procyanidin dimer**	577.1346	24.47±1.28	6.08±0.76	27.47±0.50
6.71	**Procyanidin trimer**	865.1979	42.74±0.73	22.89±0.18	28.58±3.50
6.78	**Procyanidin tetramer**	1153.2614	18.75±1.35	/	20.39±0.63
6.82	**Procyanidin pentamer**	1441.3248	42.95±0.07	/	/
	Anthocyanins (malvidin derivatives) (ESI+)				
7.10	**Malvidin 3-*O*-glucoside**	493.1346	7.40±0.64	5.48±0.59	6.00±0.51
7.70	**Malvidin 3-*O*-(6"-*O*-acetyl)hexoside**	535.1452	/	/	/
8.90	**Malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	639.1714	8.52±1.23	3.72±0.29	8.08±0.53

* Values in Table are presented as means ± standard deviation, n=3. "/"-no found phenolic compounds.

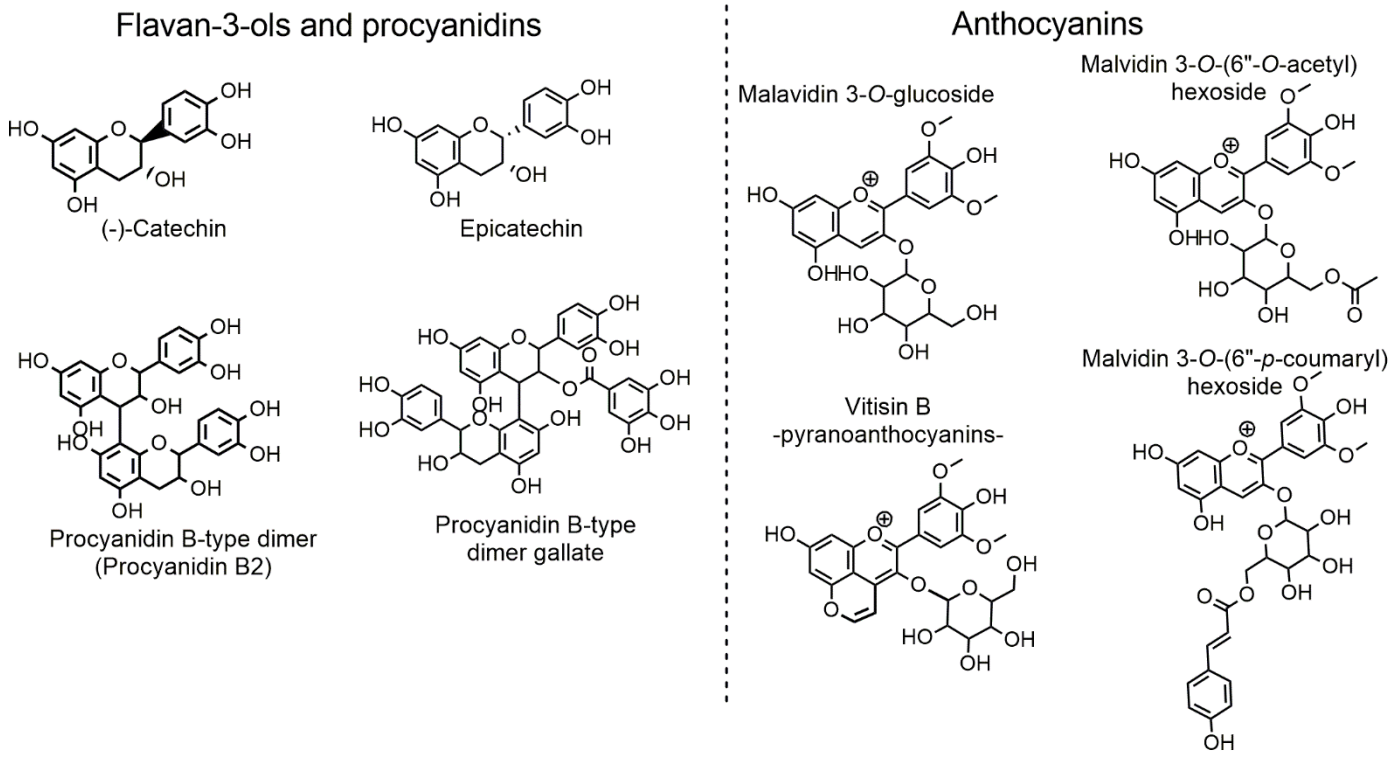

Figure 4.5. Structural formulas of main flavan-3-ols, procyanidins, and anthocyanins/pyroanthocyanin confirmed in young and/or aged 'Prokupac' wines by untargeted analysis.

4.6.1. SDS-PAGE analysis of salivary proteins before and after interactions with 'Prokupac' wine samples

To understand the binding affinity and mechanism of interactions between phenolic compounds and salivary proteins, and the tendency to form complexes between them, SDS-R-PAGE analysis of the salivary proteins was performed before and after mixing with wine/skin/seed samples (Figure 4.6a,b).

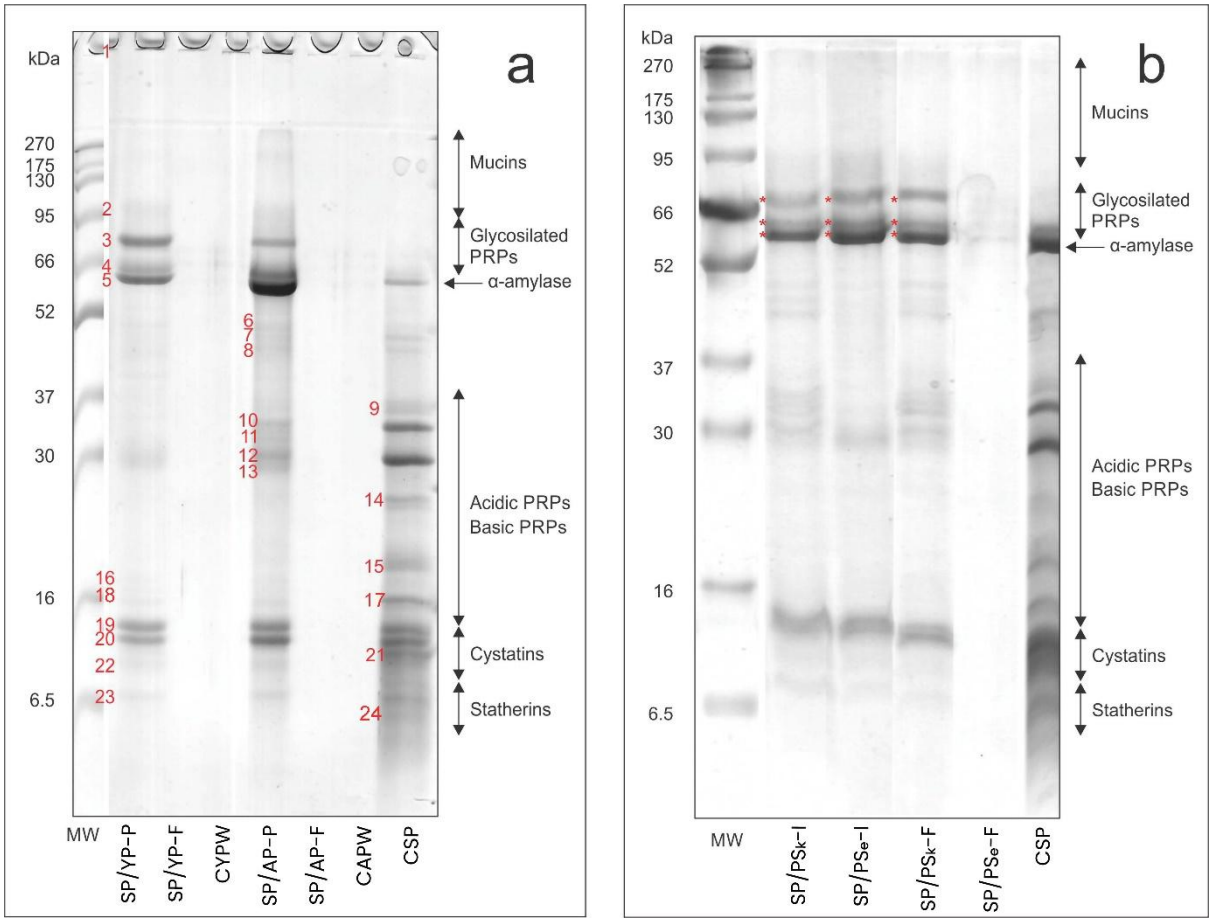

Figure 4.6. Electrophoretic patterns of salivary proteins before and after interaction with: (a) young and aged 'Prokupac' wines; and (b) 'Prokupac' grape seed and skin extracts, analyzed by SDS-PAGE under reducing conditions (SDS-R-PAGE). Abbreviations are explained in Section 3.5.; MW—molecular weight standard; PRPs—proline-rich proteins. Red numbers on the electrophoretic patterns of (a) mark bands of salivary proteins and complexes formed after interaction with phenolics. Red * on the electrophoretic patterns of (b) mark bands of complexes, GPRPs and/or complexes, and α-amylase and/or complexes, respectively.

Bands of proline-rich proteins can be observed in two regions with the following MW ranges: (a) 14 to 37 kDa (acidic and basic PRPs) and (b) 66–95 kDa (weakly glycosylated PRPs). Previous studies have shown that proline-rich proteins and statherins are the most prone to reacting with phenolics, especially with acidic PRPs (Ramos-Pineda *et al.*, 2020). The electrophoretic patterns of the salivary protein/wine filtrates (SP/YP-F and SP/AP-F) were empty, with no visible bands originating from salivary proteins (Figure 6a). This meant that salivary proteins were retained in the precipitates and were present as insoluble complexes with phenolic compounds and rarely in free form, as shown in the SP/YP-P and SP/AP-P patterns. Five intensive bands, with molecular masses of 75.9, 61.9, 58.3, 13.1, and 11.7 kDa (Figure 4.6a, red marked numbers, 3, 4, 5, 19, and 20), can be observed in the electrophoretic patterns of both precipitates (Figure 6a, lines SP/YP-P and SP/AP-P). These bands (especially bands 4, 19, and 20) showed similar electrophoretic pathways as some salivary proteins (see line CSP, Figure 4.1a), but these bands were more intensive compared to the salivary bands and could be attributed to the formation of phenolics/salivary protein complexes. Bands 3, 4, and 5 (Figure 4.6a) could be attributed to complexes between phenolics and acidic or basic PRPs, while bands 19 and 20 (Figure 4.2a) were probably

complexes of phenolics and histatins or statherins (Figure 4.6a, see lines SP/YP-P and SP/AP-P) (Rinaldi *et al.*, 2014; Ramos-Pineda *et al.*, 2020; Soares *et al.*, 2011; Azevedo *et al.*, 2020; Sun *et al.*, 2013). These bands (except band 3) were more intense for SP/AP-P than the same bands in the SP/YP-P pattern (Figure 6a), especially band 5. These differences in band intensity were probably due to the different abilities of phenolics in young and aged wines to react with salivary proteins. Procyanidins, interflavan or flavanol/anthocyanin polymers, and ellagitannins in aged wine obviously showed a high affinity to bind to salivary proteins and form insoluble complexes (Ma *et al.*, 2016; Sarni-Manchado *et al.*, 1999; Soares *et al.*, 2018; Soares *et al.*, 2019). On the other hand, procyanidins with a high affinity for salivary proteins (Sun *et al.*, 2013), as well as monomeric flavan-3-ols (catechin and epicatechin) and anthocyanins which preferentially form soluble aggregates (Ferrer-Gallego *et al.*, 2015; Soares *et al.*, 2018; Ramos-Pineda *et al.*, 2019), were predominantly detected in young wine, while polymeric phenolics were less represented or completely absent. Furthermore, in both precipitates (Figure 2a, lines SP/YP-P and SP/AP-P), numerous diffuse bands of low intensity in the MW range from 16 to 52 kDa could be observed. These bands could also be associated with complexes formed between wine phenolics and salivary proteins. A band of high-molecular-weight complexes could be noticed at the entrance to the upper gel for both precipitates (Figure 4.6a). These complexes were probably formed by phenolics and glycosylated PRPs (Sarni-Manchado *et al.*, 2008). As expected, no bands were visible in the electrophoretic patterns of CYPW and CAPW (Figure 4.6a).

To ensure a better visualization of the newly formed complexes and the decreasing/increasing band intensity of individual salivary proteins after interaction with wine phenolics, electrophoregrams of CSP and precipitates (SP/YP-P and SP/AP-P) are presented in Figure 4.6a. As can be seen, the peaks of acidic and basic PRPs on SP/YP-P and SP/AP-P electrophoregrams were significantly reduced or absent compared to the same peaks on the CSP electrophoregram, indicating that these fractions of salivary proteins were crucial for the formation of complexes with phenolic compounds. On the other hand, several high-intensity (3, 4, 5, 19, and 20, Figure 4.6a) and low-intensity (6, 11, 13, 16, 18, and 22) peaks could be seen in the SP/YP-P and SP/AP-P electrophoregrams, probably originating from newly formed complexes, as previously observed (Figure 4.6a).

The previous observations are also summarized in Table 4.10, which shows the changes (%) in the contents of the individual salivary proteins in the CSP and in the precipitates (SP/YP-P and SP/AP-P), as all salivary proteins and complexes were re-tained in the precipitates after interaction. As can be seen, the previous observations agreed with the results of the densitometric analysis (Table 4.10).

To determine the interactions between phenolics and salivary proteins, the type of aggregates/complexes formed (soluble or insoluble), and to confirm the observations made previously, a control saliva test was also carried out. This experiment was concerned with the interaction of salivary proteins with grape seed and grape skin ex-tracts, which predominantly contained flavan-3-ols/procyanidins or anthocyanins, respectively. The electrophoretic patterns of SP/PSk-I and SP/PSk-F were similar, with the same electrophoretic pathways and mobility of all identified salivary peptides and/or complexes observed, which were not disrupted under reducing conditions (Figure 4.6b). This indicated the low ability of anthocyanins and other monomeric grape skin phenolics (flavonols) to interact with salivary proteins and form soluble aggregates (Paissoni *et al.*, 2018; Ferrer-Gallego *et al.*, 2015; Soares *et al.*, 2019), as can be observed in the SP/PSe-F pattern. On the other hand, in the SP/PSe-I electrophoretic patterns, interactions could be observed mainly between grape seed procyanidins and salivary proteins, especially acidic/basic PRPs (Figure 4.1). However, only several bands of low intensity (around 66 kDa) were visible in the electrophoretic SP/SE-F pattern, demonstrating the tendency of grape seed procyanidins to form in-soluble complexes with salivary proteins (Ma *et al.*, 2016; Rinaldi *et al.*, 2014; Ramos-Pineda *et al.*, 2020; Sarni-Machado *et al.*, 2008; Soares *et al.*, 2011). The electrophoretic patterns of the filtrates (SP/YP-F and SP/AP-F) (Figure 4.6a) and SP/PSe-F (Figure 4.6b) were similar, as were the electrophoretic patterns of precipitates (SP/YP-P and SP/AP-P) (Figure 4.6a) and SP/PSe-I (Figure 4.6b). This indicated that the procyanidins and the polymeric forms of flavan-3-ols present in wine were key to the interaction with salivary proteins and the formation of oral sensations. In addition, the differences between the complexes formed (insoluble complexes or soluble aggregates) between the salivary proteins and the procyanidins

from the seeds or anthocyanins from the skins could be clearly seen in the electrophoregrams shown in Figure 4.7b.

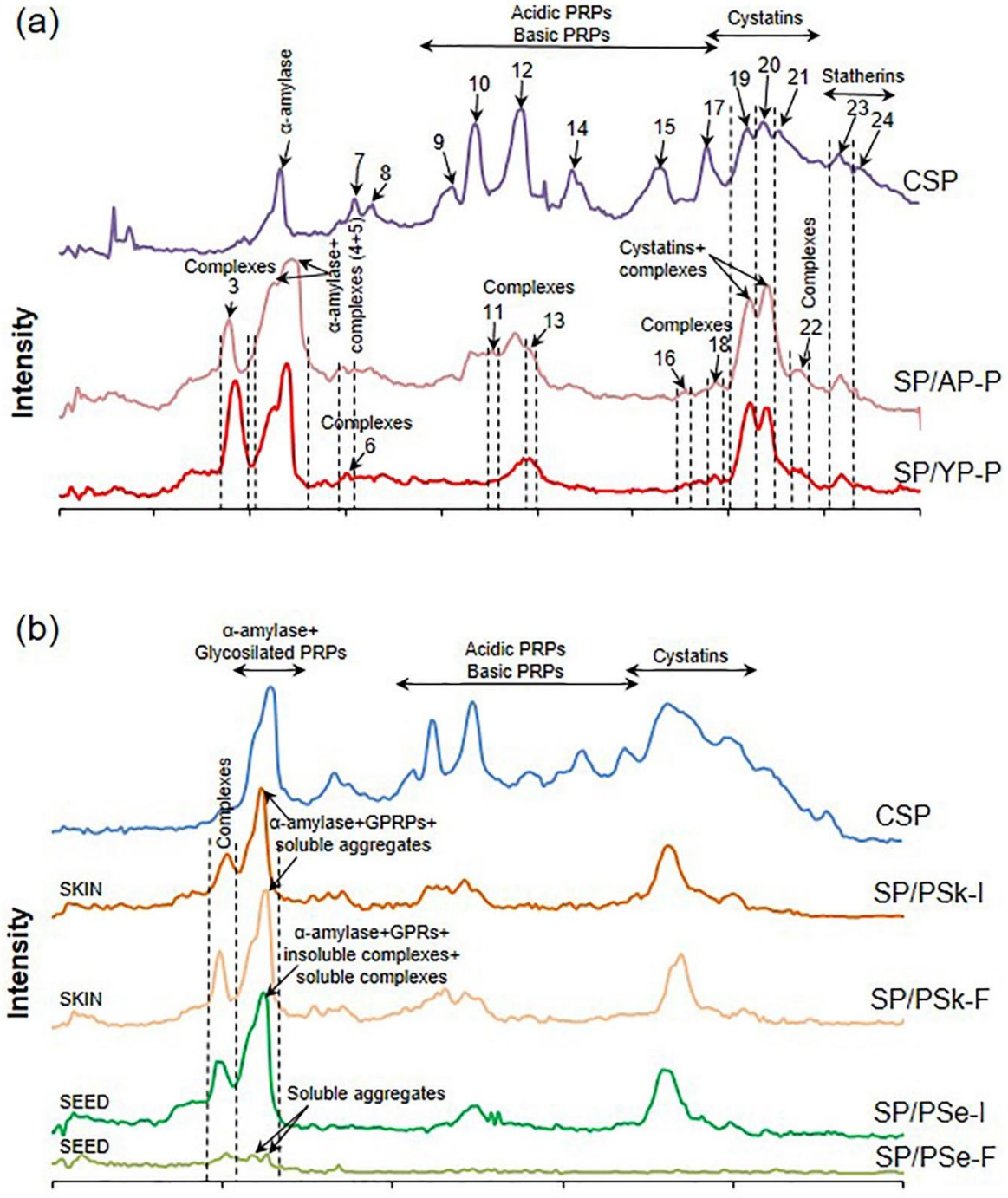

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Figure 4.7. Electrophoregrams of: (a) salivary proteins (CSP) and precipitates after interaction with young and aged wines (SP/YP-P and SP/AP-P); (b) salivary proteins (CSP), CSP/seed and CSP/skin fractions after incubation (SP/PSe-I and SP/PSk-I), and CSP/seed and CSP/skin filtrates (SP/PSe-F and SP/PSk-F). Abbreviations are also explained in Section 1.5.; PRPs—proline-rich proteins. The numbers mark peaks (Figure 4.7) in accordance with the numbers marked in Figure 6.

Table 4.10. The change (%) of individually salivary proteins content in control salivary solution (CSP), and after interaction with wine phenolics (the same band confirmed on SP/AP-P and SP/YP-P patterns).

No. Polypeptide band	CSP (%)	SP/AP-P (%)	SP/YP-P (%)	Characetrisation of identified bands (observations)	Ratio of band area (SP/AP-P)/(SP/YP-P)
2	—	+	+	Complexes	1.03
3	—	+	+	Complexes	0.65
4+5	100	643.4	346.9	α-amylase+GPRPs+complexes	1.85
6	—	+	+	Complexes	2.09
7	100	21.2	—	—	*
8	100	60.3	—	—	*
9	100	—	—	—	—
10	100	52.3	—	PRPs	*
11	—	+	+	Complexes	4.37
12	100	54.2	43.0	PRPs	1.26
13	—	+	+	Complexes	0.78
14	100	—	—	PRPs	—
15	100	—	—	PRPs	—
16	—	+	+	Complexes	0.93
17	100	—	—	PRPs	—
18	—	+	+	Complexes	1.60
19	100	150.4	137.0	Cystatins+complexes	1.09
20	100	120.7	75.8	Cystatins+complexes	1.59
21	100	—	—	Cystatins	—
22	—	+	+	Complexes	2.86
23	100	43.6	19.2	Statherins	2.27
24	100	0	0	Statherins	—

Abbreviations: Content of each salivary proteins in CSP labeled as 100%. „—„ nonidentified polypeptides; „+“ identified polypeptide band only on SP/AP-P and SP/YP-P patterns (complexes). Ratio of band area (SP/AP-P)/(SP/YP-P)-Ratio of areas of the same polypeptide bands confirmed on SP/AP-P and SP/YP-P patterns. *Polypeptide confirmed only at SP/AP-P pattern.

4.6.2. Binding affinity of salivary proteins for selected young/aged wine phenolics

Targeted analysis of selected procyanidins and anthocyanins before and after interaction with salivary proteins revealed their individual affinities to bind to salivary proteins and their contributions to sensory perceptions and astringency. As can be seen, (epi)catechin, procyanidins, and anthocyanins in young and aged Prokupac wines showed different chemical affinities to salivary proteins (Table 4.11).

Table 4.11. Binding affinity (%) of salivary proteins for selected anthocyanins, flavan-3-ols and procyanidins from young and aged Prokupac wine, analysed by targeted UHPLC Q-ToF MS.

Target compounds	*m/z* exact mass	SP/YP	SP/AP
		Percentage of bound phenolics (%)	
Monomeric flavan-3-ol and procyanidins (ESI-)			
(Epi)catechin	289.0712	4.78±0.95	54.00±1.10
Procyanidin dimer (procyanidin B1)	577.1346	28.51±0.85	71.25±0.31
Procyanidin trimer (procyanidin C1)	865.1979	29.86±1.94	77.87±0.24
Procyanidin tetramer	1153.2614	23.44±1.21	74.99±0.95
Procyanidin pentamer	1441.3248	32.16±3.37	100
Anthocyanins (malvidin derivatives) and pyranoanthocyanin (ESI+)			
Malavidin 3-*O*-glucoside	493.1346	3.91±0.35	90.01±0.03
Malvidin 3-*O*-(6"-*O*-acetyl)hexoside	535.1452	/	100
Malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside	639.1714	15.86±0.97	100
Malvidin 3-*O*-hexoside-acetaldehyde (Vitisin B)	517.1346	/	78.31±0.48

* Values in Table are presented as means ± standard deviation, n=3.

Flavan-3-ols and all procyanidin oligomers (from dimer to pentamer) showed a tendency to bind to salivary proteins. The lowest binding affinity was observed for (epi)catechin, while the binding ability of procyanidins increased from dimer to pen-tamer in both wines. These results agreed with the observations of other studies (Ma *et al.*, 2016; Sarni-Manchado *et al.*, 1999; Sun *et al.*, 2013; Soares *et al.*, 2015; Brandao *et al.*, 2020) investigating the interactions between grape seed/wine procyanidins and salivary proteins. In similarity to our results, Ma *et al.* (2016) showed that larger procyanidins oligomers (trimers, tetramers, and pentamers) had a stronger affinity for salivary proteins. The increased binding affinity of the procyanidin pentamer and other oligomers may be attributed to their enhanced ability to form multiple hydrogen bonds and hydrophobic interactions (Figure 4.4a) with salivary proteins, mainly PRPs (McRae *et al.*, 2010). However, the percentages of bound (epi)catechin and procyanidins were significantly higher in aged Prokupac wine (54.00% epicatechin to 100% procyanidin pentamer) than in young wine (4.78% epicatechin to 32.16% procyanidin pentamer). The transformation of procyanidins during wine aging probably affects their composition and structure, which could increase their binding efficiency for salivary proteins. However, the binding affinities of tannins (polymerized forms) in aged wine for poly-prolines were variable and depended on the vintage and number of years of aging of the wine (McRae *et al.*, 2010).

Interestingly, the results of the binding affinity of anthocyanins in young and aged wines showed significant differences. Anthocyanins in young wine showed little or no binding affinity for salivary proteins (0 to 15.86%). Previous studies have also indicated that poor electrostatic (ionic) interactions between anthocyanins and salivary proteins (Paissoni *et al.*, 2018; Soares *et al.*, 2019). and the formation of soluble aggregates (Figure 4a) contribute to the perception of astringency (Ferrer-Gallego *et al.*, 2015). On the other hand, the same anthocyanins and vitisin B in aged wine were almost completely retained in the precipitate, apparently showing a "high" binding affinity. This can also be explained by the fact that the contents of total and individual anthocyanins in AP were significantly lower than in YP (Table S2). For example, the content of malvidin 3-*O*-glucoside was almost 8-fold lower in AP than in YP (Table 4.10). As aforementioned, these differences in the binding affinities of selected phenolics (epicatechin, procyanidins, and anthocyanins) can be explained by the different compositions of young and aged wines, as well as by the synergistic effects of other wine phenolics (Soares *et al.*, 2019). In the case of aged Prokupac wine, procyanidins, high-molecular-weight tannins, and ellagitannins showed high binding affinities for salivary proteins (Sarni-Manchado et al., 1999; Soares *et al.*, 2011; Sun *et al.*, 2013; Soares *et al.*, 2019) and formed insoluble complexes that probably collected and intensively bound other phenolics during precipitation. The results of the targeted analysis supported the conclusions and results of the electro-phoretic analyses.

Taking into account previous interpretations (Soares *et al.*, 2019, Charlton *et al.*, 2002; Helmerhorst *et al.*, 2010; Rashwan *et al.*, 2025) and the results of this study, the mechanism of interactions between proline-rich proteins and the major wine phenolics is illustrated (Figure 4.8a), along with a schematic representation of the formation of insoluble complexes between PRPs and phenolic compounds in young (Figure 4.8b) and aged (Figure 4.8c) wines.

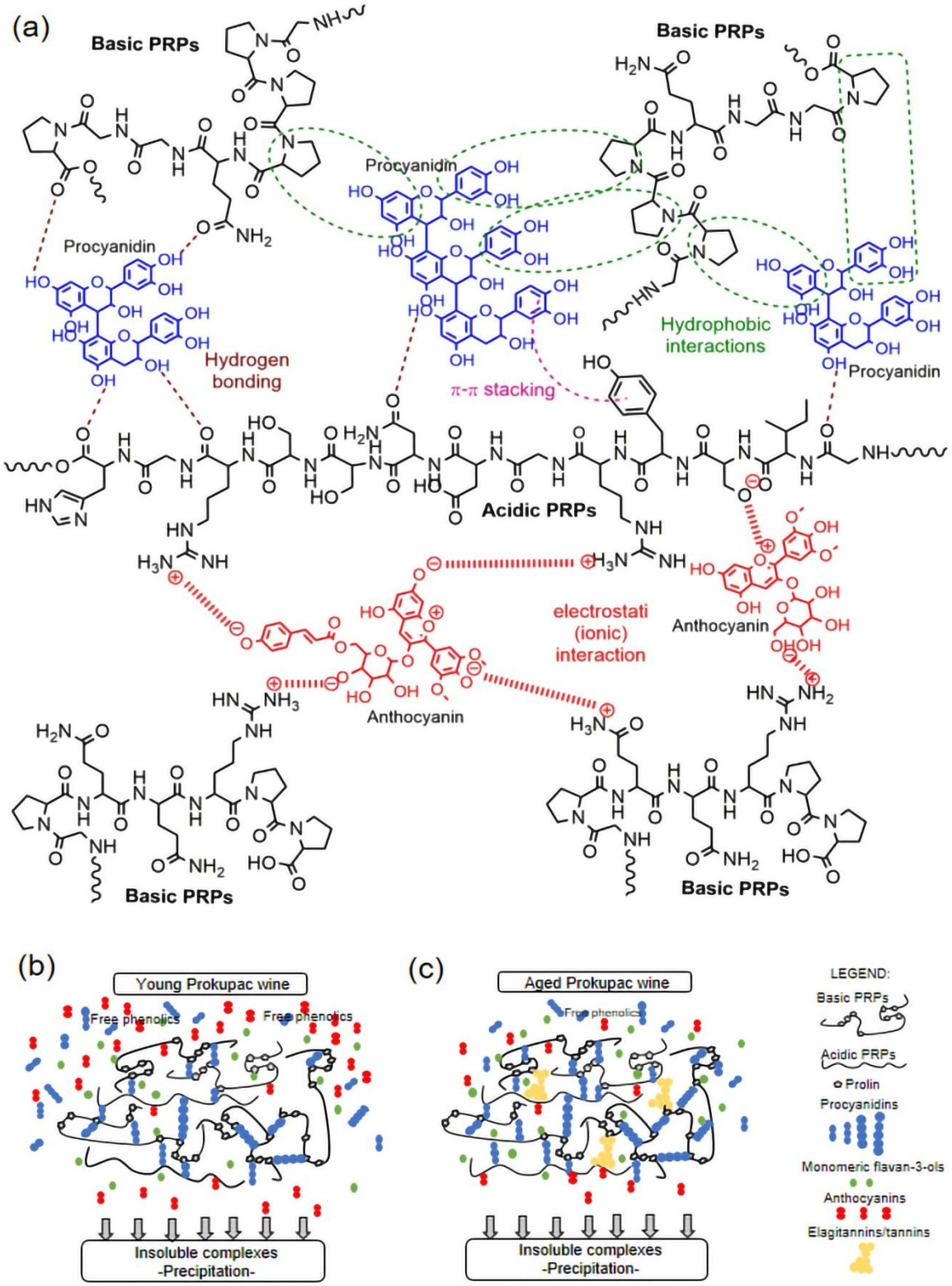

Figure 4.8. A schematic representation of: (a) Interactions between proline rich proteins and procyanidins/anthocyanins; (b) formation of insoluble complexes between PRPs and phenolic compounds from young wine; (c) formation of insoluble complexes between PRPs and phenolic compounds from aged wine.

4.7. Sensory analysis

The results of the sensory analysis are summarized in Figure 4.9, which visually highlights the perceptual differences between young and aged wines analyzed. This radar chart highlights that aging has a pronounced impact on bitterness and tannin quality while varietal character strongly dictates acidity and

astringency levels. Prokupac shows distinctively higher acidity across both age groups, whereas Merlot consistently expresses softer acidity and bitterness. Aging processes (barrel and bottle maturation) tend to enhance the perceived quality of tannins, especially in Prokupac and Cabernet Sauvignon, while simultaneously increasing bitterness. The increased perception of tannin quality in aged wines could be attributed to the polymerization and structural changes of tannins over time, which alter their interactions with salivary proteins. These findings were consistent with the results of the electrophoretic and targeted UHPLC QTOF-MS analyses, which showed more intensive interactions between aged wine phenolics and salivary proteins, especially procyanidins and other polymeric molecules. In addition, soluble complexes of anthocyanin glucosides and acyl derivatives were recognized as carriers of bitterness (Paissoni *et al.*, 2018; Soares *et al.*, 2013), but the sensory analysis showed that there were no differences in bitterness between young and aged wines. By contrast, the proportion of bound anthocyanins in aged wines was obviously high and was caused by their precipitation with insoluble tannin-procyanidin-ellagitannin/salivary protein complexes. These results contribute to a deeper understanding of the development of red wines and its impact on consumer perception.

In addition, the perception of each sensory parameter for young and aged wines are thoroughly presented in the Supplementary Figures S1-4. These sensory trends were further supported by statistical analyses (Tables S7-S9). Sensory analysis revealed that both varietal differences and wine age significantly influenced panel perception, with additional variability occasionally introduced by individual panelists. Among young wines (Table S7), one-way ANOVA showed that varietal differences had strong impact on acidity, bitterness and tannin quality, while astringency was also significantly affected, though to a lesser extent. Panelists were generally consistent in their scoring, except for bitterness, where significant variability was observed. A complementary view of the same dataset confirmed that panelist disagreement was most pronounced for acidity and astringency, whereas varietal influence on these parameters was less dominant in that context.

For aged wines (Table S8), wine age emerged as a key factor significantly altering the perception of acidity, bitterness, and tannin quality, while bitterness showed the strongest age-related differentiation. In, contrast astringency perception was not significantly affected by wine age but varied among panelists, indicating that this attribute was judged subjectively, rather than driven by compositional changes. Two-way ANOVA (Table S9) further emphasized that the combined influence of wine age and panelists shaped sensory evaluation, confirming that while maturation processes modulate bitterness and tannin quality, panelist-related variation plays an important role in the assessment of acidity and astringency.

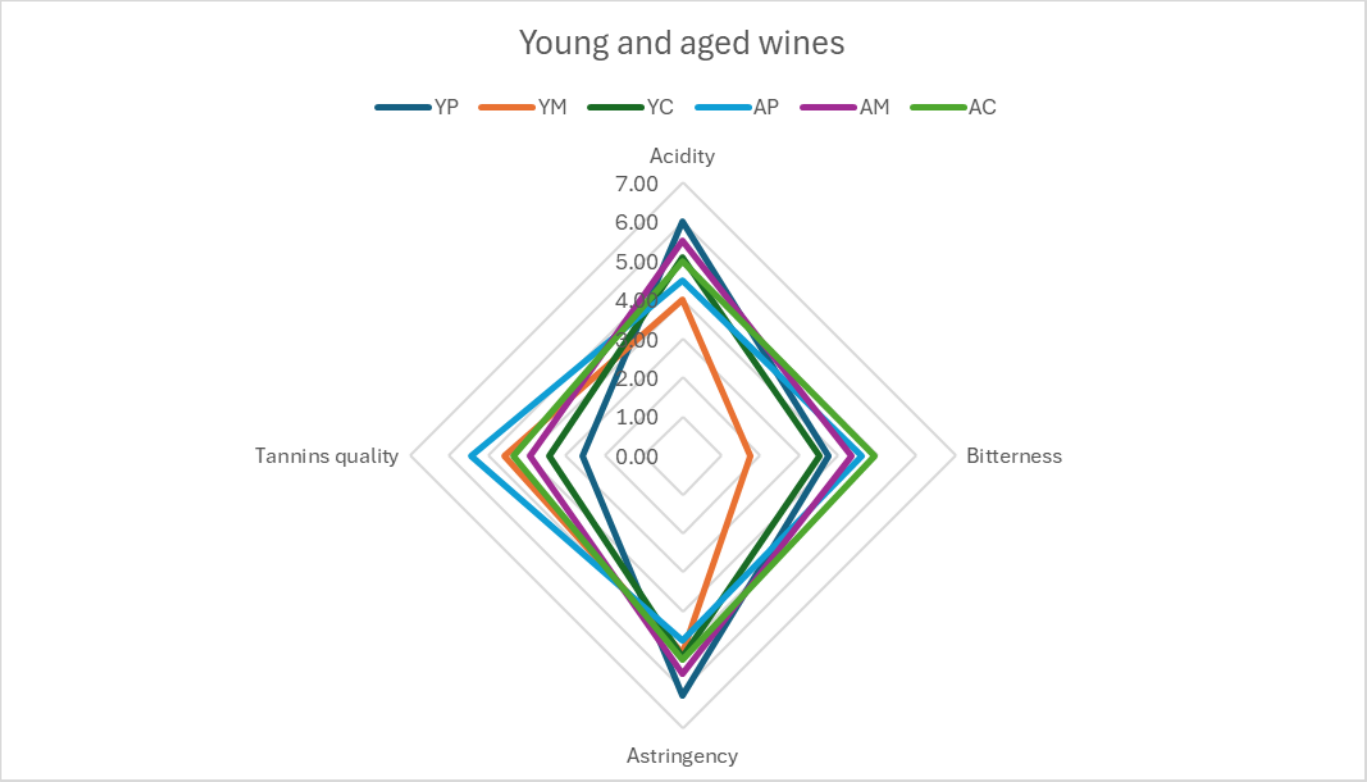

Figure 4.9. Sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines ;
Abbreviations : YP – young 'Prokupac' ; YM – young 'Merlot'; YC – young 'Cabernet Sauvignon'; AP – aged 'Prokupac'; AM – aged 'Merlot'; AC – aged 'Cabernet Sauvignon.'

To complement the statistical findings, a descriptive sensory evaluation based on Boxbaum's model was conducted, further illustrating the sensory distinctions between the wines, in regards to varieties (Table S10). Young 'Prokupac' wine exhibited a closed red color and slight opalescence due to the presence of colloids, with moderate fluidity in the glass. The aroma showed signs of slight degradation of aromatic compounds along with notes of overripe berry notes (bereton). The overall impression was clean, varietal, and typical, with moderate intensity. On the palate, the wine was moderately full-bodied, lacking balance in the finish due to the cumulative effect of acidity and tannins with moderate persistence. Aged 'Prokupac' wine displayed a closed ruby color with clarity. The aroma was clean, of moderate intensity, evoking ripe red fruit. On the palate, the wine showed a well-balanced interplay of alcoholic sweetness, tannins, and acidity, with a moderately persistence aroma.

Deep red color with bluish, navy hues, moderate mobility in the glass, slightly higher alcohol fullness at 14.7% vol, aroma typical of the variety, moderately intense, with a note of overripe grapes. On the palate, it shows a nice balance of alcoholic sweetness, tannins, and acids. After several years of barrel and bottle aging, the deep red with bluish and navy hues had evolved into a garnet to brick-red tone, reflecting anthocyanin polymerization and gradual pigment oxidation. The moderate mobility in the glass became slightly more pronounced due to the natural softening of tannins and a slight reduction in viscosity. The aromatic profile had shifted from the fresh, varietal note of overripe grapes toward a more complex bouquet, integrating dried fruit (fig, prune), chocolate, tobacco leaf, and subtle balsamic tones, because of slow oxidative reactions and ester formation. On the palate, the initial balance of alcoholic sweetness, tannins, and acids was preserved, but the tannins had become rounder and velvetier, while the acidity had integrated more fully, giving a smoother mouthfeel and a long, harmonious finish.

Deep red color with purple tones, moderate mobility in the glass, alcohol around 14.5% vol, clean varietal aroma of moderate intensity; on the palate, moderately full-bodied, with acids standing out from the extract and acting cumulatively with the tannins. With maturation, the intense purple red of young wines had transitioned to deep garnet with brick-red reflections along the rim. The initially prominent acids and firm tannins had gradually integrated due to polymerization processes, creating a more supple and layered structure. Aromas had evolved from pure varietal notes toward a complex bouquet of blackcurrant

preserves, cedar, leather, graphite, and delicate spice, underpinned by tertiary nuances such as forest floor and cigar box. On the palate, the wine's moderate fullness had gained depth and breadth, with the cumulative effect of softened tannins and mellowed acidity producing a rounder, more elegant texture, while maintaining Cabernet Sauvignon's hallmark persistence and structure.

5. Conclusion

In this study thorough research of grape and red wine anthocyanins and flavan-3-ols (procyanidins) interactions with salivary proteins have been conducted. The methodology applied included: (I) targeted UHPLC Q-ToF-MS identification, characterization and semi-quantification of phenolic compounds of skin and seed extracts of indigenous Serbian grape varieties 'Kadarka' and 'Prokupac'; (II) untargeted UHPLC Q-ToF-MS identification, characterization and semi-quantification of phenolic compounds of young and aged wines of 'Prokupac', 'Cabernet Sauvignon' and 'Merlot' varieties; (III) monitoring of binding affinity of grape and young and aged wine phenolics with salivary proteins; (IV) LC/MS characterization of aged red wine phenolics; (V) SDS-R-PAGE analysis of monitoring interaction between grape skin and seed and young and aged wine phenolics with salivary proteins; (VI) sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines.

Main objectives were to further investigate the potential of influence of anthocyanins on the red wine astringency perception, through their binding affinity and mechanism of reactions with salivary proteins (especially PRPs).

Different results were obtained, depending on the materials used in the investigation. Previous statements of grape skin and wine anthocyanins reacting with salivary proteins have been proven and confirmed. However, although there are interactions between anthocyanins and salivary proteins, their biding affinity might be incremental concerning the astringency development and perception. Anthocyanins build soluble aggregates with salivary proteins, showing little to no potential and influence over astringency phenomenon. Furthermore, the binding affinity of anthocyanins depends on their relative content and composition. Regarding the flavan-3-ols and procyanidins, their binding affinity with salivary proteins has been proven to be much higher than in anthocyanins, especially when it comes to real, live systems such as young and aged wines. The complexes that flavan-3-ols and procyanidins build with salivary proteins are insoluble, due to the polymerization of flavan-3-ols and higher molecular masses and big surface of electrical charge. Since anthocyanins are going through the reactions of co-pigmentation with flavan-3-ols, it might be that they indirectly affect the binding of salivary proteins to flavan-3-ols, to a negligible extent, but this needs further investigation.

This research has made a scientific contribution through structured methodology, using both controlled and real systems as materials, building new research paths and scientific body for red wine astringency investigations.

Although it gave answers to some of the questions posed, there are some limitations in regards to further research, such as complexity of anthocyanin-salivary proteins interactions, methodological limitations, the influence of wine matrix, sensory perception and subqualities, development of advanced analytical techniques, integration of in vivo and in vitro studies, impact of anthocyanin derivatives, standardization of sensory evaluation and role of co-factors in the wine matrix.

Given the inherent limitations and complexity of the research, future investigations into the influence of anthocyanins and other phenolic compounds on astringency development and perception may proceed along several distinct directions:

1) effects should be directed toward elucidating the molecular mechanisms underlying anthocyanin-protein interactions, including the role of biochemical transformations such as polymerization, acylation, and glycosylation in binding affinity.
2) expanding the integration of advanced analytical platforms such as multi-omics approaches, molecular docking and *in situ* spectroscopy will be essential to capture the dynamic nature of these interactions withing the complex wine environment.
3) sensory studies should aim for greater refinement, with standardized protocols and larger, diverse panels that allow the disentangling of perceptual subqualities of astringency, bitterness, and related tactile sensations.
4) A stronger link between *in vitro* and *in vivo* investigation is required, bridging biochemical reactivity with human oral physiology to achieve more physiologically relevant interpretations.

5) Future research should consider the role of the entire phenolic spectrum, flavan-3-ol, procyanidins, ellagitannins, and anthocyanin-derived pigments in shaping wine astringency, while also accounting for the modulatory impact of non-phenolic co-factors, such as polysacharides and proteins present in the wine matrix.

In conclusion, the present findings contribute to the growing understanding of how anthocyanins and flavan-3-ols participate in the perception of red wine astringency but also highlights that this remains a multifaceted phenomenon requiring multidisciplinary approaches. Future work that integrates chemistry, sensory science, and physiology will be essential for building a more comprehensive model of astringency development, ultimately advancing both fundamental knowledge and practical applications in winemaking and wine quality optimization.

6. References

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7. Supplementary

7.1. Tables

Table S1. Detailed characteristics of the Serbian red wine samples, including the varietal composition, vintage, appellation and the geographical zone of both countries

Sample Names	Varietal Composition	Vintage	Appellation	Zone
1PK	Prokupac	2017	Tri Morave	West
2PK	Prokupac	2018	Tri Morave	West
3PK	Prokupac	2019	Tri Morave	West
4PK	Prokupac	2017	Tri Morave	West
5PK	Prokupac	2015	Beograd	Central
6PK	Prokupac	2016	Beograd	Central
7PK	Prokupac	2018	Beograd	Central
8PK	Prokupac	2016	Toplica	South
9PK	Prokupac	2017	Toplica	South
10PK	Prokupac	2018	Toplica	South
11PK	Prokupac	2017	Mlava	East
12PK	Prokupac	2018	Mlava	East
13PK	Prokupac	2019	Mlava	East
14PK	Prokupac	2016	Šumadija	Central
15PK	Prokupac	2017	Šumadija	Central
16PK	Prokupac	2018	Šumadija	Central
1TM	Black Tamjanika	2016	Negotinska krajina	East
2TM	Black Tamjanika	2018	Negotinska krajina	East
3TM	Black Tamjanika	2015	Negotinska krajina	East
4TM	Black Tamjanika	2017	Negotinska krajina	East
5TM	Black Tamjanika	2019	Negotinska krajina	East
1ME	Merlot	2017	Šumadija	Central
2ME	Merlot	2018	Srem	Central
3ME	Merlot	2019	Srem	Central
4ME	Merlot	2020	Srem	Central
5ME	Merlot	2016	Šumadija	Central
6ME	Merlot	2017	Šumadija	Central
7ME	Merlot	2019	Šumadija	Central
8ME	Merlot	2019	Beograd	Central
9ME	Merlot	2020	Beograd	Central
10ME	Merlot	2013	Beograd	Central

11ME	Merlot	2015	Beograd	Central
12ME	Merlot	2017	Beograd	Central
1CS	Cabernet Sauvignon	2015	Table wine	East
2CS	Cabernet Sauvignon	2016	Table wine	East
3CS	Cabernet Sauvignon	2017	Table wine	East
4CS	Cabernet Sauvignon	2016	Šumadija	Central
5CS	Cabernet Sauvignon	2017	Šumadija	Central
6CS	Cabernet Sauvignon	2018	Šumadija	Central
7CS	Cabernet Sauvignon	2019	Beograd	Central
8CS	Cabernet Sauvignon	2020	Beograd	Central
9CS	Cabernet Sauvignon	2017	Negotinska krajina	East
10CS	Cabernet Sauvignon	2018	Negotinska krajina	East
1CU	Cabernet Sauvignon, Merlot	2016	Toplica	South
2CU	Cabernet Sauvignon, Merlot	2018	Toplica	South
3CU	Cabernet Franc, Cabernet Sauvignon	2017	Šumadija	Central
4CU	Cabernet Sauvignon, Merlot	2019	Šumadija	Central
5CU	Cabernet Sauvignon, Merlot	2015	Tri Morave	West
6CU	Cabernet Sauvignon, Merlot	2017	Tri Morave	West
7CU	Cabernet Sauvignon, Merlot	2019	Šumadija	Central
8CU	Cabernet Sauvignon, Merlot	2020	Šumadija	Central
9CU	Cabernet Sauvignon, Prokupac	2019	Šumadija	Central
10CU	Cabernet Sauvignon, Cabernet Franc, Merlot	2017	Šumadija	Central
1KA	Kadarka	2017	Palić	North
CS	Cabernet Sauvignon	2024	Toplica	South
ME	Merlot	2024	Toplica	South
PK	Prokupac	2024	Toplica	South

Table S2. Identification, characterisation and relative content (%) of main anthocyanins in control skin extract. Target compounds, mean expected retention times (RT), molecular formula, calculated mass, *m/z* exact mass, mean mass accuracy (mDa), base peak and MS fragments are presented.

RT	Compound name	Formula	Calculated mass	*m/z* exact mass	mDa	MS fragments (% of base peaks)	Samples		
							KArc	PArc	CMArc
							*Relative content (%)		
Malvidin derivatives									
6.94	**Malvidin 3-*O*-hexoside**	$C_{23}H_{25}O_{12}^+$	493.13405	493.13458	-0.53	315.05055(2), **331.08219(100)**, 332.08572(26)	31.59	36.44	21.95
7.88	**Malvidin 3-*O*-(6''-acetyl)hexoside**	$C_{25}H_{27}O_{13}^+$	535.14462	535.14648	-1.87	315.05176(2), **331.08315(100)**, 332.08636(28)	17.35	16.64	16.40
8.55	**Malvidin 3-*O*-(6''-*p*-coumaroyl)hexoside**	$C_{32}H_{31}O_{14}^+$	639.17083	639.17502	-4.19	**331.08357(100)**, 332.08681(29)	29.65	13.90	10.45
							78.58	**66.98**	**48.80**
Peonidin derivatives									

6.94	**Peonidin 3-*O*-glucoside**	$C_{22}H_{23}O_{11}^+$	463.12400	463.12671	-2.71	286.04868(6), **301.07228(100)**, 302.07514(27)	2.96	13.65	12.29
7.82	**Peonidin 3-*O*-(6"-acetyl)hexoside**	$C_{24}H_{25}O_{12}^+$	505.13460	505.13741	-2.81	286.04843(4), **301.07188(100)**, 302.07481(24)	1.08	5.07	6.14
8.48	**Peonidin 3-*O*-(6''-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{13}^+$	609.16027	609.16038	-0.11	286.04937(2), **301.07315(100)**, 302.07632(25)	3.60	6.15	4.86
							7.64	**24.88**	**23.29**
Delphinidin derivatives									
6.33	**Delphinidin 3-*O*-glucoside**	$C_{21}H_{21}O_{12}^+$	465.10330	465.10670	-3.40	**303.04924(100)**, 304.05233(23), 305.05479(4)	1.11	0.97	8.68
7.14	**Delphinidin 3-*O*-(6"-acetyl)hexoside**	$C_{23}H_{23}O_{13}^+$	507.11390	507.11487	-0.97	**303.05128(100)**, 304.05517(20), 305.05653(3)	0.30	0.25	1.97
7.82	**Delphinidin 3-*O*-(6''-*p*-coumaroyl)hexoside**	$C_{30}H_{27}O_{14}^+$	611.14019	611.14021	-0.02	**303.04854(100)**	0.54	0.31	0.78
							1.94	**1.47**	**11.43**
Petunidin derivatives									
6.70	**Petunidin 3-*O*-hexoside**	$C_{22}H_{23}O_{12}^+$	479.11900	479.12255	-3.55	302.04075(4), **317.06423(100)**, 318.06838(20), 319.07(4)	3.98	3.50	8.54
7.55	**Petunidin 3-*O*-(6"-acetyl)hexoside**	$C_{24}H_{25}O_{13}^+$	521.12950	521.13127	-1.77	302.04379(3), **317.06724(100)**, 318.07077(25)	1.65	1.32	4.69
8.29	**Petunidin 3-*O*-(6''-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{14}^+$	625.15518	625.15898	-3.80	302.04841(2), **317.06820(100)**, 318.07184(25)	6.20	1.78	3.25
							11.84	**6.60**	**16.47**
							100	**100**	**100**
			**				**21.69**	**25.81**	**100**

*Relative content of individual anthocyanins were determined in control skin extracts. **Comparison of total identified anthocyanins among control skin extracts. **Abbreviations:** **KArc**-Relative content of individual anthocyanins in Kadarka skin control extract; **PArc**-Relative content of individual anthocyanins in Prokupac skin control extract; **CMArc**-Relative content of individual anthocyanins in Cabernet Sauvignon/Merlo control extract/fraction.

Table S3. Identification, characterisation and relative content (%) of flavan-3-ols/procyanidins in control seed extracts. Target compounds, mean expected retention times (RT), molecular formula, calculated mass, *m/z* exact mass, mean mass accuracy (mDa), base peak and MS fragments are presented.

RT	Compound name	Formula	Calculated mass	*m/z* exact mass	mDa	MS fragments (% of base peaks)	Samples	
							KSrc	PSrc
							*Relative content (%)	
					Flavan-3-ols and derivatives			
6.26	**Catechin**	$C_{15}H_{13}O_6$—	289.07120	289.07340	-2.20	109.03032(98), 121.03006(29), **123.0458(100)**, 125.02503(43), 137.02512(29), 151.04058(32), 161.05995(14), 187.04083(11), 203.0721(21), 221.0827(14)	27.59	24.64
7.01	**Epicatechin**	$C_{15}H_{13}O_6$—	289.07120	289.07336	-2.16	109.0307(98), 121.03057(29), **123.04638(100)**, 125.02579(45), 137.02572(29), 151.04117(33), 161.06064(13), 187.04131(11),	11.48	14.53
7.94	**Epicatechin gallate**	$C_{22}H_{17}O_{10}$—	441.08220	441.08502	-2.82	124.01754(10), 125.02541(44), **169.01477(100)**, 203.07238(7), 205.05137(5), 245.08318(15), **289.07335(21)**, 290.07629(4)	9.30	9.23
5.93	**Epicatechin-3-*O*-hexoside**	$C_{21}H_{23}O_{11}$—	451.12400	451.12446	-0.46	109.03025(17), 125.02528(20), 137.02621(16), 151.04144(13), 179.03522(13), 203.07211(21), 245.08363(65), **289.07342(100)**, 290.07762(20)	0.20	0.36
							48.56	**48.76**
					Procyanidins and derivatives			
6.06	**Procyanidin dimer B type is. I**	$C_{30}H_{25}O_{12}$—	577.13460	577.13750	-2.90	125.02539(27), 137.02513(8), 205.05145(8), 245.08309(29), **289.07287(100)**, 407.07950(25)	14.48	13.39
6.80	**Procyanidin dimer B type is. II**	$C_{30}H_{25}O_{12}$—	577.13460	577.13757	-2.97	125.02566(75), 137.02552(12), 205.04934(7), 245.08179(24), **289.07379(100)**, 407.08029(85)	9.22	13.07
7.41	**Procyanidin dimer B type gallate is. I**	$C_{37}H_{29}O_{16}$—	729.14560	729.15031	-4.71	125.02468(36), 169.01437(17), 287.0561(10), **289.07211(70)**, 290.07535(12), **407.07826(100)**, 408.08131(27), **441.08332(10)**, 451.10391(19)	11.04	10.24
8.49	**Procyanidin dimer B type gallate is. II**	$C_{37}H_{29}O_{16}$—	729.14560	729.14945	-3.85	125.02563(29), 169.01464(16), 287.05658(8), **289.07305(61)**, **407.07926(100)**, 408.08265(27), **441.08476(8)**, 451.10601(17)	2.84	2.32
3.03	**Procyanidin trimer B type is. III**	$C_{45}H_{37}O_{18}$—	865.19854	865.19894	-0.40	125.02585(71), **287.05762(100)**, 289.07289(67), 407.07805(61), 413.08896(31), 425.08901(48), 451.10494(23), 575.12191(49), **577.13581(48)**, 695.14338(45)	0.63	—
6.60	**Procyanidin trimer B type is. I**	$C_{45}H_{37}O_{18}$—	865.19800	865.20300	-5.00	125.02517(81), 245.04822(23), **287.05689(100)**, 289.07273(71), 407.07837(72), 425.08899(50), 451.10501(33), **577.13679(61)**, 695.14413(38), 713.15485(23)	5.46	4.73
7.21	**Procyanidin trimer B type is. II**	$C_{45}H_{37}O_{18}$—	865.19800	865.20243	-4.43	125.02499(92), **287.05689(100)**, 289.07238(75), 407.07904(78), 425.08842(54), 451.10468(37), **577.13687(61)**, 695.14428(41), 713.15334(27), 865.20045(34)	4.94	4.66
8.02	**Procyanidin trimer B type gallate is. I**	$C_{52}H_{41}O_{22}$—	1017.20890	1017.21522	-6.32	125.02538(51), 287.05706(50), **289.07211(43)**, 407.07822(58), 575.12011(39), 577.12837(31), 695.13841(39), **729.14861(100)**, 865.18153(30), 1017.21149(96)	1.33	0.93
8.29	**Procyanidin trimer B type gallate is. II**	$C_{52}H_{41}O_{22}$—	1017.20890	1017.21414	-5.24	125.0247(24), 287.05669(40), **289.07221(27)**, 407.0815(38), 451.10545(21), 575.12633(27), 695.13195(22), **729.15073(67)**, 847.15966(37), **1017.21085(100)**	1.49	1.90
							51.44	**51.24**
							100	**100**
					**		**74.77**	**100**

*Relative content of individual grape seed phenolics were determined in control seed extracts. **Comparison of total identified grape seed phenolics among analysed extracts. **Abbreviations:** **KSrc**-Relative content of individual phenolics in Kadarka seed control extract; **PSrc**-Relative content of individual phenolics in Prokupac seed control extract. „—„ nonidentified compound.

Table S4. Equation parameters of standards used for quantification of wine phenolics (Table X) and semi-quantification od skin and seed phenolics (Table X).

Compounds	y=ax±b	R2	LOD (µg/mL)	LOQ (µg/mL)
Coumaric acid	y = 25243511.3354x - 866412.4214	0.9961	0.38	1.25
Vanillic acid	y = 913191.4989x + 31480.0391	0.9985	0.27	0.89
Gallic acid	y = 13648904.1369x - 768789.9173	0.9972	0.26	0.88
Caffeic acid	y = 59357328.4218x + 6466375.9189	0.9959	0.27	0.91
Ferulic acid	y = 6680790.5687x + 216092.6620	0.9977	0.18	0.61
Epicatechin	y = 17883934.8558x + 1151944.3976	0.9962	0.16	0.54
Catechin	y = 14379602.5826x + 578077.8336	0.996	0.31	1.04
Kaempferol	y = 54512598.2797x + 4271269.9233	0.9864	0.17	0.57
Myricetin	y = 24260634.2632x + 722991.6082	0.9904	0.43	1.44
Naringenin	y = 30822249.0218x + 7852515.7889	0.99	0.32	1.07
Resveratrol	y = 7123109.0475x + 1067695.8248	0.9981	0.31	1.03
Ellagic acid	y = 13775863.4526x + 578964.5772	0.9966	0.16	0.52
Procyanidin B2	y = 12351809.4676x - 258435.3175	0.9988	0.14	0.45
Malvidin-3-*O*-glucoside	y = 11838421.7623x + 1430652.8398	0.9963	0.3	1.01
Procyanidin C1	y = 3688585.1231x + 364447.9105	0.99	0.59	1.97
Epicatechin gallate	y = 17579661.3297x - 1711418.9997	0.9927	0.23	0.76
Luteolin	y = 40348411.9748x + 7228244.4086	0.9886	0.16	0.52

Table S5. Untargeted UHPLC Q-ToF MS phenolic profile of young and aging Prokupac red wine, and ratio of each compound identified in young and aged wine.

No.	RT	Compounds	Formulas	Calculated mass	*m/z* exact mass	mDa	MS fragments (main fragment)	Ratio YP/AP
				Phenolic acid and derivatives				
1	2.80	**Coumaric acid**	$C_9H_7O_3^-$	163.0395	163.0401	0.58	**119.0497(100)**	**–**
2	7.38	**Vanillic acid**	$C_8H_7O_4^-$	167.0344	167.0356	1.17	**123.0439(100)**, 107.0133	**–**
3	1.00	**Gallic acid**	$C_7H_5O_5^-$	169.0137	169.0148	1.10	**125.0239(100)**, 124.0163	**1.2**
4	4.37	**Caffeic acid**	$C_9H_7O_4^-$	179.0344	179.0356	1.17	**135.0445(100)**, 134.0371, 107.0499	**1.4**
5	3.92	**Ferulic acid**	$C_{10}H_9O_4^-$	193.0501	193.0503	0.22	**134.0365(100)**, 133.0283, 117.0342, 148.0133, 164.0119	**–**
6	6.59	**Ethyl gallate**	$C_9H_9O_5^-$	197.045	197.0465	1.50	**124.0162(100)**, 125.0227, **169.0144**	**1.1**
7	9.42	**Ethyl caffeic acid**	$C_{11}H_{11}O_4^-$	207.0657	207.0670	1.27	**133.0292(100)**, 135.0446, 134.036, 161.0244, **179.0343**	**0.25**
8	3.20	**Coutaric acid**	$C_{13}H_{11}O_8^-$	295.0454	295.0470	1.61	**119.0501(100)**, **163.0400**	**2.2**
9	7.52	**Ellagic acid**	$C_{14}H_5O_8^-$	300.9984	301.0001	1.66	**300.9992(100)**, 299.9913, 283.9966, 229.016, 201.0202, 151.0033, 245.0144, 185.0251, 173.0229, 257.0103	**0.51**
10	1.54	**Caftaric acid**	$C_{13}H_{11}O_9^-$	311.0403	311.0421	1.79	**135.0447(100)**, 149.0089, **179.0352**, 134.0372	**0.27**
11	4.17	**Fertaric acid**	$C_{14}H_{13}O_9^-$	325.056	325.0600	4.04	**134.0368(100)**, **193.0506**, 178.027, 149.0089	**1.4**
12	7.81	**Aesculin**	$C_{15}H_{15}O_9^-$	339.0716	339.0734	1.79	**161.0241(100)**, 159.0295, 133.0285, **177.0398**, 115.0392	**–**
13	3.84	**Caffeoylquinic acid (like Chlorogenic acid)**	$C_{16}H_{17}O_9^-$	353.0873	353.0887	1.44	**191.0559(100)**, 161.0239, 127.0395, 173.0451, **135.0449**	**–**
				Flavan-3-ols and procyanidins				

14	3.42	**Catechin**	$C_{15}H_{13}O_6^-$	289.0712	289.0727	1.49	**123.045(100)**, 109.0294, 125.0244, 151.0398, 137.0244, 203.0712, 149.025, 221.0821, 187.0402, 245.0813	**1.4**
15	6.13	**Epicatechin**	$C_{15}H_{13}O_6^-$	289.0712	289.0727	1.49	**123.045(100)**, 109.0294, 125.0244, 151.0399, 137.0243, 203.0713, 149.0253, 221.0819, 187.0403, 245.0820	**2.5**
16	2.48	**Procyanidin B type dimer is. I**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0724(100)**, **407.0780**, 125.0243, 245.0805, 161.0248, 137.0242, 273.0408, **425.0884**, **451.1036**, 255.0339, 229.0511	**2.9**
17	4.11	**Procyanidin B type dimer is. II**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0718(100)**, **407.0776**, 125.0241, 245.0798, 161.0249, 137.0239, 273.0404, **425.0885**, **451.1047**, 255.0377, 229.0512, 205.0485	–
18	5.38	**Procyanidin B type dimer is. III**	$C_{30}H_{25}O_{12}^-$	577.1346	577.1365	1.90	**289.0722(100)**, **407.0778**, 125.0242, 245.0803, 161.0250, 137.0242, 273.0407, **425.0882**, **451.1031**, 229.0512, 205.0476, 109.0291	**2.0**
19	3.41	**Chalcan-flavan 3-ol dimer is. I (like Gambiriin A1)**	$C_{30}H_{27}O_{12}^-$	579.1503	579.1522	1.95	**289.0720(100)**, 245.0824, 271.0607, 179.0352, 205.0510, 165.0187, 151.0400, 137.0245, 125.0242, 109.0293	–
20	6.07	**Chalcan-flavan 3-ol dimer is. II**	$C_{30}H_{27}O_{12}^-$	579.1503	579.1522	1.95	**289.0719(100)**, 245.0824, 271.060719, 179.0352, 205.0510, 165.0188, 151.0397, 137.0241, 125.0241, 109.0293, 221.0825	–
21	6.84	**Procyanidin dimer B type gallate**	$C_{37}H_{29}O_{16}^-$	729.1456	729.1481	2.54	**407.0772(100)**, **289.0716**, 125.0239, 451.1023, **169.0141**, 577.1319, 271.0612, 287.0567, 441.0825, 161.0246, 245.0591, 203.0206	–
				Flavonols and glycosides				
22	10.1	**Kaempferol**	$C_{15}H_9O_6^-$	285.0399	285.0411	1.19	**285.0405(100)**, 185.0609, 229.0515, 239.035, 159.0447, 211.0396, 143.0497, 151.0038, 227.0347, 255.0301, 268.0370	–
23	9.30	**Quercetin**	$C_{15}H_9O_7^-$	301.0348	301.0368	1.97	**151.0036(100)**, 121.0292, **178.9984**, 149.0237, 301.0334, 245.0456, 273.0400, 229.0500, 201.0549	**3.5**
24	10.3	**Isorhamnetin**	$C_{16}H_{11}O_7^-$	315.0505	315.0516	1.12	**300.0276(100)**, 151.0033, 301.031, 107.0133, 271.0251, 283.0259, 255.0293, 227.0344, 243.0301, 179.0001	**8.6**
25	8.41	**Myricetin**	$C_{15}H_9O_8^-$	317.0297	317.0315	1.76	**151.0036(100)**, 137.0241, 107.0137, 178.9987, 165.0191, 227.0349, 243.0311, 271.0247, 317.0306	**1.3**
26	9.27	**Laricitrin**	$C_{16}H_{11}O_8^-$	331.0454	331.0473	1.91	**151.0062(100)**, 316.0231, 178.9995, 271.0243, **317.0257**, 287.0179, 259.0252, 243.0300, 107.0135	–
27	7.72	**Syringetin**	$C_{17}H_{13}O_8^-$	345.061	345.0634	2.36	**190.9994(100)**, **315.0144**, 163.0028, 287.0211, **330.0383**, 316.019, 271.0243, 259.0244, 243.0282, 345.0607	–
28	7.60	**Quercetin 3-*O*-hexuronide**	$C_{21}H_{17}O_{13}^-$	477.0669	477.0687	1.78	**301.0358(100)**, 151.0034, 178.9984, 283.0251, 273.0403, 255.0301, 245.0451	**2.1**
29	7.13	**Myricetin 3-*O*-hexoside**	$C_{21}H_{19}O_{13}^-$	479.0826	479.0847	2.13	**316.0229(100)**, 271.0245, 287.0194, 178.9982, 151.0035, 479.0832	**11.9**
30	7.05	**Myricetin 3-*O*-hexuronide**	$C_{21}H_{17}O_{14}^-$	493.0618	493.0647	2.87	**317.0304(100)**, 318.0312, 178.9971, 151.0049, 137.0232, 271.0281, 299.0174	–
31	7.65	**Laricitrin 3-*O*-hexoside**	$C_{22}H_{21}O_{13}^-$	493.0982	493.0988	0.58	**330.0382(100)**, 331.0446, **315.0150**, 316.0201, 287.02, 493.1013, 271.0245, 243.0285, 151.0055, 178.9975	–
32	8.11	**Syringetin 3-*O*-hexoside**	$C_{23}H_{23}O_{13}^-$	507.1139	507.1156	1.73	**344.0541(100)**, 345.0591, 507.1147, 273.0405, 301.0369, **316.0588**, **329.0321**, 258.0160, 151.0034	**1.7**
				Other detected non-anthocyanin flavonoids				

33	9.83	**Naringenin**	$C_{15}H_{11}O_5^-$	271.0606	271.0622	1.55	**119.0501(100)**, 151.0034, 107.0133, 177.0182, 161.0586, 145.0275, 229.0541	**1.4**
34	7.39	**Taxifolin**	$C_{15}H_{11}O_7^-$	303.0505	303.0522	1.72	**125.0249(100)**, 151.0216, 174.0312, 199.0390, 137.0211, 193.0515, 243.0271	**–**
35	5.05	**Dihydromyricetin**	$C_{15}H_{11}O_8^-$	319.0454	319.0469	1.51	**125.0242(100)**, 165.019, 151.0038, 167.0346, 137.0241, 175.0040, 193.0137, 205.0501, 233.0457	**–**
36	8.40	**Phloretin 2'-*O*-hexoside (like Phlorizin)**	$C_{21}H_{23}O_{10}^-$	435.1291	435.1316	2.48	**167.0351(100)**, **273.0778**, 125.0238, 274.0802, 179.0348, 123.0452, 168.0388	**–**
				Stilbenoids				
37	9.34	**Resveratrol**	$C_{14}H_{11}O_3^-$	227.0708	227.0721	1.28	**143.0501(100)**, 185.0593, 117.0347, 157.0655, 167.0535	**2.0**
38	8.22	**Resveratrol hexoside (like Piceid)**	$C_{20}H_{21}O_8^-$	389.1236	389.1253	1.66	**227.0711(100)**, 185.0605, 143.0499, 159.0811	**1.1**
				Anthocyanins and pyranoanthocyanins				
				Malvidin derivatives				
39	6.59	**Malvidin 3-*O*-glucoside**	$C_{23}H_{25}O_{12}^+$	493.1346	493.1375	2.9	**331.0831(100)**, 332.0854, 315.0508, 316.0578, 287.0555	**8.0**
40	7.13	**Malvidin 3-*O*-hexoside-acetaldehyde (Vitisin B)**	$C_{25}H_{25}O_{12}^+$	517.1346	517.1367	2.1	**355.0819(100)**, 356.0854, 317.0662	**3.4**
41	7.40	**Malvidin 3-*O*-(6"-acetyl)hexoside**	$C_{25}H_{27}O_{13}^+$	535.1452	535.1475	2.33	**331.0819(100)**, 332.085, 333.0878, 315.0505	**–**
42	7.45	**Malvidin-3-O-(6"- acetyl)hexoside-acetaldehyde (Malvidin-acetaldehyde adduct I)**	$C_{27}H_{27}O_{13}^+$	559.1452	559.147	1.83	**355.0822(100)**, 356.0848, 397.0921	**–**
43	7.12	**Malvidin 3-*O*-hexoside-pyruvate (Vitisin A)**	$C_{26}H_{25}O_{14}^+$	561.1244	561.1266	2.17	**399.0722(100)**, 400.0754	**1.7**
44	8.64	**Malvidin 3-*O*-hexoside-4-vinylphenol**	$C_{31}H_{29}O_{13}^+$	609.1608	609.1626	1.78	**447.1079(100)**, 448.1112, 431.0755	**–**
45	8.39	**Malvidin 3-*O*-hexoside-4-vinylcatechol (Pinotin A)**	$C_{31}H_{29}O_{14}^+$	625.1557	625.1577	1.97	**463.1026(100)**, 464.1059, 447.0745	**–**
46	8.22	**Malvidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{32}H_{31}O_{14}^+$	639.1714	639.1739	2.52	**331.0819(100)**, 332.085, 333.0876	**–**
47	8.11	**Malvidin-3-O-(6"- coumaroyl)hexoside-acetaldehyde (Malvidin-acetaldehyde adduct II)**	$C_{34}H_{31}O_{14}^+$	663.1714	663.1737	2.32	**355.0811(100)**, 356.0852, 357.087	**–**
				Other detected anthocyanins				
48	6.06	**Petunidin 3-*O*-glucoside**	$C_{22}H_{23}O_{12}^+$	479.119	479.1205	1.55	**317.0657(100)**, 318.0698, 302.0423	**–**
49	7.59	**Peonidin 3-*O*-(6"- acetyl)hexoside**	$C_{24}H_{25}O_{12}^+$	505.1346	505.1362	1.6	**301.0704(100)**, 302.0746, 286.048	**–**
50	8.30	**Peonidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{13}^+$	609.1608	609.1635	2.68	**301.0708(100)**, 302.0744, 303.076, 286.0477	**–**
51	8.06	**Petunidin 3-*O*-(6"-*p*-coumaroyl)hexoside**	$C_{31}H_{29}O_{14}^+$	625.1557	625.1581	2.37	**317.0661(100)**, 318.0689, 302.0466	**–**

* **Abbreviations:** is.-isomers; "–" compounds identified only in young or aged wine; **YP**-Young Prokupac wine; **AP**-Aging Prokupac wine; Ratio of each compound identified in young and aged wine, calculated as ratio of their areas (**Ratio YP/AP**).

Table S6. Polypetide composition (%) of saliva proteins

No. Saliva Band	LMW (kDa)	CSP (%)
4	61.9	4.02
7	46.8	4.49
8	44.5	4.06
9	35.4	5.40
10	33.2	9.25
12	29.4	13.42
14	25.3	7.28
15	19.1	7.50
17	17.4	7.67
∑ 9-17 (PRPs)		**50.52**
19	13.1	6.45
20	11.7	8.52
21	10.9	8.06
∑ 19-21 (Cystatins)		**23.02**
23	6.9	7.98
24	5.5	5.90
∑ 23-24 (Statherins)		**13.88**
-	-	**100**

Table S7. One-way ANOVA for sensory parameters "acidity", "bitterness", "astringency", and "tannin quality" (factors varieties and panelists among young wines)

Sensory parameter	*SS*	*MS*	*F*	*p*-value	*SS*	*MS*	*F*	*p*-value
	Varieties				**Panelists**			
Acidity	24.0556	12.0278	433	2.08×10^{-24}	0.3056	0.2778	0.0270	1
Bitterness	28.5	14.25	62.7	5.75×10^{-12}	42.5833	3.8712	2.3280	0.0234
Astringency	8.7222	4.3611	11.7483	0.00014	3.6389	0.3308	0.4580	0.9111
Tannin Quality	24	24	90.5143	2.97×10^{-9}	6.3056	0.5732	0.5159	0.8736

Table S8. One-way ANOVA for sensory parameters "acidity", "bitterness", "astringency", and "tannin quality" (factors varieties and panelists among aged wines)

Sensory parameter	*SS*	*MS*	*F*	*p*-value	*SS*	*MS*	*F*	*p*-value
	Varieties				**Panelists**			
Acidity	6	3	0.7857	0.4641	88	8	4.3636	0.0012
Bitterness	2.0556	1.0278	0.3069	0.7378	3.3333	0.3030	0.2226	0.9936
Astringency	4.2222	2.1111	0.4858	0.6196	91.6389	8.3308	3.5703	0.0044
Tannin Quality	14.3889	7.1944	1.8476	0.1735	68.8889	6.2626	2.0311	0.0711

Table S9. Two-way ANOVA for sensory parameters "acidity", "bitterness", "astringency" and "tannin quality" (factors wine age and panelists)

Sensory parameter	***SS***	***MS***	***F***	***p*-value**	***SS***	***MS***	***F***	***p*-value**
	Wine age				**Panelists**			
Acidity	16.0667	4.0167	2.4034	0.0369	53.3833	4.8530	2.9039	0.0584
Bitterness	77.2333	19.3083	11.6114	1.6×10^{-6}	42.5833	3.8712	2.3280	0.0234
Astringency	4.3333	1.0833	0.4921	0.7415	57.1333	5.1939	2.3593	0.0217
Tannin Quality	27	6.75	3.0244	0.0274	36.1333	3.2848	1.4718	0.1767

Table S10. Results of sensory analysis of wines by *Buxbaum* method

Sensory parameter	**Wine sample**					
	YP	**YM**	**YC**	**AP**	**AM**	**AC**
Colour	2.0	2.0	2.0	2.0	2.0	2.0
Clearness	2.0	2.0	2.0	2.0	2.0	2.0
Aroma	3.2	3.6	3.5	3.6	3.4	4.0
Taste	10.4	10.7	10.5	10.4	10.1	10.2
Total	17.6	18.3	18.0	18.0	17.5	18.2

7.2. Figures

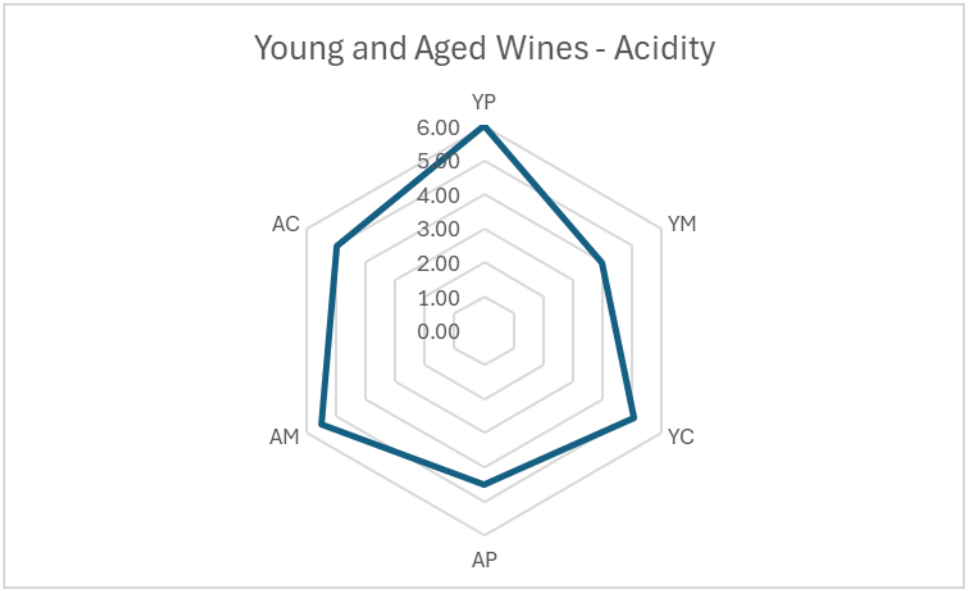

Figure S1. Sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines for acidity; Abbreviations : see Section 4.7

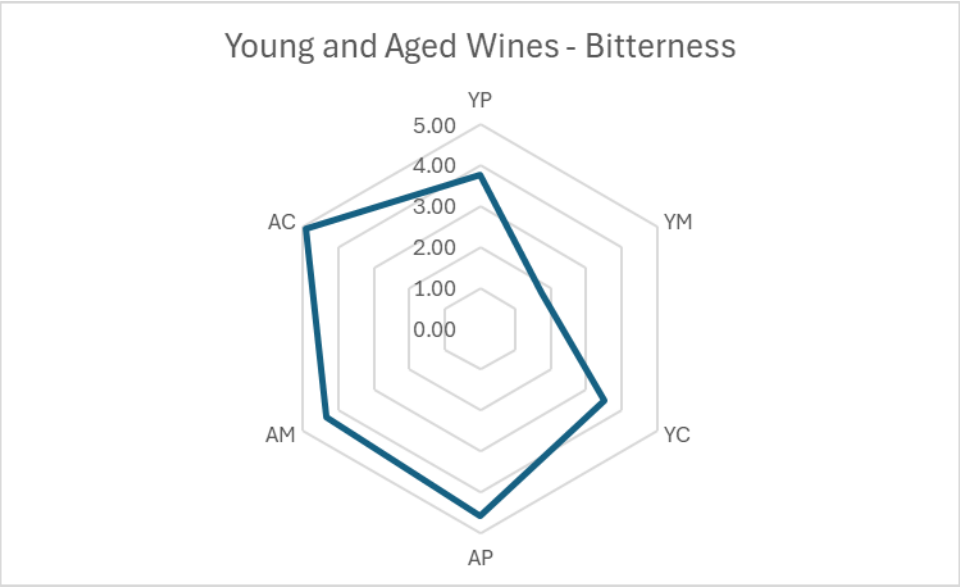

Figure S2. Sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines for bitterness
Abbreviations : see Section 4.7

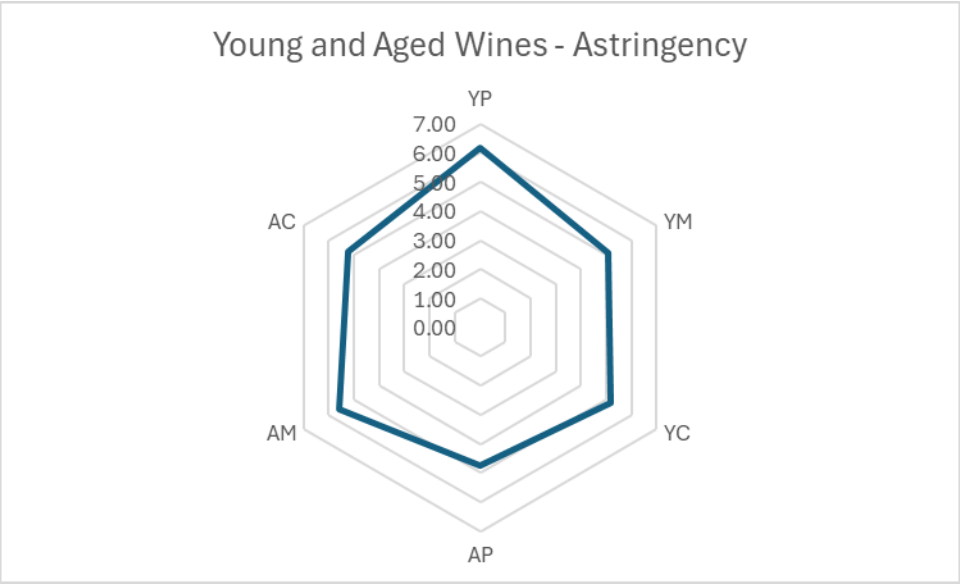

Figure S3. Sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines for astringency; Abbreviations : see Section 4.7

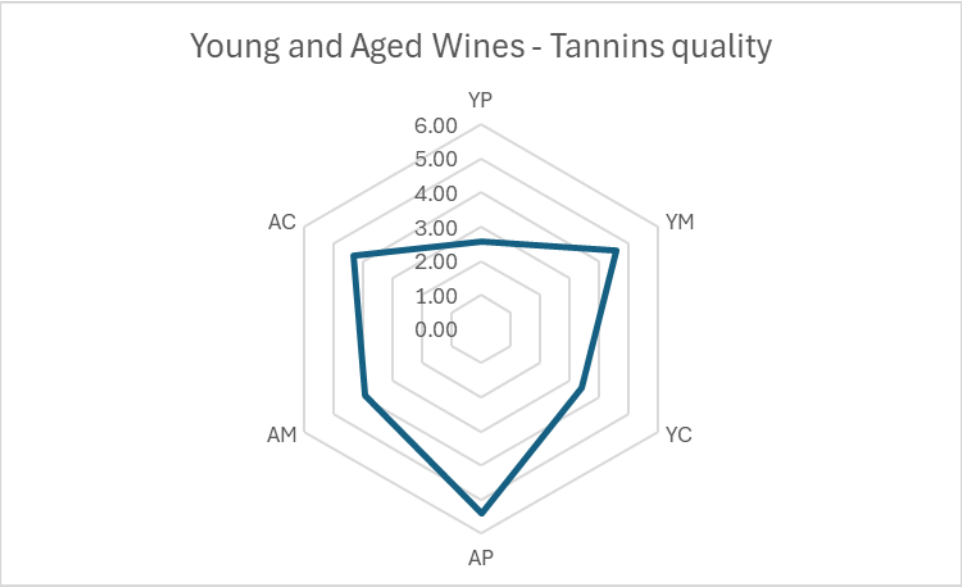

Figure S4. Sensory analysis of young and aged 'Prokupac', 'Cabernet Sauvignon', and 'Merlot' wines for tannin quality; Abbreviations : see Section 4.7

8. Author's Biography

Katarina R. Delić, borned on 20.12.1992, in Požarevac, Republic of Serbia.
She graduated from Economic-marketing and mechanical high school in Kučevo, as a technician in tourism. After graduating high school, she has inscripted to the Faculty of Agriculture, University of Belgrade, Department of Fruit Science and Viticulture, in the academic year 2011/2012.
She obtained her Bachelor of Agriculture in 2016, graduating while defending her bachelor thesis, "Characteristics of Smederevka clones in Grocka vine-growing region," on 21 March 2016, with an average grade of 9,63, acquiring the professional title of graduate agricultural engineer for fruit growing and viticulture.
During her studies, she distinguished herself as one of the top students at the Faculty of Agriculture, University of Belgrade, and represented the institution in the morning program Generation 2015 on Serbian Radio and Television. As a distinguished member of the graduating class, she was invited to deliver the ceremonial speech on Faculty Day in 2015.
After completing her Bachelor's studies, she volunteered in the Local Self-Government of the Municipality of Kučevo, Department of Agriculture. During her volunteer work, she self-initiated the education of agricultural producers in the territory of Kučevo municipality, in the field of fruit growing and viticulture. She gained experience in performing administrative and legal tasks, planning, organizing, and executing projects, financial analysis of projects, risk assessment, as well as market research. In 2017, she assumed the role of Director at the Agricultural Cooperative "Soare" in Neresnica, inspiring young people to contribute to rural development and encouraging them to remain in the countryside. Under her leadership, the Cooperative established a dedicated registry office for maintaining records on animal husbandry. Following the establishment of the registry office, she stepped down as Director of the Cooperative to further enhance her expertise in viticulture and winemaking. In July 2019, she joined the PIK Oplenac winery in Topola as a B.Sc. Eng. Viticulture and Wine Production Technologist.
In the 2019/20 academic year, she enrolled in the Master's program in Agriculture, specializing in the Fruit Growing and Viticulture module at the Faculty of Agriculture, University of Belgrade. On March 11, 2021, she successfully defended her master's thesis, titled "Economic-Technological Characteristics of Wine Varieties in the Plantations of the Holy Monastery of Koporin." In the same year, she commenced dual doctoral studies at the Faculty of Agriculture, University of Belgrade, in the Department of Food Technology, and at the University of Bordeaux, Institute for the vine and the wine. She was awarded a scholarship from the Government of France for her doctoral studies, along with the "Dositej" Fund for Young Talents scholarship for studies abroad. Her doctorate is conducted under a Co-Mentoring Doctorate Agreement established between the University of Belgrade and the University of Bordeaux.
In June 2022, she was appointed as a Research Trainee at the Faculty of Agriculture, University of Belgrade, in the Department of Chemistry and Biochemistry at the Institute for Food Technology.
She is proficient in speaking, reading, and writing in English, Russian, and French, with a working knowledge of German and Spanish.
She currently works as Programs and Project Associate at the Science fund of Republic of Serbia.

Statement of Authorship

Author's Name and Surname Katarina R. Delić

Index Number TH210001

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