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CYCLE: XXXV

INNOVATION IN OENOLOGY TO IMPROVE THE PHENOLIC COMPOSITION OF ITALIAN RED WINES

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“Meravigliarsi di tutto è il primo passo della ragione verso la scoperta”

Louis Pasteur

Alla mia famiglia,

che mi ha insegnato a guardare il mondo con curiosità e meraviglia

ABSTRACT

This research aimed at implementing the knowledge needed to increase the production of high-quality and typical red wines by exploiting and preserving the intrinsic varietal features of different Italian grapes. The PhD thesis is composed by two parts. The first focuses on the withering of red winegrapes, through the study of techniques currently used in order to introduce innovations in traditional processes. The results obtained contribute to the comprehension of the complexity of the withering process of red winegrapes. In particular, a Systematic Review on phenolic acids in dehydrated grapes was performed, giving a full view of the worthwhile recent findings on these often-overlooked compounds. The importance of the harvest time for grapes destined to withering process was highlighted, studying the combined effect of different ripeness degree and withering rates on the standard chemical composition and phenolic profile of Nebbiolo grapes destined to the production of 'Sforzato' di Valtellina DOCG reinforced wine. Moreover, the effects of different withering techniques were assessed on the Italian winegrape variety Aleatico. The results provided new insights in the phenolic profile extractability after partial dehydration, which resulted differently affected from skins and seeds and strongly influenced by the withering conditions. The second part deals with the exploration of new processing aids and the impact of additives on traceability techniques. In this part, a corn-derived biosurfactant extract was evaluated as a novel processing aid and compared with oenological tannins as a solubilizing and stabilizing agent of anthocyanins in red wine on cv. Nebbiolo and Cabernet sauvignon. The results

showed that biosurfactant addition improved the colour properties of skin extracts in simulated macerations, with a variety-dependent effectiveness. Finally, the impact of oenological processing aids and additives on the genetic traceability of Nebbiolo wines have been studied, contributing to explaining the causes of the reduced varietal identification efficiency in commercial wines.

Keywords: Winegrapes, Phenolic compounds, Withering, Extractability, Processing aids

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1. General introduction

Wine is a product with high cultural, social, economic, and territorial importance (Beckert et al., 2017; Merkyté et al., 2020). In particular, the wine sector is one of the most representative economic activities in Italy, a country very rich in terms of grape cultivars and ampelographic heritages (Bavaresco et al., 2014; Giacosa et al., 2021; Vrontis et al., 2016). Indeed, in recent years, the interest by the consumers towards authenticity and territorial identity of wines was increased (Rocchi et al., 2013). Red wines are peculiar in terms of winemaking techniques, mainly due to the presence of the maceration phase in the production process, which allow to extract phenolic compounds from the solid parts of the grapes (i.e. skins and seeds) (Morata, 2018). Indeed, grapes skins, flesh, and seeds contain several classes of phenolic compounds, which are strictly associated with red wines quality and "typicity" (Vidal et al., 2004, Harrison et al., 2018). The extraction and preservation of polyphenols in wine depends on several factors, such as genetic features of grape variety, growing area, vineyard management, climate conditions of the year and winemaking techniques (Bosso et al., 2009; Ortega-Regules et al., 2006). The new oenological trends focus on two main aspects: the valorization of grape potentialities moving towards a variety-dependent approach and the exploration of new paths to produce typical wines.

This research aimed at implementing the knowledge needed to increase the production of high-quality and typical red wines by exploiting and preserving the intrinsic varietal features of different Italian grapes.

To carry out this purpose, two main lines of research were followed: on one hand (A) traditional techniques were deeply studied to introduce innovations and allow winemakers to manage traditional processes with new awareness, while on another hand (B) processing aids and additives were assessed to improve the phenolic composition and typicity of wines.

The first research line (EXPERIMENTAL SECTION – PART A) was particularly focused on the withering of winegrapes, a complex process for which a complete understanding is still missing, particularly for red winegrape varieties. The bibliographic research on the evolution of phenolic profile during withering processes produced a Systematic Review on phenolic acids in withered grapes (Chapter I), giving a full view of the worthwhile recent findings on these often-overlooked compounds. To the best of our knowledge, for the first time a three-year study evaluated the combined effect of different ripeness degree and withering rates on the standard chemical composition, mechanical properties, and extractable phenolic profile of Nebbiolo grapes destined to the production of 'Sforzato' di Valtellina DOCG reinforced wine (Chapter II). Moreover, to go deeper in the comprehension of the extractability of phenolics in withered red grapes, the effect on mechanical properties, phenolic profile, and cell wall polysaccharides composition have been studied, assessing two different off-vine withering techniques on the Italian winegrape variety Aleatico (Chapter III).

For the second research line (EXPERIMENTAL SESSION – PART B), a corn-derived biosurfactant extract was evaluated as a novel processing aid during the

maceration phase of two red cultivars (Nebbiolo and Cabernet sauvignon) and compared with oenological tannins as a solubilizing and stabilizing agent of anthocyanins in red wine (Chapter IV). Finally, the impact of oenological additives and processing aids on the genetic traceability of 'Nebbiolo' wines was studied, contributing to explaining the causes of the reduced varietal identification efficiency in commercial wines (Chapter V).

EXPERIMENTAL SECTION

A - The withering of red winegrapes

Chapter I

Effect of withering process on the evolution of phenolic acids in winegrapes: A systematic review

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ABSTRACT

Background: Withering processes have a strong impact on the phenolic composition of winegrapes and related wines. Even if attention has often been focused on other phenolics, a great number of studies have included phenolic acids because of their quantitative and qualitative modifications during dehydration-withering processes. A systematic review that provides a concise overview of the extensive literature available on this important topic is lacking.

Scope and approach: This review identified 39 articles to answer the research question: What changes occur in phenolic acids after the withering process of winegrapes? The expected contribution of this systematic review is to have a full view of the worthwhile recent findings on these often-overlooked compounds to manage the technological process with new awareness and to find eventual weaknesses in this field to highlight new questions and research directions.

Key findings and conclusions: The research yielded useful results for withering and winemaking management. Phenolic acids are well represented in certain special productions to be proposed as markers of wine authenticity. Hydroxycinnamic and hydroxybenzoic acids and their derivatives are often affected differently by the withering process. Their evolution during grape withering is a complex phenomenon that is affected by many varietal and technological variables. In particular, the withering conditions applied and grape genotype play an important role in the changing amounts of phenolic acids, but there is still much to be understood, especially related to the combined effect of

both factors through genetic responses to environmental stresses and their respective chemical implications.

1. Introduction

The techniques currently utilized in winemaking and grape treatment for wine production are the result of decades of history, experience, and tradition. Scientific research has answered many practical questions, introduced important innovations, and allowed winemakers to manage traditional processes with new awareness.

Among the oenological techniques, the withering of winegrapes is one of the most strongly related to the cultural history and the climate peculiarities of the territories (Mencarelli & Tonutti, 2013). As a consequence, this process may produce wines with very different sensory features: (i) sweet wines, such as the botrytized Sauternes and Tokaj, the Mediterranean Italian Passito, the special icewines from Canada, the traditional wines made from Pedro Ximenez sun-dried grapes, or the straw wines called Vin de Paille and produced in the French Jura region; (ii) fortified wines, sweet but more alcoholic than conventional wines, produced in territories, such as Marsala, where the traditional wines were historically added with alcohol during overseas shipping for the English trade; (iii) reinforced wines, dry and full-bodied, such as the Sforzato or Sfursat from the heroic viticulture of Valtellina slopes and the Amarone from the Italian Valpolicella region (Kallitsounakis & Catarino, 2020; Scienza, 2013).

The withering process can be conducted on-vine, leaving the grapes on the plant subject to atmospheric phenomena for a long time (e.g., botrytized wine, late

harvest wines, or icewines), or off-vine by subjecting the grapes to postharvest dehydration, which may occur outdoors or indoors, under controlled conditions or not (Figueiredo-Gonza´lez et al., 2013a). In recent decades, many authors have extensively studied wines produced from withered grapes because of their cultural and economic relevance, highlighting important changes in berry metabolism throughout the withering process (D'Onofrio et al., 2019; Esmaiili et al., 2007; Zoccatelli et al., 2013).

Polyphenols, which are crucial compounds for wine quality, change during the grape dehydration process (Harrison, 2018; Mencarelli et al., 2010). Several studies have been carried out to better understand the changes in the content and composition of phenolic compounds during the on- or off-vine withering of winegrapes (Corradini & Nicoletti, 2013; Figueiredo-Gonza´lez et al., 2013a; Torchio et al., 2016). These authors highlighted the complexity of the evolution of phenolics over the process, which seems to be the result of a composite balance between synthesis, oxidation/loss, and concentration (Bonghi et al., 2012; De Rosso et al., 2016). This balance varies greatly depending on the genetic features of each cultivar (Zenoni et al., 2016), the ripeness level and mechanical properties of grapes (i.e., skin hardness; Rolle et al., 2009), the different dehydration techniques applied (Constantinou et al., 2018), the management of three important environmental factors (i.e., temperature, humidity, and airflow), and berry weight loss rate (Rolle et al., 2013). Additionally, specific classes of phenolic compounds, although subjected to the same conditions, are not affected in the same manner by the withering process (Toffali et al., 2011).

Phenolic acids are an important fraction of non-flavonoid compounds (Baderschneider & Winterhalter, 2001) present in grapes and wines, which have attracted increasing interest over the past ten years because of their potential health benefits (e.g., antioxidant, antibacterial, antiviral, anticancerogenic, anti-inflammatory, and vasodilatatory actions) (Babbar et al., 2015; Morales-Prieto et al., 2020). Phenolic acids consist predominantly of two subgroups: hydroxycinnamic acids (HCAs) and hydroxybenzoic acids (HBAs), of types C6–C3 and C6–C1, respectively (Monagas et al., 2005). The chemical structures of the most represented phenolic acids and their average contents in the wines are presented in Table S1.

HCAs represent the main class of phenolic compounds in white wines and the most represented class of non-flavonoid phenolics in red wines, averaging approximately 130 and 60 mg/L, respectively (Vanzo et al., 2007). They are synthesized through the shikimate pathway from phenylalanine and tyrosine through the action of phenylalanine ammonia lyase (PAL), which is a key enzyme that exhibits a relevant interconnection with the synthesis of other phenolic compounds (Laura et al., 2019). Some of the most represented HCAs are p-coumaric, caffeic, ferulic, and sinapic acids. They are usually present in grapes and wines as tartaric acid esters and diesters (HCTAs), such as caftaric acid (caffeoyltartaric acid), coutaric acid (p-coumaroyltartaric acid), and fertaric acid (feruloytartaric acid) and rarely as sugar esters (Buiarelli et al., 2010; Ferrandino et al., 2012; Ong & Nagel, 1978). In grapes, they are mainly located in the pulp and slightly in the skin (Garrido & Borges, 2013). The contents of HCA esters

detected in grapes vary greatly for different cultivars; however, caftaric acid is generally the major compound, followed by coutaric and fertaric acids (Waterhouse, 2002). During the fermentation process, the partial hydrolysis of esters occurs, with a reaction rate depending on the pH and presence of specific enzymes, and free HCAs can be released and partially esterified with ethanol, forming HCA ethyl esters (Somers et al., 1987). The oxidation of caftaric acid by grape polyphenol oxidase (PPO) to the corresponding o-quinone and subsequent reaction with glutathione through the –SH groups form a stable and colorless compound named GRP (Grape Reaction Product), which plays a key role in the oxidation of other phenolic compounds. This oxidation reaction starts to take place during the crushing and pressing of the grapes, causing must browning (Cheynier, Rigaud, et al., 1989). HCAs and their derivatives are also known to have antimicrobial properties, particularly against the wine spoilage lactic acid bacteria and yeasts (e.g., Brettanomyces bruxellensis) (Harris et al., 2010; Sabel et al., 2017; Stivala et al., 2017), increasing cell membrane permeability from wine (Campos et al., 2009) and interfering with the intracellular pH and metabolism (Carmona et al., 2016). The presence of HCAs in wines markedly affects color quality features in different ways: on one hand, they can contribute significantly to the browning of musts due to their oxidation (Cheynier, Basire, & Rigaud, 1989); on the other hand, they can contribute to the color stabilization of red wines due to the copigmentation effect or participate in the formation of acylated anthocyanins and anthocyanin-derived pigments (i.e., pinotins and portisins) (Bloomfield et al., 2003; He et al., 2012). Therefore, the decrease in the content of coumaroylated anthocyanins during winemaking and aging processes

due to hydrolytic and enzymatic reactions can release HCAs in musts and wines (Monagas et al., 2005). In terms of wine mouthfeel qualities, these compounds have been associated with astringency and bitterness perceptions (Garrido & Borges, 2013; Oka- mura & Watanabe, 1981), but their levels seem not to be perceptible in wine (Vérette et al., 1988). However, some more recent studies have pointed out that "puckering" astringency may be elicited by these compounds and evidenced a synergism on bitterness perception (Gonzalo-Diago et al., 2014; Hufnagel & Hofmann, 2008). Moreover, HCAs were identified as potential volatile precursors of ethyl phenols produced by Brettanomyces metabolism, conferring unpleasant flavors to wine (Vanbeneden et al., 2008). In particular, p-coumaric, ferulic, and caffeic acids could be enzymatically decarboxylated to vinyl derivatives by a cinnamate decarboxylase and subsequently reduced by a vinylphenol reductase, leading to the typical off-flavors described as “horse sweat,” “medicinal,” “rancid,” and “barnyard” (Malfeito-Ferreira, 2018). While several microorganisms, including Saccharomyces cerevisiae, can perform the enzymatic decarboxylation of HCAs, the populations of Brettanomyces spp. have a particularly effective activity in the reduction step (Kheir et al., 2013).

HBAs represent a minor component in grapes and wines, averaging approximately 10–20 mg/L in white wines and approximately 70 mg/L in red wines (Waterhouse, 2002; Waterhouse & Teissedre, 1997). Unlike HCAs, HBAs are not phenylpropanoids; they can be synthesized directly from the shikimic acid pathway, even if PAL is not active (Laura et al., 2019). Para-hydroxybenzoic,

protocatechuic, vanillic, gallic, and syringic acids are the most abundant HBAs detected (Kallithraka et al., 2009). Among them, gallic acid is considered the most important, as it is the precursor of all hydrolyzable tannins (Niculescu et al., 2018) and for its relevant antioxidant activity (Kallithraka et al., 2009). HBAs are mainly present in grape skins and pulp as glycosides, but gallic acid can also be extracted from grape seeds in free form through the hydrolysis of tannin galloyl esters or encompassed in condensed tannins (Revilla & Gonzalez-SanJose, 2003; Zou et al., 2002). In wines, HBAs are mostly found in free form (Monagas et al., 2005), even though some authors have also reported ethyl and methyl esters and glucose esters (Baderschneider & Winterhalter, 2001). With regard to sensory contributions, some recent studies have pointed out a synergic astringency effect with other phenols as reported for HCAs, but HBAs showed a higher affinity for salivary proteins than HCAs (Ferrer-Gallego et al., 2014, 2017). Moreover, Wang et al. (2020) highlighted a relevant matrix effect of phenolic acids on wine aroma modulation, particularly focusing on the interaction between gallic acid and free terpene compounds that inhibits volatile release.

When discussing the outcome of grape withering processes on phenolic compounds, attention is often focused on major molecules, such as anthocyanins and tannins. Nevertheless, many significant changes occur in the phenolic acids. In fact, their evolution throughout the withering process is complex and affected by many variables. Many studies have addressed phenolic acid evolution during grape withering, considering their importance in terms of abundance and wine

quality implications; however, a systematic review to summarize these findings is lacking.

Wines made from withered grapes are often identified as unique products of specific, limited geographical areas. Considering their growing interest in competitive and global markets, several studies have tried, through chemical analysis, to define characteristic compounds as quality markers to differentiate these wines from other products. In many cases, the most abundant classes of phenolic compounds that can be used as markers are HBAs or HCAs (Figueiredo-González, Regueiro, et al., 2014; Loizzo et al., 2013; Panceri et al., 2015). The purpose of this paper is to provide an overview of the scientific literature on this challenging topic. The approach used was aimed to focus on the evolution of phenolic acids from both qualitative and quantitative points of view in withered grapes and wines as objectively as possible. The expected contribution of this systematic review is twofold: (i) to have a systematic full view of the worthwhile recent findings to manage the technological process with new awareness, concerning the importance of these compounds, which are often overlooked; (ii) to find eventual research gaps in this field to highlight new questions and research directions.

2. Method

To produce an accurate and concise overview of the extensive literature available on this theme, a systematic review was performed on February 24, 2021, using

the procedure based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Moher et al., 2009).

A protocol was developed to provide explicit, rigorous, and transparent planning proceedings and eligibility criteria for the identification and selection of the studies of interest.

2.1. Research question

The review addressed the following close-framed question:

What changes occur in phenolic acids after the withering process of winegrapes?

The question has been deemed suitable for carrying out a systematic review, with all the PICO key elements specified, as illustrated in Table 1.

Table 1
Identification of the question key elements (PICO).

Key element	Response
P – population of interest	Phenolic acids of withered winegrape
I – intervention of interest	Withering process
C– comparator	Phenolic acids of fresh winegrape
O– outcome	Changes and evolution of phenolic acids

2.2. Search strategy

The research was performed simultaneously on three digital sources of information: "Scopus" (https://www.scopus.com/), "ScienceDirect"

(https://www.sciencedirect.com/), and "Web of Science" (https: //webofscience.com/).

An initial list of search terms was developed, including keywords, synonyms, plural/singular forms, and alternative words (Table 2).

Table 2
List of keywords useful to construct strings with Boolean operators.

Keyword	Synonyms	Plural/ singular forms	Alternative words/different spellings
Withering			Dehydration, drying, raisining
Withered grapes		Withered grape	Dehydrated grapes, dried grapes, raisin grapes
Grape	Winegrape	Grapes, winegrapes	
Sweet wines	Passito wines, withered wines, raisin wines,	Sweet wine	Straw wines, Icewines, Reinforced wines, Fortified wines, Dessert wines, Botrytized wines, Marsala, Porto, Sauternes, Tokaji, Sforzato, Sfusat, Amarone, Recioto, Aleatico, Vin Santo, Vin de Paille
Phenolic acids			HCA, HCAs, HCTAs, HBA, HBAs, hydroxycinnamic acids, hydroxycinnamics. hydroxybenzoic acids, hydroxybenzoics

The set of terms was used to create the search strings, matching them with Boolean operators. The search strings used for all the selected sources of information are listed in Table 3.

Table 3
Search strings used in all the selected sources of information.

Search string	Databases		
	Scopus	ScienceDirect	Web of Science
(phenolic acids) AND (withered grapes)	36	94	3
"phenolic acids" AND "sweet wines"	98	88	2
("phenolic acids") AND ("ice wine" OR "ice wines" OR "icewine" OR "icewines")	47	33	4
("HCAs" OR "HCTAs" OR "hydroxycinnamics") AND ("withering" OR "dehydration" OR "drying" OR "raisining") AND ("grape" OR "winegrape")	54	2115	0
("HCA" OR "hydroxycinnamic" OR "hydroxybenzoic") AND ("withering" OR "withered" OR "raisin" OR "dehydration") AND ("grape" OR "winegrape")	370	717	14
("HBA" OR "HBAs" OR "hydroxybenzoics") AND ("withering" OR "withered" OR "raisin" OR "dehydration") AND ("grape" OR "winegrape")	6	430	0
("HCAs" OR "HCTAa" OR "hydroxyinnamics") AND ("withered" OR "dehydrated" OR "dried" OR "raisin") AND ("grape" OR "winegrape")	56	391	0
("HCA" OR "hydroxycinnamic" OR "hydroxybenzoic") AND ("withered" OR "dehydrated" OR "dried" OR "raisin") AND ("grape" OR "winegrape")	849	2721	14
("HBA" OR "HBAs" OR "hydroxybenzoics") AND ("withered" OR "dehydrated" OR "dried" OR "raisin") AND ("grape" OR "winegrape")	24	1541	0
("phenolic acids") AND ("reinforced" OR "fortified" OR "dessert" OR "straw" OR "botrytized" OR "passito") AND ("wine" OR "wines")	950	976	7
("phenolic acids") AND ("Sauternes" OR "Tokaji" OR "Sforzato" OR "Sfursat" OR "Amarone" OR "Recioto" OR "Aleatico" OR "Vin Santo")	53	44	1
("phenolic acids") AND ("Marsala" OR "Porto" OR "Vin de Paille" OR "sweet wine" OR "withered wine" OR "raisin wine")	1593	861	146
("hydroxycinnamic" OR "HCA" OR "HCTAs" OR "hydroxybenzoic" OR "HBA") AND ("reinforced" OR "fortified" OR "passito") AND "wines"	244	599	6
("hydroxycinnamic" OR "hydroxybenzoic") AND ("Sauternes" OR "Tokaji" OR "Sfursat" OR "Amarone" OR "Recioto" OR "Aleatico" OR "Vin Santo")	38	56	1
("hydroxycinnamic" OR "hydroxybenzoic") AND ("Marsala" OR "Porto" OR "Vin de Paille" OR "sweet wine" OR "withered wine" OR "raisin wine")	810	660	131

The search fields selected were “all fields,” “find articles with these terms” (i.e., all parts of the documents excluding references), and “all fields” for “Scopus,” “ScienceDirect,” and “Web of Science,” respectively. The date range selected focused on articles published "from 2007 to the present".

2.3. Selection of relevant studies

This query yielded 16,883 results, which were preliminarily selected and deduplicated. The potentially relevant citations identified were 94, and a two-step screening was carried out.

Step 1: Initial screening. The first stage consisted of an initial screening of the retrieved articles based on the Title and Abstract, removing those not related to the evolution of phenolic acids during the withering process of winegrapes.

Step 2: Full-text review and data extraction. The full texts of the studies selected in the first stage were then fully reviewed to determine if they were still eligible to undergo data extraction.

The inclusion criteria applied for the article selection were:

- study on the phenolic fraction of withered winegrapes (Vitis vinifera L.) and wines;
- focus on phenolic acids;

- evolution of phenolic acids during on-vine and off-vine withering processes and winemaking implications.

The flow diagram of the relevant study selection process, shown in Fig. 1, allowed to obtain 39 suitable documents.

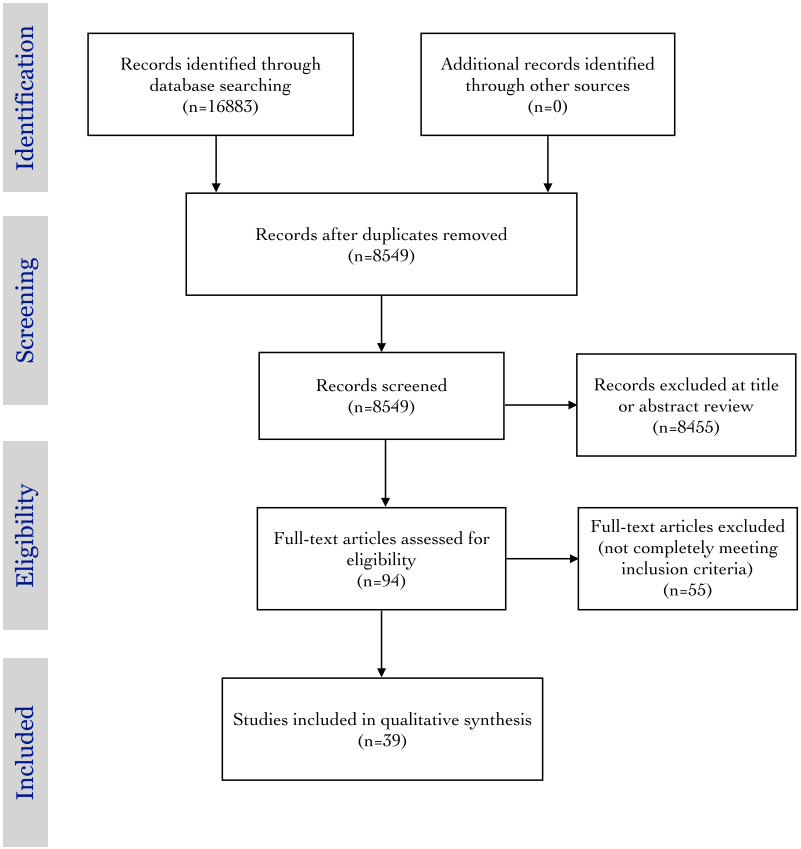

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of the study selection process: evolution of phenolic acids in winegrapes undergoing a withering process.

2.4. Data extraction, quality assessment, and reporting phase

In the extraction stage, all selected articles were assessed for methodological quality. The main results were collected, described, and critically examined to extract the common trends, comparing the findings regarding winegrapes used for on-vine withering and off-vine natural and controlled withering, genetic-dependent effects toward thermohygrometric environmental conditions, and winemaking implications. To produce a synthetic overview of the main findings pointed out by the recent literature (from 2007 to the present) about withering conditions applied, three extraction tables have been elaborated, ordering the studies based on the withering technique. Table 4 includes the 14 most relevant findings on the modification of phenolic acids over the on-vine winegrape withering process, with most of them regarding the production of ice or botrytized wines. Table 5 briefly describes the main outcomes of the 18 studies on the off-vine withering process under controlled conditions, while Table 6 shows a synthesis of the results of the seven studies regarding off-vine natural withering conditions.

3. Results and discussion

As grape berry composition is affected by several metabolic and physicochemical mechanisms during withering and the findings can vary depending on the withering process, the results were divided into on- and off-vine and the latter further separated under controlled conditions or natural dehydration (Tables 4–6). This section highlights the different conclusions reported in the selected

studies regarding the changes in phenolic acids during the winegrape withering process.

The results are discussed in an attempt to understand the causes and establish trends.

Table 4
Extraction table of the main outcomes on the evolution of phenolic acids in winegrapes during the **On-vine** withering process.

Author, Year	Title/purpose	On-vine/Off-vine withering	Variety	Outcomes/main findings on phenolic acids of withered grapes
Avizcuri-Inac et al. (2018)	Chemical and sensory characterization of sweet wines obtained by different techniques	**On-vine** natural dehydration *vs* **off-vine**	Tempranillo, Grenache, Viura, Cagazal, Riesling Gewürztraminer, Verdil	**Sweet wines obtained using different techniques were classified in three clusters based on phenolic compounds**: the first one was characterized by the **highest contents of HBAs and HCAs**; the second cluster was by **intermediate amounts of phenolic compounds**; and the third, including four artificial icewines, was described by the **lowest amounts of phenolic compounds, particularly HBAs**.
Blanco-Ulate et al. (2015)	Developmental and metabolic plasticity of white-skinned grape berries in response to *Botrytis cinerea* during noble rot	**On-vine:** harvest of grapes with symptoms of noble rot caused by *Botrytis cinerea* strain BcDW1 and other strains present in the field in three different stages of infection	Sémillon	The **increased levels of HCAs derivatives** were probably due to the **activation of** the **shikimate and phenylpropanoid metabolic pathways** by noble rot infection, normally associated with the ripening of red-berry cultivars.
Figueiredo-González et al. (2013a)	Effects on color and phenolic composition of sugar concentration processes in dried-on-or dried-off-vine grapes and their aged or not natural sweet wines.	**On-vine natural withering** vs **Off-vine withering**	All varieties studied in reviewed articles	*Review on the effect of withering on the phenolic composition of grapes and wines*
Hong et al. (2012)	Influence of plant defense system and fungal growth in *Botrytis cinerea*- infected Chardonnay berries	**On-vine:** harvest of botrytized grapes	Chardonnay	Metabolomic investigation revealed a **decrease in the concentration of HCTAs** as ***Botrytis cinerea*** infection increased.
Ivanova et al. (2011)	Identification and changes of polyphenolic compounds in red and white grape varieties grown in R. Macedonia during ripening	**On-vine:** late harvest	Vranec, Smederevka, Merlot, Chardonnay	The **decrease in** the content of **total phenols** in the overripening and late-harvest phase **could be the result of a change in the HCA ester concentration in skins,** due to **oxidation/polymerization reactions** for white and red varieties and the **participation in acetylated anthocyanin synthesis** in red varieties.
Kallitsounakis and Catarino (2020)	An overview on botrytized wines	**On-vine:** botrytized wine	All varieties investigated in the reviewed studies	*Review on the different stages of botrytized wine production. The authors provided a section on **HCAs** and their **esters**.*
Kilmartin et al. (2007)	Polyphenol content and browning of Canadian icewines	**On-vine: icewine** production process *vs* **cryogenic extraction method** ("REAL" vs "FAUX" Icewines)	Riesling and Vidal	**Differences** in the amounts of **HCAs were observed** between "REAL" and "FAUX" icewines in 1999–2000 vintages. **The overall concentration of HCAs in "FAUX" icewines was up to 11-times and 21-times higher than that of the "REAL"** Riesling and Vidal icewines, respectively. In the 2002 vintage, instead, the authors found relatively high concentrations of **HCAs** both in "REAL" and "FAUX" icewines, probably owing to the matching of the earliest harvest dates in that year.
Loizzo et al. (2013)	Phenolics, aroma profile, and in vitro antioxidant activity of Italian dessert Passito wine from Saracena (Italy)	**On-vine:** overripe grapes (Malvasia and Guarnaccia) + **Off-vine:** in the typical *cannizza* under the sun, covered during the night (Moscato, not macerated grapes collected few weeks before harvest)	Malvasia, Guarnaccia, and Moscato	**HBAs were the most abundant class of marker phenolic compounds,** particularly **gallic acid,** which showed the highest content (**376.5 mg/L**).
Lukić et al. (2016)	Changes in physicochemical and volatile aroma compound composition of Gewürztraminer wine as a result of late and ice harvest	**On-vine**: **late harvest** and **icewine** production process	Gewürztraminer	**Higher amounts of HCAs ethyl esters** were found **in late harvest and icewines than those in regular harvest** and of **4-vinylguaiacol in late harvest ones** (but not in icewines), suggesting a **concentration effect of HCAs** in the special wines compared to the control.
Mikulic-Petrovsek et al. (2017)	Effects of partial dehydration techniques on the metabolite composition in 'Refosk' grape berries and wine	**On-vine**: *double maturation raisonnée* (DMR) *vs* **off-vine:** berry partial dehydration in the chamber (PDC: 22–23 °C, 60% relative humidity, and ventilation)	Refosk	Only one HCA compound was detected in grape skins of Refosk (***p*-coumaric acid hexose, 0.72–1.48 mg/100 g dry weight**) and its content **decreased over the withering process**, particularly for PDC-treated berries. Seven HCA derivatives were detected in wines, **caftaric acid being the most abundant (11.43–20.68 mg/L)**. Wines obtained with **PDC-withered grapes showed the highest amounts of HCAs and derivatives compared to those of the DMR wines and the control (1.8 and 1.4-times higher, respectively)**.
Rusjan and Mikulic-Petkovsek (2017)	Double maturation raisonnée: the impact of on-vine dehydration on the berry and wine composition of Merlot	**On-vine:** *double maturation raisonnée* (DMR), cutting 1-year old canes with fruitful shoots during berry ripening	Merlot	**A reduction in the contents of HCA and derivatives in DMR grapes** was observed **compared to that of the control**; instead, **a**

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Table 4 (*continued*)

Author, Year	Title/purpose	On-vine/Off-vine withering	Variety	Outcomes/main findings on phenolic acids of withered grapes
				high presence of these compounds was found **in DMR wines**, in some cases significantly higher than that in control wines.
Tang et al. (2013)	Evaluation of nonvolatile flavor compounds in Vidal icewines from China	**On-vine:** icewine production process	Vidal	**HBAs were the major phenolic acids in icewines**, representing more than 74% of the total amount of the class: **protocatechuic acid (7.43–9.48 mg/L)** showed the highest content and **syringic acid (0.20–0.36 mg/L)** the lowest. As regards **HCAs**, the main compounds were **ferulic (1.07–2.14 mg/L)** and ***p*-coumaric (0.20–1.39 mg/L) acids**. **No significant differences** were found **in phenolic acid concentrations between wines characterized by different pressing levels.**
Tian, Li, et al. (2009)	Comparative study of 11 phenolic acids and five flavan-3-ols in cv. Vidal: impact of natural icewine making versus concentration technology	**On vine:** naturally frozen juice *vs* artificially produced (refrigerator-frozen and concentrated-juice)	Vidal	**The presence and concentration of phenolic acids could differentiate between icewine production methods**. At the end of experimental fermentations, the phenolic acid content **increased by 20% in the wine produced from refrigerator-frozen juice** and **increased by 2.5–2.7 times in the wine from concentrated juice** compared to that of the wine made from naturally frozen juice.
Tian, Pan, et al. (2009)	Comparison of phenolic acids and flavan-3-ols during wine fermentation of grapes with different harvest times	**On-vine:** dry wine, semi-sweet wine, icewine with low alcohol levels	Vidal	For the production of icewines, **increased contents of HBAs and HCAs** were observed when grape **harvest time was delayed**.

Table 5
Extraction table of the main outcomes on the evolution of phenolic acids in winegrapes during the **Off-vine controlled** withering process.

Author, Year	Title/purpose	On-vine/Off-vine withering	Variety	Outcomes/main findings on phenolic acids of withered grapes
Bellincontro et al. (2009)	Study of postharvest water stress of winegrape using non-destructive techniques	**Off-vine:** in perforated plastic trays inside a ventilated small tunnel at different controlled conditions (10 °C with 1.5 m/s airflow; 10 °C with 2.5 m/s airflow; 20 °C with 1.5 m/s airflow. Relative humidity: 45%)	Cesanese	Among the HCAs detected, **caftaric acid was very sensitive to atmosphere variation**. Its content was **368.95**, **513.40**, and **393.28 mg/kg** for grapes withered at 10 °C-1.5 m/s airflow, 10 °C-2.5 m/s airflow, and 20 °C-1.5 m/s airflow, respectively, and in all the cases it was significantly lower than that of the control (644.99 mg/kg).
Bonghi et al. (2012)	Phenol compound metabolism and gene expression in the skin of winegrape berries subjected to partial postharvest dehydration	**Off-vine:** in controlled conditions, slow and rapid rates of up to 10% and 30% weight loss	Raboso Piave	**A slight increase in caffeic acid contents** was observed **in rapidly dehydrated samples at 10% weight loss** and **in *p*-coumaric acid content under slow dehydration at 30% weight loss**. **Reduced content** of **gallic acid** was observed in **rapidly dehydrated samples**. The results obtained on gene expression patterns suggested a complex expression regulation of the **PAL multigene family.**
Budić-Leto et al. (2017)	Differentiation between Croatian dessert wine Prosek and dry wines based on phenolic composition	**Off-vine** with a system for temperature control and ventilation (maximum daily temperature of 40 °C)	Prosek, Plavac, and Posip	Prošek **dessert wines resulted in significantly lower concentrations of HCAs than those of dry wines**.
Frangipane et al. (2007)	Study of phenolic compounds in Aleatico grapes dried in a forced air tunnel	**Off-vine:** in a forced air tunnel with different temperatures (from 15 to 30 °C) throughout 30 days of withering	Aleatico	In the **skin**, ***trans*-caftaric acid** content **increased** at temperatures **between 17 and 23 °C**, but it **declined** when the temperature increased **to 30 °C, evidencing no substantial differences compared to the initial value**. **In the juice**, ***trans*-caftaric acid content** remained almost constant until midway through the process, after which **its concentration tripled**. Contents of **syringic** and **gallic acids in grape juice**, instead, **decreased during the withering process**.
Frangipane et al. (2012)	Effect of drying process in chamber at controlled temperature on the grape phenolic compounds	**Off-vine**, in a chamber in controlled conditions (18–21 °C, 68% relative humidity, 1 m/s airspeed) for 16 days	Roscetto	**A slight decrease** in the content of **HCTAs** (expressed as **mg/1000 berries**) was observed in the juice, probably because of **oxidation reactions that occurred during the withering process**. **HBA content showed the largest decrease**.
Marquez et al. (2012)	Colour and phenolic compounds in sweet red wines from Merlot and Tempranillo grapes chamber-dried under controlled conditions	**Off-vine:** in chamber drying under controlled conditions at a constant temperature of 40 °C and initial relative humidity of 30%	Tempranillo and Merlot	**A great increase in HBA and HCTA amounts** was detected **in withered Merlot and Tempranillo grapes.** The concentration of **HBAs increased during the first 24–48 h of maceration, and then no significant differences or slight decrease** were found **in Merlot and Tempranillo, respectively**; moreover, contents of **HCTA esters increased over the first 24 h of maceration and then decreased**.
Marquez et al. (2014)	Antioxidant activity and phenolic profile changes during the winemaking of Cabernet sauvignon sweet wines	**Off-vine**: in controlled condition at a constant temperature of 40 °C and an initial relative humidity of 20%.	Cabernet sauvignon	**The overall amounts of phenolic acids increased** during off-vine withering and subsequent experimental winemaking**, namely, for HBAs.** The observed increase peaked at **48 h of skin maceration.** Additionally, **contents of HCAs also increased** for all the compounds detected **except for *cis*-fertaric acid, which showed the opposite trend.** Moreover, the evolution of the total amount of phenolic acids was correlated to the **antioxidant activity**.
Mencarelli et al. (2010)	Chemical and biochemical change of healthy phenolic fractions in winegrape by means of postharvest dehydration	**Off-vine**: in different controlled conditions (10, 20, or 30 °C and 30% relative humidity)	Aleatico	**The highest increase in the overall content of phenolic acids (caftaric and coutaric acids) in withered winegrapes was observed at 20 °C and 20% weight loss**, while at 10 °C, the increase was lower, and at 30 °C, it decreased for grapes at 20% and 30% weight loss. **An upregulation in the expression of the PAL gene was detected** in the berries exposed to temperatures of 10 and 20 °C at 10% weight loss. Nevertheless, **transcriptomic abundance did not correlate well with metabolomic abundance**.
Negri et al. (2017)	The induction of noble rot (*Botrytis cinerea*) infection during postharvest withering changes the metabolome of Garganega grapevine berries	**Off-vine:** in perforated plastic boxes in a ventilated withering facility under natural conditions (**17–20 °C, 79% relative humidity**) **until the grapes reached about 30% weight loss**; then half number of plastic boxes were covered **to increase**	Garganega	The authors observed **a decrease in HCTA** concentrations (coutaric, caftaric, and fertaric acids) in ***Botrytis*-induced grapes**, probably owing to **the degradation reactions by fungal metabolism**.

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Table 5 (*continued*)

Author, Year	Title/purpose	On-vine/Off-vine withering	Variety	Outcomes/main findings on phenolic acids of withered grapes
		relative humidity (88–94%) to induce noble rot development, whereas the remaining boxes were maintained at **68–75% relative humidity** for 32 days. The length of the process was 61 days.		
Nicoletti et al. (2013)	Postharvest dehydration of Nebbiolo grapes grown at altitude for *Sfursat* wine production is affected by time of defoliation	**Off-vine:** in controlled-environment rooms (10, 20, or 30 °C; 60% relative humidity; 1 m/s air flow)	Nebbiolo	**During withering, all samples lost phenolic acids** (caftaric and coutaric acids) **except for grapes harvested from not-defoliated vines and withered at 10 °C**, in which the amounts of phenolic acids increased.
Panceri et al. (2013)	Effect of dehydration process on mineral content, phenolic compounds, and antioxidant activity of Cabernet sauvignon and Merlot grapes	**Off-vine:** under controlled conditions of 7 °C, 35% relative humidity, and volumetric airflow of 12 m^3/s (dehydration until 30% and 40% weight loss)	Cabernet sauvignon and Merlot	Among **HBAs** detected, **gallic acid was the predominant** compound in grapes and wines. While **contents of HBAs increased in Cabernet sauvignon, a decrease was observed in Merlot grapes**, suggesting an effect induced by genotype. **Increased contents of caffeic acid** in free form were reported **after 40% dehydration for both varieties.**
Panceri et al. (2015)	Effect of grape dehydration under controlled conditions on chemical composition and sensory characteristics of Cabernet sauvignon and Merlot wines	**Off-vine:** under controlled conditions with a constant temperature of 7 °C, 35% relative humidity, and volumetric airflow of 12 m^3/s (dehydration until 30% and 40% weight loss)	Cabernet sauvignon and Merlot	**Total content of HCAs was generally higher only for Merlot wines made from grapes at 30% weight loss** than that of the control wine. **Higher contents of HBAs** were found **in the wines produced from Cabernet sauvignon and Merlot grapes at 30% and 40% weight loss**.
Panceri and Bordignon-Luiz (2017)	Impact of grape dehydration process on the phenolic composition of wines during bottle ageing	**Off-vine:** under controlled conditions with a constant temperature of 7 °C, 35% relative humidity, and airflow of 12 m^3/s (dehydration until 30% and 40% weight loss)	Cabernet sauvignon and Merlot	The wines produced from dehydrated grapes evidenced **higher amounts of gallic, protocatechuic, vanillic, and caffeic acids** than that of the control. An increase in the concentration of phenolic acids (gallic, protocatechuic, and *p*-coumaric acids) was observed during 22 months of bottle ageing in all Cabernet sauvignon and Merlot wines made from withered grapes.
Piano et al. (2013)	Focusing on bioactive compounds in Uvalino grapes	**Off-vine:** in a room at a constant temperature of 24 °C and 30% humidity *vs* **On-vine:** not-harvested overripe grapes (control)	Uvalino	**HCTA contents** (caftaric, coutaric, fertaric acids) slightly **increased after 7 days of withering/overripening**, and this increase was maintained up to the 21st day (final point).
Serratosa et al. (2008b)	Drying of Pedro Ximenez grapes in chamber at controlled temperature and with dipping pretreatments	**Off-**vine: at a controlled temperature of 40 and 50 °C *vs.* traditional sun-drying	Pedro Ximenez	An **increase** in the concentration of **gallic acid in the juice from withered grapes** was observed **during the withering process**, **from 2.33 to 7.24 mg/L** at a temperature of 40 °C and **10.2 mg/L** at 50 °C. Similarly, also **HCA concentrations increased by 50% or doubled** after withering at 40 or 50 °C, respectively.
Toffali et al. (2011)	Novel aspects of grape berry ripening and post-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis	**Off-vine:** in controlled conditions (13–17 °C and about 65% relative humidity)	Corvina	**Anthocyanins acylated with *p*-coumaric acid** started to accumulate after veraison but continued to **increase** thereafter, **during the withering process.** Consequently, **the ratio of non-acylated anthocyanins to coumaroyl anthocyanins fell from 6.4 in ripening and ripe berries to 3.7 in withering and withered berries**.
Torchio et al. (2016)	Influence of different withering conditions on phenolic composition of Avanà, Chatus and Nebbiolo grapes for the production of 'Reinforced' wines	**Off-vine:** different controlled conditions such as slow withering (18 °C, 40% relative humidity, and 0.9 m/s airflow) and fast withering (28 °C, 40% relative humidity and 0.9 m/s airflow)	Avanà, Chatus, Nebbiolo	**The concentration effect due to withering prevailed over the degradation reactions for grape pulp HCTAs** in two varieties out of the three tested, **particularly for Chatus**. These amounts were not influenced by the withering rate.
Zenoni et al. (2016)	Disclosing the molecular basis of the postharvest life of berry in different grapevine genotypes	**Off-vine**: in controlled conditions with gradually decreasing temperature (from 16° to 7 °C) and gradually increasing relative humidity (from 55% to 80%)	Corvina, Sangiovese, Merlot, Oseleta, Syrah, and Cabernet sauvignon	The positive or negative **final balance of HCAs/HBAs depended on genotype,** and it was negative in Corvina.

Table 6
Extraction table of the main outcomes on the evolution of phenolic acids in winegrapes during the **Off-vine natural** withering process.

Author, Year	Title/purpose	On-vine/Off-vine withering	Variety	Outcomes/main findings on phenolic acids of withered grapes
Constantinou et al. (2017)	Metabolic fingerprinting of must obtained from sun-dried grapes of two indigenous Cypriot cultivars destined for the production of 'Commandaria': a protected designation of origin product	**Off-vine:** sun-drying process, during which the grapes were manually turned over periodically. The sun-drying process lasted 10–12 days.	Xynisteri and Mavro	Among phenolic compounds, **HBAs represented the most predominant group in Xynisteri** grapes (62.1% of total phenolics) **and were an important group in Mavro** (42.3%). **Total amounts of HBAs increased in musts of both varieties during sun-drying**. As regards **HCAs and derivatives,** they were the second major group of phenolic compounds, contributing to 25.7% and 39.8% for Xynisteri and Mavro, respectively. During withering, **a six-times increase in their content was observed**, even if the concentration of some **singular compounds** such as caffeic acid dihexoside and fertaric acid isomer **decreased**.
Constantinou et al. (2018)	The impact of postharvest dehydration methods on qualitative attributes and chemical composition of 'Xynisteri' grape must	**Off-vine:** traditional sun-drying method *vs*. alternative dehydration methods (multiple horizontal wires, multiple vertical pallets, low greenhouse, hot-air dryer treatment)	Xynisteri	**Total HBA contents** in musts from grapes withered with multiple horizontal wires, low greenhouse, and hot-air treatments **increased by 1.8-, 2.3- and 2.7-times that of fresh grapes**, respectively. **Traditional sun-drying revealed the lowest effect on HCAs (1.5-times)**, while other **withering methods showed a strong increase (from 1.7 to 6.0 times), which was higher than that of the concentration effect.**
Figueiredo-González et al. (2013b)	Evolution of colour and phenolic compounds during Garnacha Tintorera grape raisining	**Off-vine:** in a room with natural ventilation over 83 days, grapes were placed in a single layer in plastic boxes, turned over, and checked weekly	Garnacha tintorera	The overall concentration of **HCA esters** decreased from **32 to 14 mg/kg of berry during 83 days of withering,** and this decrease began after the 16th day. A **decreasing trend** in HCA esters was observed during withering **for the two isomers**: ***cis*-caftaric** (from 0.38 to 0.07 mg/kg of berry), ***trans*-caftaric** (from 19 to 9.7 mg/kg of berry), ***cis*-coutaric** (from 4.0 to 1.1 mg/kg of berry) and ***trans*-coutaric** (from 9.0 to 3.2 mg/kg of berry).
Figueiredo-González, Regueiro, et al. (2014)	Garnacha-Tintorera based sweet wines: detailed phenolic composition by HPLC/DAD - ESI/MS analysis	**Off-vine**: for naturally sweet wine, in plastic boxes at natural conditions of temperature and humidity	Garnacha tintorera	**The concentration of HBAs was lower in the two sweet wines than that of the dry wine (control), particularly for the fortified one**, mainly driven by the steep decrease in gallic acid content**. HCTAs** and free **HCAs** were present at high concentrations (45 mg/L) in dry base wine, but their presence **decreased** probably **owing to enzymatic browning reactions**.
Figueiredo-González, Cancho-Grande, et al. (2014)	The phenolic chemistry and spectrochemistry of red sweet winemaking and oak-aging	**Off-vine**: in plastic boxes at natural conditions of temperature and humidity *vs*. fortified sweet wine	Garnacha tintorera	An **increase in phenolic acid amounts** was observed **for** both **natural** and **fortified sweet wines during** the **oak-ageing** period of 6 and 12 months. **The concentration of HBAs increased**, starting from 32 to 40 mg/L at the beginning in natural and fortified sweet wines, respectively, and averaging about **50 mg/L in both sweet wines at the end of the ageing**, probably because of the **transfer from the oak wood**. Moreover, **a decreasing trend** was found **in the contents of HCA esters** (caftaric and coutaric acids). The **simultaneous increase of HCA free forms** suggested a **slow hydrolysis** process, but this increase was not proportional to the decrease of the contents of esters.
Nievierowski et al. (2021)	Role of partial dehydration in a naturally ventilated room on the mycobiota, ochratoxins, volatile and phenolic composition of Merlot grapes	**Off-vine:** in a naturally ventilated room for 21 days	Merlot	The main differences were observed for **four HBAs** (ethyl gallate, *p*-hydroxybenzoic acid, gallic acid-hexose, and gallic acid) and **two HCAs** (caffeic and caftaric acids). **The amounts of these compounds increased more than two times during withering compared to that of fresh grapes.**
Serratosa et al. (2008a)	Changes in color and phenolic compounds during the raisining of grape cv. Pedro Ximenez (for the production of sweet wines)	**Off-vine**: sun-drying grape raisining on mats in slight hills facing south (40–50 °C) for 7 days	Pedro Ximenez	**The withering process enhanced the concentration of HBAs in grape must**. Particularly, ***p*-hydroxybenzoic acid amount strongly increased by 188%**, suggesting an additional increase by means other than concentration. Meanwhile **the content of gallic acid increased by 33%**, suggesting that this compound can participate in other reactions during the withering process. As regards **HCTAs**, **the amounts of caftaric, coutaric, and fertaric acids increased more for *cis*- isomers than for *trans*-isomers.** These increases were lower than those expected, indicating a **simultaneous degradation of these compounds during withering** that contribute to the typical browning of the sun-dried grapes.

3.1. Evolution of phenolic acid content over withering: the result of a complex balance

In all the studies considered, phenolic acids were somehow affected by the withering process, but the analysis of the literature revealed different positions among authors about the balance between concentration, hydrolysis, synthesis, and oxidation/catabolism processes in winegrapes.

Some authors considered the increase in the content of phenolic acids as a consequence of the prevalence of the concentration effect on the degradation reactions. Among these authors, Marquez et al. (2012), assessing grape juice from off-vine dehydration at a constant temperature of 40 °C in Tempranillo and Merlot grapes (Table 5), reported a substantial increase in the contents of HBAs and selected HCTAs due to water evaporation and, possibly, the release from solid portions of the grapes during withering, even if some of these compounds were degraded through enzymatic browning reactions. Additionally, Torchio et al. (2016) studied the evolution of phenolic compounds in grape pulp over the off-vine dehydration process at two different controlled temperature conditions (18 and 28 °C) on cultivars Avanà, Chatus, and Nebbiolo (Table 5) and concluded that contents of HCTAs increased in withered grapes compared to those of fresh samples significantly for Chatus and Nebbiolo winegrapes. These results were probably due to a balance in favor of the concentration effect, as suggested by the findings of Frangipane et al. (2007). Later, to avoid the weight loss variable during the withering process, Frangipane et al. (2012) expressed the content of

HCA in Roscetto juice as mg/number of berries (Table 5), demonstrating in their experiment how the degradation effect prevailed over the concentration effect. Regarding HBAs, contents of syringic and gallic acids decreased during the withering process when expressed as mg/L juice, in agreement with previous observations on Aleatico grapes (Frangipane et al., 2007, Table 5).

Lukić et al. (2016) (Table 4), focusing on the change in the physicochemical composition of Gewürztraminer wines as a result of late and icewine harvest, hypothesized that the higher amounts of ethyl cinnamates found in these wines could have been due to the concentration effect of HCAs as a consequence of water loss. Accordingly, Panceri et al. (2013) observed an increase in the contents of HCAs (mainly caffeic acid) in Merlot and Cabernet sauvignon grapes (Table 5). These authors suggested that off-vine withering under controlled conditions at 7 °C, and low relative humidity could promote the activity of cinnamoyl esterase, similar to that in the maturation of wines in bottles, where HCA esters are hydrolyzed to the free form. However, in the same study, the authors observed a divergence in the HBA amounts for the two grape varieties: Cabernet sauvignon showed an increase in the content of these compounds during withering, whereas Merlot showed a decrease, suggesting a genotype effect rather than a change in the metabolic pathways. More recently, Nievierowski et al. (2021), studying the impact of off-vine withering in a naturally ventilated room on the quality of Merlot grapes, found a significant increase in phenolic acid amounts, both for HBAs and HCAs, particularly for gallic, p-hydroxybenzoic, caffeic, and caftaric acids (Table 6). Additionally, Constantinou et al. (2017) estimated a sixfold

increase in the HCA content and total amounts of HBAs in both traditional Cypriot cultivars Xynisteri and Mavro undergoing an off-vine sun-drying short process (Table 6). Interestingly, these authors pointed out some differences among the contents of single HCAs within the subgroup. Many significant changes were observed in individual phenolic compounds in a comprehensive comparative study on Xynisteri grapes (Constantinou et al., 2018), and the increasing trend was common for all phenolic acids, but with different intensities and prevalence of singular compounds within the HBA and HCA classes concerning the various postharvest withering methods tested (Table 6). Additionally, the authors interpreted the significant increase in phenolic acid content observed during withering to be higher than that caused by the concentration effect, using four alternative dehydration methods, namely, multiple horizontal wires, multiple vertical pallets, low greenhouse, and hot-air dryer. Serratosa et al. (2008a) also suggested the possibility of an additional increase in the amounts of these compounds in ways other than concentration. Studying Pedro Ximenez grapes withered at tem- peratures of 40–50 °C (Table 6), as in the typical sweet wine production in southern Spanish regions, the authors observed a 188% increase in p-hydroxybenzoic acid content, suggesting an additional increase in the amount of these compounds during off-vine sun-drying. However, other phenolic acids (i.e., gallic acid and HCTAs) showed simultaneous degradation, probably due to oxidation or polymerization processes, leading to a final increase lower than expected. In these peculiar conditions of high temperature, several authors have highlighted the occurrence of browning enzymatic reactions involving phenolic acids (Figueiredo-González

Regueiro, et al, 2014; Marquez et al.,2012; Serratosa et al., 2008b). It is assumed that the first step of the PPO enzymatic browning reaction consists of the oxidation of caftaric acid (Serratosa et al., 2011), which is very sensitive to atmospheric variation, particularly related to water stress and UV light (Bellincontro et al., 2009).

Although numerous studies have shown an increase in the contents of phenolic acids, supporting the assumption of the prevalence of the concentration effect or suggesting the possibility of new formation over the withering process, other studies pointed out a decrease in their contents in both on-vine and off-vine, controlled or not, withering conditions. Among the off-vine withering studies (Table 5), Negri et al. (2017) found a decrease in amounts of many grape metabolites, including HCTAs, in noble-rot botrytized Garganega grapes, suggesting that they were degraded by fungal metabolism. The loss of phenolic acids in winegrapes infected with noble rot has been reported in other white varieties. A reduction in the content of caftaric acid (− 69%) was observed in Chenin blanc grapes (Carbajal-Ida et al., 2016), and of HCAs in skins of botrytized Chardonnay grapes (Hong et al., 2012), probably because of laccase and tyrosinase activities that could be responsible for the oxidation of HCTAs (Dubernet et al., 1977). Nevertheless, in the early stages of the phenylpropanoid pathway during Botrytis cinerea infection, increased production of HCAs has been highlighted (Blanco-Ulate et al., 2015; Kallitsounakis & Catarino, 2020). These results confirmed the complexity of the balance that determines the final content of phenolic acids in these special wines.

Piano et al. (2013) observed a very slight increase in the HCTA content in the Piedmontese variety Uvalino undergoing off-vine withering in a room at a constant temperature of 24 °C and relative humidity of 30% (Table 5); however, fewer differences were observed in the contents of these compounds in overripened grapes, all during a 21-day withering period. However, Nicoletti et al. (2013) observed a decrease in the contents of phenolic acids during off-vine withering under all the controlled conditions tested on Nebbiolo grapes harvested from vines defoliated at different times (Table 5), except for the non-defoliated sample withered at 10 °C, confirming how the agronomic techniques also affect the features of the withered grapes and wines. As a probable explanation for the decrease in the contents of esters of HCAs observed during the off-vine natural withering process of Garnacha tintorera (Table 6), Figueiredo-González et al. (2013b) hypothesized the participation of these compounds in degradation reactions owing to their high suitability as substrates in some other types of reactions (e.g., copigmentation or PPO browning), as well as anthocyanin acylation for the free forms, as previously presumed in studies performed on-vine by Ivanova et al. (2011) and off-vine by Toffali et al. (2011) (Tables 4 and 5). Nevertheless, lower contents in grapes did not lead to lower contents in the final wines. Rusjan and Mikulic-Petkovsek (2017), who studied for the first time the impact of the on-vine cutting of 1-year old canes with fruitful shoots during ripening, − the so-called double maturation raisonnée (DMR)− on Merlot grapes (Table 4), found the lowest concentration of HCAs in DMR-subjected grape skins when compared to that of the control, despite the presence of high amounts of these compounds in the wine made from those grapes. By contrast, Budić-Leto et

al. (2017), in a study on the differentiation between Croatian dessert wine Prošek and dry wines based on phenolic composition (Table5), found significantly lower concentrations of phenolic acids in Prošek dessert wines than in the dry wines.

These heterogeneous results can be explained by several factors: first, it has been shown that the positive or negative final balance of HCAs and HBAs depends on the genotype (May´en et al., 1997; Zenoni et al., 2016), and several authors have indicated that different varieties subjected to the same withering conditions can be affected differently by the process (Kilmartin et al., 2007; Marquez et al., 2012; Torchio et al., 2016). Additionally, a very important part of the phenomenon explanation is certainly attributable to the different withering conditions applied, both on-vine (Avizcuri-Inac et al., 2018; Mikulic-Petrovsek et al., 2017) and off-vine (Bellincontro et al., 2009; Constantinou et al., 2018; Frangipane et al., 2007; Nicoletti et al., 2013). Furthermore, the rate of weight loss can significantly affect the complex balance dynamics (Bonghi et al., 2012; Mencarelli et al., 2010; Panceri et al., 2015).

3.2. Grape genetic regulation

During withering, berry tissues are metabolically active, and several specific-activated processes may affect the grape compositional features (Rizzini et al., 2009). Several studies have examined the specific changes in gene expression and the complex integration of transcriptomic, metabolomic, and proteomic levels during grape withering (Di Carli et al., 2011; Zamboni et al., 2008, 2010). However, little information is available on the weight of genetic regulation in

determining the final concentration of phenolic acids in withered grapes. During the study of chemical and biochemical changes in the off-vine process of Aleatico grapes at different temperatures and weight loss rates, Mencarelli et al. (2010) found an upregulation of the expression of PAL gene, which is active at the beginning of the phenolic acid pathway, in berries exposed to temperatures of 10 and 20 °C, over dehydration at 10% weight loss. However, transcript abundance of the PAL gene did not correlate well with metabolite abundance. Additionally, Bonghi et al. (2012) evaluated phenolic compound metabolism and gene expression in the skins of Raboso Piave winegrapes under off-vine controlled withering conditions related to slow and rapid rates of up to 10 and 30% weight loss. These authors highlighted the importance of the complex expression regulation of numerous members of the *Vitis vinifera* L. PAL multigene family as a key step in the multiple physiological responses to environmental stresses. In a comprehensive genetic/metabolomic study, Zenoni et al. (2016) highlighted the complexity of the metabolomic and transcriptomic changes during postharvest dehydration. Even though they found an upregulation of the expression of genes encoding important enzymes that catalyze the earliest steps of phenylpropanoid biosynthesis, the negative or positive final balance of HCAs/HBAs in several winegrape cultivars (Corvina, Sangiovese, Merlot, Syrah, Oseleta, and Cabernet sauvignon) ongoing withering process was genotype-dependent. This balance was negative in Corvina. Furthermore, the six varieties studied were characterized by distinct gene expression profiles and modulation intensities: Corvina berries showed the strongest and fastest response to postharvest withering, whereas Cabernet sauvignon showed the weakest and

slowest gene induction. In accordance with these findings, more recently, the same authors assessed two different thermo-hygrometric conditions of off-vine withering with natural or forced airflow on cultivar Corvina and found no significant differences in the accumulation of HCAs/HBAs and their derivatives in response to different environmental stresses (Zenoni et al., 2020).

Regarding the decrease in the concentration of phenolic acids observed by some authors during the grape withering process, a possible explanation could be found from a genetic point of view. In the transcriptomic studies performed by Zamboni et al. (2008) on Corvina winegrapes, the authors described the upregulation of the expression of a chalcone isomerase gene, suggesting the activation of the flavonoid pathway during the withering process, and two tags of polyphenol oxidase, indicating a probable oxidation/polymerization of phenolic compounds. In accordance with the results of Zamboni et al., Di Carli et al. (2011) described an upregulation of the activity of reactive oxygen species (ROS)-scavenging enzymes during the slow withering of Corvina winegrapes. In particular, different trends in ROS accumulation were observed between the veraison and ripening phases, and a decrease in the abundance of PPO involved in browning reactions was observed during withering with respect to the veraison stage. Moreover, as mentioned above, some authors hypothesized that the decrease in the contents of HCAs in the late harvest phase is a result of their functions in some types of reactions such as the synthesis of acylated anthocyanins (Figueiredo-González et al., 2013b; Ivanova et al., 2011; Toffali et al., 2011). Although some genes involved in the first part of anthocyanin acylation mechanisms have been found

in transcriptomic studies (Bontpart et al., 2018; Zamboni et al., 2008), further studies are needed to gain a better understanding of the role of genetic regulation in these reactions and their important chemical implications.

3.3. Winemaking implications: extraction and evolution

Qualitative and quantitative evolution of phenolic acids has also been observed during the winemaking of withered grapes. In particular, Marquez et al. (2014) evaluated the antioxidant activity in relation to the phenolic profile during the winemaking of Cabernet sauvignon grapes off-vine withered at a constant temperature of 40 °C and initial relative humidity of 20%. They observed an overall increase in the concentration of HBAs, probably due to the early diffusion from the solid parts of the grapes to their pulp during the withering process and later during subsequent maceration of the skins. The peaks of the amounts of these compounds were observed after 48 h of maceration, as was observed for HCA esters. These results are consistent with those previously reported for Tempranillo and Merlot grapes by Marquez et al. (2012), in which small amounts of HCA esters were present in the final wines, possibly owing to degradation reactions toward free forms and their suitability as substrates for major reactions during maceration.

Few studies have been conducted to evaluate the influence of the grape withering process on the phenolic composition of wines during aging and bottle storage. Figueiredo-González et al. (2014) studied the phenolic composition of naturally sweet and sweet fortified Garnacha tintorera-based wines during 6 and 12 months

of oak aging and observed an increase in the amounts of HBAs and HCAs in both wines. This increase was probably due to release from oak wood and transfer to the wine during the aging period or a consequence of the decrease in the contents of coumaroyl anthocyanin forms to release HCAs. The authors also highlighted a simultaneous decrease in HCTA amounts in the wines, suggesting the occurrence of slow hydrolysis reactions during the aging period. Additionally, the esters could have participated in other reactions such as polymerization or copigmentation with anthocyanins. These findings are in agreement with the previous results reported by Karagiannis et al. (2000), who conducted one of the first studies on this topic on cultivar Muscat lefko grapes from the island of Samos.

Panceri and Bordignon-Luiz (2017) observed an increase in the concentration of phenolic acids (gallic, protocatechuic, p-coumaric acids) during 22 months of bottle ageing for Cabernet sauvignon and Merlot wines made from grapes withered under controlled conditions, in accordance with the findings of Issa-Issa et al. (2020) in Fondillón wines made from Monastrell on-vine overripe grapes. The increased availability of free forms of HCAs may result in potential risk for the production of unpleasant flavors because of their suitability as substrates for spoilage microorganisms (Kheir et al., 2013). This possible risk, enhanced by the presence of residual sugars, necessitates rigorous microbiological control by applying appropriate inactivation measures. Nevertheless, wines made from withered grapes are often characterized by higher alcohol content than that of

other styles of wine, making the proliferation of spoilage microorganisms more difficult (Malfeito-Ferreira, 2018).

3.4. The case of icewines: a discussed topic

The evolution of phenolic acid content during the production of icewines is a peculiar and often discussed topic. The first study on this issue was conducted by Kilmartin et al. (2007) on the polyphenol composition of Canadian icewines to identify the quality markers of authentic icewines. For example, "Faux" Riesling and Vidal icewines (harvested above −8 °C) had up to 11- and 21-times higher concentrations of total HCAs than those of "Real" icewines (harvested at temperatures of −8 °C or below). These findings agree with those reported by Tian, Li, et al. (2009) who introduced a further kind of "Faux" icewine for cultivar Vidal: a refrigerator-frozen juice, artificially refrigerated at −8 ◦C, and a concentrated juice made using rotary evaporation at 66 °C. The artificially concentrated juice presented a concentration of phenolic acids 2.1-times higher than that of the refrigerator-frozen juice, and notably, approximately 3.6-times higher than that of naturally frozen juice. These differences were quite preserved in the icewine types produced from them, with little influence from the yeast strain used. Furthermore, the influence of the harvest date on the phenolic acid content of icewines was discussed by the same authors. Kilmartin et al. (2007) pointed out that the earliest harvest dates produced icewines with higher concentrations of HCAs in Riesling and Vidal winegrapes harvested in Canada in 1999, 2000, and 2002, while Tian, Pan, et al. (2009) argued that the contents of HBAs and HCAs

increased as grape harvest time was delayed to produce icewines from the Vidal variety harvested in China in 2005 and 2006. With regard to the considerations previously made on the influence of withering conditions, it should be recalled that the traditional icewine production technique is an on-vine withering process and is thus subjected to changes due to weather conditions (e.g., freeze-thaw cycles) that could markedly affect the balance of phenolic acids in the berries.

Avizcuri-Inac et al. (2018) focused their efforts on the chemical and sensorial characterization of sweet wines to understand the influence of the dehydration process on the features of several wines made from on-vine withered grapes. The authors analyzed sweet wines obtained by using different varieties and techniques and classified them into three clusters based on the phenolic compound characteristics. The first cluster, characterized by the highest contents of HBAs, HCAs, and other phenolic compounds, such as flavonols, included a late harvest wine and a natural icewine (both involving Tempranillo red grapes). The second cluster, with intermediate amounts of phenolic compounds, included two natural icewines (one from white and one from red grapes), one artificial icewine (from Tempranillo red grapes), and a supurao wine (from Tempranillo and Grenache off-vine dehydrated red grapes). The third cluster, which was described by the lowest values of phenolic compounds, particularly for HBAs, included four icewines obtained by grape freezing in the chamber, three produced from white grapes and one from red grapes. Although these findings could have been influenced by variety, environmental and technological factors (such as grape pressing), they show that the extraction and concentration of these compounds

produced by freezing winegrapes were lower than those in wines belonging to the other two clusters, produced with more traditional techniques. The concentration of phenolic acids has been associated with the quality and authenticity of icewines (Kilmartin et al., 2007; Tang et al., 2013; Tian, Li, et al., 2009, Tian, Pan, et al., 2009). However, new studies are needed to better understand the link between weather conditions and phenolic acid features in the production of these special wines. This alternative approach can be challenging and technically difficult, but will have very useful practical applications, such as a more focused choice of the harvest period.

4. Conclusive remarks and future perspectives

The aim of the present systematic review was to produce a concise overview of the extensive literature available on the evolution of phenolic acids in withering grapes and the wines produced from them. Although phenolic acids are structurally simple molecules, their evolution over the withering process is complex. In most cases, the amount of phenolic acids increased after withering, both at high and low temperatures. However, in some studies, their concentrations decreased or remained almost constant in grapes. In these previous studies, the authors considered a possible cause of the decrease as the high suitability of phenolic acids as substrates for other types of reactions (e.g., copigmentation or PPO browning). Indeed, it is of fundamental importance to remember that the final contents of phenolic compounds in withered grapes and the resulting wines are strongly dependent on the balance between concentration, synthesis, and loss

or degradation of these compounds. This balance is particularly evident for phenolic acids in botrytized grapes (reported in section 3.1). Even if at the end of the process the overall balance resulted in the loss of phenolic acids, probably owing to tyrosinase and laccase activities, in the early stages of the phenylpropanoid pathway during Botrytis cinerea infection an increased production of HCAs was highlighted. Moreover, HCAs, HBAs, and their derivatives are often affected differently by the withering process, and in some cases, even single compounds within the same class can present different trends. The contents of these compounds are affected by many factors, including genotype and withering conditions. There is still much to understand about the weight of the genetic control of the withering process, and new studies are needed to investigate the berry genetic response to environmental stresses and the related chemical re- percussions. In this sense, the modulation of the water loss rate and intensity may be used to increase the concentration of specific compounds. This knowledge can help manage the withering process according to the desired oenological objective.

The winemaking implications are very interesting and well-studied: HBA and HCA ester release seems to start during withering, from the solid parts of the grapes to the pulp, and to continue thereafter during maceration, with a peak after 48 h of skin maceration. The free forms of HCAs tend to increase throughout winemaking and aging. This information may be useful for practical applications, allowing the management of technological processes with improved awareness. On one hand, the increase in the contents of these compounds could involve

copigmentation effects, improved antioxidant activity, and health benefits; on the other hand, it may lead to increased risks, such as must browning or off-flavor production, because of their role as substrates for spoilage microorganisms.

Finally, several authors associated the concentration of phenolic acids with a quality and authenticity marker in icewines, but further research should be carried out to better understand the link between the weather/environmental conditions and the phenolic acid features of winegrapes in on-vine withering processes.

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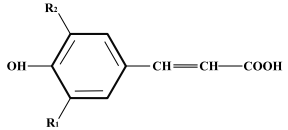
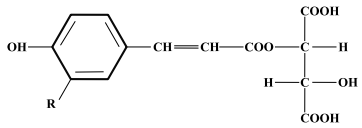
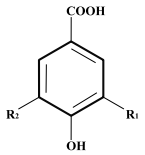
Table S1 – Structure and contents of the main phenolic acids present in wines.

Phenolic acids	Chemical structure			Indicative contents in wine (mg/L)			
				white wines	red wines	white wines from withered grapes	red wines from withered grapes
Hydroxycinnamic acids (HCAs)							
	**R_1**	**R_2**	**compound**				
	H	H	*p*-Coumaric acid	0.22–5.94	0.40–19.30	0.02–2.90	0.04–7.50
	OH	H	Caffeic acid	0.74–13.30	0.60–70.70	0.30–27.90	1.60–23.00
	OCH_3	H	Ferulic acid	0.20–3.87	0.00–2.90	0.20–11.50	0.07–2.21
	OCH_3	OCH_3	Sinapic acid	0.00–0.10	0.00–1.80	0.25–1.20	–
Tartaric esters of hydroxycinnamic acids (HCTAs)							
		**R**	**compound**				
		H	Coutaric acid	0.32–127.00	1.00–32.00	0.12–24.40	0.49–22.00
		OH	Caftaric acid	0.16–304.00	2.00–106.00	0.35–68.20	0.28–74.95
		OCH_3	Fertaric acid	0.10–3.97	0.70–6.50	0.92–10.20	0.74–9.45
Hydroxybenzoic acids (HBAs)							
	**R_1**	**R_2**	**compound**				
	H	H	*p*-Hydroxybenzoic acid	0.02–0.22	0.20–2.00	0.40–2.81	0.11–3.40
	OH	H	Protocatechuic acid	0.50–2.31	0.20–7.00	0.09–37.30	0.03–15.77
	OCH_3	H	Vanillic acid	0.00–1.52	0.30–10.00	0.03–7.05	0.01–19.41
	OCH_3	OCH_3	Syringic acid	0.00–0.88	1.30–10.00	0.03–0.36	0.02–18.00
	OH	OH	Gallic acid	0.29–6.80	0.47–95.00	0.09–376.00	0.45–75.56

Compiled from: Avizcuri-Inac et al. (2018); Budić-Leto et al. (2017); Buiarelli et al. (2010); Cheynier et al. (1989a); Figueiredo-González et al. (2014a); Figueiredo-González et al. (2014b); Kilmartin et al. (2007); Loizzo et al. (2013); Marquez et al. (2012); Marquez et al. (2014); Mikulic-Petrovsek et al. (2017); Rusjan et al. (2017); Salagoïty-Auguste and Bertrand (1984); Tang et al. (2013).

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Chapter II

Combined effect of harvest time and postharvest dehydration length on the composition of withered grapes for Sforzato di Valtellina DOCG wine production

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ABSTRACT

Sforzato di Valtellina (*Sfursat*) is a PDO reinforced red wine produced in Valtellina (northern Italy) from partially withered red grapes (*Vitis vinifera* L.) cv. Nebbiolo. The grape ripeness degree and the withering process strongly influence wine quality. The aim of this research was to evaluate the combined influence of different grape ripeness levels and withering length on the chemical composition, mechanical properties, and phenolic profile of Nebbiolo winegrapes. During three consecutive vintages (2019, 2020, and 2021), three different technological binomials have been tested on grapes grown in different locations (Valtellina upper- and lower-valley vineyards): early harvest/long withering (EL), medium-term harvest/medium-term withering (MM), and late harvest/short withering (LS). At the end of the withering process, EL thesis usually presented the highest values of sugars and acidity. Extractable seed polyphenols showed a decreasing trend by leaving the grapes on the plant longer, and this effect increased considerably after withering with respect to fresh samples. The EL and MM thesis evidenced the greater concentration of these compounds expressed on grape weight, particularly for tannins. Instead, skin-extracted total phenolics were less influenced by the harvest time, whereas their concentration increased after withering. The harvest time appears to have a higher impact than the withering length on the final extractable anthocyanin content, although the trend was no stable during the vintages or common for the two vineyards evaluated. EL and MM experienced the highest contents of grape skin tannins in most cases, suggesting that a longer withering increases their concentration. In conclusion, harvest time and withering length can be modulated

according to the desired oenological objective, promoting the valorization of grape potentialities. The choice to anticipate the harvest time for grapes destined to withering could be interesting in view of performing long wine ageing, starting from grapes characterized by higher acidity and phenolic contents, as well as to avoid the impact of adverse climate conditions.

Keywords: Grape postharvest, phenolic compounds, withering process, red winegrapes, reinforced wines, Sforzato di Valtellina DOCG

1. Introduction

Territorial identity represents an added value for the wine-growing activities. It has a central role for the wine market, not only for economic aspects, but also for cultural and social development reasons.[1, 2] Therefore, it's essential to preserve the quality of unique and typical wines such as the Sforzato di Valtellina DOCG (Denominazione di Origine Controllata e Garantita or Protected Designation of Origin, PDO), one of the main identifying results of the so-called heroic steep slope viticulture and winemaking of Valtellina alpine valley (Northern Italy). This type of wine, which is also locally called '*Sfursat',* is a reinforced dry red wine produced with partially withered cv. Nebbiolo red winegrapes (*Vitis vinifera* L.). The Designation of Origin guidelines for this wine stipulates that the postharvest withering process must take place in uncontrolled conditions in fresh and dry dehydration rooms named *fruttai*. The grapes are usually placed in single-layer crates or on reed mats, and the withering starts immediately after the harvest and lasts until the grape crushing, which happens no earlier than the 1st of December of the same year.[3]

In the production process of these special wines, there are two determining variables that can influence the chemical-physical features of the dehydrated grapes and, consequently, the quality of the wines: the ripeness degree at the harvest, and the withering process length and conditions such as temperature, relative humidity, and air flow speed.[4, 5, 6, 7]

The importance of the ripeness degree and the withering conditions on grape phenolic composition have been separately studied in recent years,[8, 9, 10, 11] but to

the best of our knowledge there is little information available in literature on the combined effect of these two variables on the grape quality features and phenolic profile. Grape skin and seeds contain several classes of phenolic compounds, which are significantly affected by these factors and strictly associated with red wines quality.[12, 13, 14]

The attempt to get a better understanding on this topic represents a considerable challenge due to the several other factors to consider, such as the different climatic conditions of the year or the vineyard location and management.[15, 16, 17] To carry out this purpose, a three-year experimental plan (vintages 2019, 2020, and 2021) was designed to answer the question: "what is the best time to harvest red grapes destined to withering?". The influence of three different binomials of grape ripeness degree and withering length have been studied, comparing their effects on grape must composition, grape skin and seed potential phenolic content, and grape skin mechanical properties of fresh and withered Nebbiolo grapes from vineyards with different locations in the Valtellina valley for the three vintages.

2. Materials and methods

2.1. Grape samples and withering process

Grape samples of cv. Nebbiolo (*Vitis vinifera* L.) were harvested from two commercial vineyards located at the two opposite ends of the vine growing area in the Valtellina valley (northern Italy): the upper-valley vineyard (A), set in the western part (Villa di Tirano, 46°12'N, 10°8'E, 400 m asl), and the lower-valley

vineyard (B), located in the eastern end of the valley (Berbenno di Valtellina, 46°10'N, 09°45'E, 370 m asl). For each vineyard, over the three consecutive years of experimentation (vintages 2019, 2020, 2021), three different binomials have been tested: early harvest/long withering (EL), medium-term harvest/medium-term withering (MM), and late harvest/short withering (LS). To accomplish this task, every year the grapes were harvested for each vineyard at three different ripeness degrees according to the grape soluble solid content reached, with a target soluble solid contents of 21.5% (w/w) for EL thesis. Each year, MM target was 1% more than EL thesis, while LS was either targeted at 1% increment from MM or lower if the climate did not allow reaching this target. At each stage, about 300 kg of grapes were harvested. A sample of 10 kg of these grapes was randomly collected for the analysis on fresh material before withering, and the remaining grapes were placed in single-layer plastic crates in a typical *fruttaio* (uncontrolled dehydration room). For all the samples, as established by the DOCG product regulation guidelines, the withering lasted until the 1st December of the same harvest year. Consequently, the length of the dehydration process depended on the harvest date, as illustrated in Figure 1.

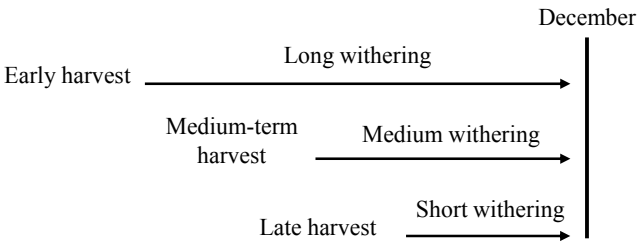

Figure 1. Experimental plan.

Harvest date and total days of withering are shown in Table S1. Long withering process lasted in total about 70 days and among the three different withering periods there were around 10 days of difference.

Eight randomised single-layer crates for each binomial have been weighted before and after the withering process to estimate the effective weight loss percentage (WL%), calculated as: [1−(net weight of withered grapes in kg/net weight of fresh grapes in kg)]. A sample of withered grapes has been collected for each binomial/vineyard tested for the laboratory analyses.

2.2. Weather data

The meteorological data of temperature (°C) and precipitation (mm) were recorded at the weather station of Sondrio (SO, Italy) and provided by ARPA Lombardia[18] for the three entire consecutive harvest years of the study (2019, 2020, and 2021).

2.3 Chemical analysis

2.3.1. Chemical reagents and Standards

Malvidin-3-O-glucoside chloride standard was provided by Extrasynthese (Genay, France). Methylcellulose, standards of (+)-catechin and (−)-epicatechin, and HPLC-gradient grade solvents were supplied by Sigma-Aldrich (St. Louis, MO, USA). Deionized water used for preparing the solutions was produced by a Milli-Q system (Merck Millipore, Darmstadt, Germany).

2.3.2. Sample preparation and standard parameters determination

In the laboratory, for each sample of fresh or withered grapes, the berries were handpicked from the stalk without detaching the pedicel and visually inspected to eliminate the damaged ones. Three replicates of about 100 g of berries were collected and manually crushed for 2 minutes. The grape must obtained was centrifuged at 3000 × g for 15 minutes at 20 °C using a Hettich 32R centrifuge (Tuttlingen, Germany) and the supernatant was analyzed. Total soluble solids were determined using a refractometer with automatic temperature compensation (Atago Palette 0-32, Atago Corporation, Tokyo, Japan). A pHmeter InoLab 730 (WTW, Weilhelm, Germany) was used to measure pH by potentiometry, and total acidity (as g/L of tartaric acid) was determined by titration with sodium hydroxide 0.1 mol/L according to OIV-MA-AS313-01 method.[19] Reducing sugars (as sum of glucose and fructose), glycerol, and organic acids (citric, tartaric, and malic acids) were determined using a HPLC system (Agilent Technologies, Santa Clara, USA) equipped with a refractive index and a UV detector.[20]

2.3.3. Extraction and determination of phenolic compounds from grape skins and seeds

For each sample, three sets of 40 g of berries were randomly selected and weighted. The evaluation of extractable phenolic compounds was carried out separately for the different grape berry components. Grape skins and seeds were separated for each set, cleaned from the pulp with the aid of a laboratory spatula. Once cleaned, the flesh was discarded and each set of skins or seeds was immediately immersed in 50 mL of a wine-like solution (15% v/v ethanol, 5 g/L tartaric acid, 100 mg/L $Na_2S_2O_5$, and adjusted to pH 3.20 with NaOH 1 mol/L),

following the proportions described by Mattivi et al.[21] to mimic a winemaking condition of a reinforced wine. The same weight of berries (40 g in 50 mL of wine-like solution) was maintained for both fresh and dehydrated grape samples in order to take into account the modifications of solid-to-liquid proportion due to weight loss. The flasks were placed at 25 °C controlled temperature and daily mixed for 5 min with the aid of internal magnetic stirring bars (20x6 mm). After 7 days of extraction, the liquid extracts were collected and used for the analyses.

Phenolic compounds were determined with a UV-1800 spectrophotometer (Shimazdu Corp., Kyoto, Japan) by spectrophotometric methods.[22,23] Total phenolic index (TPI, expressed as mg (−)-epicatechin/kg berries) was obtained measuring the absorbance at 280 nm of the sample diluted 100 times in water and quantified using a (−)-epicatechin calibration curve ($y=82.158x$, $R^2=0.999$). A dilution with ethanol:water:[37% hydrochloric acid] (70:30:1 v/v) solution was performed to determine total anthocyanins (TA, expressed as mg malvidin-3-glucoside chloride/kg berries) and total flavonoids (TF, mg (+)-catechin/kg berries), measuring the maximum absorbance at 536-540 nm for the former, and applying a graphical correction to the absorbance at 280 nm for the latter.[22] Condensed tannins (MCP, mg (−)-epicatechin/kg of grapes) were quantified by precipitation with methyl cellulose, using a 0.04% methyl cellulose solution and a sample dilution factor of 20.[24]

2.4. Grape skin mechanical properties

Grape skin mechanical properties were evaluated using a TA.XTplus Universal Testing Machine (Stable Micro Systems, Godalming, Surrey, UK). The Texture

Analyzer was equipped with an HDP/90 platform, a SMS P/2 N needle probe used for skin hardness evaluation (berry skin break force, F_{sk}, N; berry skin break energy, W_{sk}, mJ; berry skin resistance against deformation, E_{sk}, N/mm) or a flat cylindrical probe (Ø 2 mm) used for skin thickness evaluation (Sp_{sk}, μm), and a 5 kg load cell.[25] For each binomial/vineyard studied, 30 fresh or withered berries were randomly selected and individually subjected to the compression and penetration/puncture tests. The data were acquired using the Texture Exponent software (Stable Micro Systems).

2.5. Statistical analysis

Statistical analysis was executed using the R software, version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). The Tukey-b post-hoc test at $p<0.05$ was used to define significant differences among the three binomials tested by one-way ANOVA analysis of variance. T-test was used to discriminate significant differences among fresh and withered grapes.

3. Results and Discussion

3.1. Weather conditions

The climate of the east-west oriented alpine Valley of Valtellina (46°10'N, Lombardy, Northern Italy) is classified as endo-alpine, with an average of 800-1200 mm of yearly rainfall mainly distributed in the western part of the valley, and a windy regime characterized by breeze and *Föhn* phenomena.[26] As it can be seen in the Figure 2, the weather conditions of the three vintages were very different from each other. The year 2019 was characterized by a dry and warm

summer (with a maximum of 39.5 °C reached at the end of June) and a rainy autumn (466 mm), being the hottest of the three years in the period close to the harvest. The first half of 2020 was cooler than the previous year, whereas the summer was slightly hotter, and in the second half of the year rainfalls were significantly above average (314 mm in summer and 446 mm in autumn), especially over harvest time. In 2021, the beginning of the year was dry, the cool spring was followed by a very hot summer with a rainy July and a warm autumn. In general, the data recorded in the period close to the harvest time (from August to October, Figure 2b) show that 2019 was the hottest of the three years considered (18.4 vs 17.6 vs 17.6 °C of average yearly temperature for 2019, 2020, and 2021, respectively), 2020 was the wettest vintage (497 mm from August to October *vs* 339 and 333 mm of vintages 2019 and 2021, respectively), and 2021 resulted the driest harvest year (with a decrease of 200 mm with respect to the total amount of rainfall of the previous years).

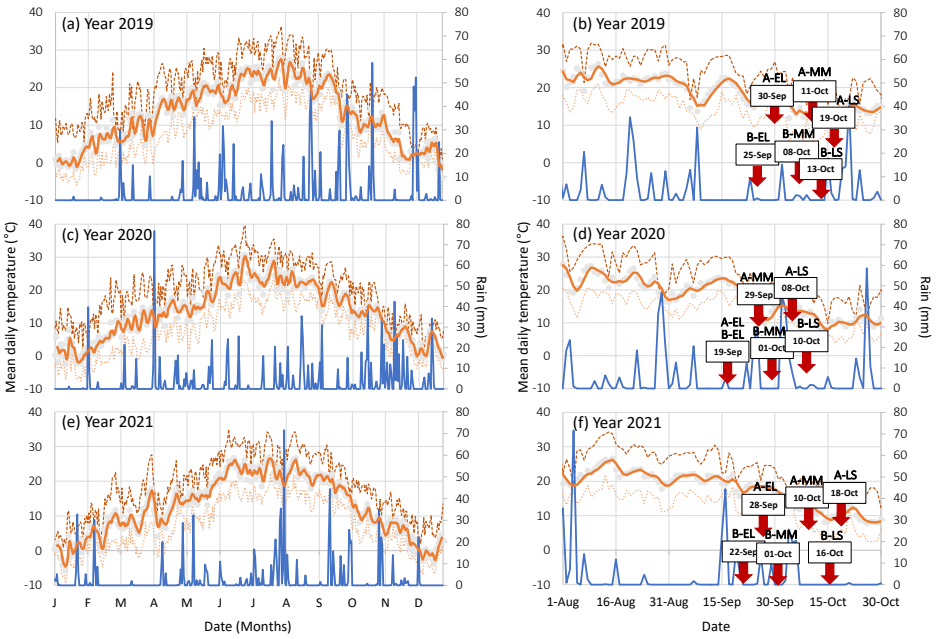

Figure 2. Minimum (dotted orange), maximum (dashed orange), and average (solid orange) daily temperature and rainfall (blue lines) of the three consecutive harvest years studied (2019a, 2020a, 2021b), harvest times and weather conditions of the months near the harvest (2019b, 2020b, 2021b) from the weather station located in Sondrio. Data: ARPA Lombardia (2022).

3.2 Grape must chemical composition

3.2.1. Technological parameters of fresh grapes

The standard parameters of fresh grapes for the years 2019, 2020, and 2021 are shown in Table 1. As provided by the research plan, higher sugars levels were found in late harvested grapes (224-258 g/L) with respect to the earliest ones (208-230 g/L). The glucose/fructose ratio in fresh Nebbiolo grapes was almost 1, which is the typical ratio for ripe grapes,[27] and experienced the tendency to decrease or remained almost constant leaving the grapes on the plant longer (0.96-1.00 for MM; 0.94-0.98 for LS), in accordance with the literature.[28]

Total acidity (expressed as g/L of tartaric acid) tended to decrease in fresh grapes from early to late harvest by an average of 1.9 and 0.9 g/L for vineyard A and B, respectively. Malic and tartaric acids tend to decrease progressively with the ripening process in fresh grapes, due to respiratory metabolism and dilution, respectively.[29,30] In this case, this behavior was observed mainly in 2019-2020, with the exception of malic acid content in 2019 vintage for vineyard A and tartaric acid in 2019 for vineyard B. In the acidic composition described above, late harvested grapes of vintage 2021 were not in line, presenting a higher tartaric acid content in fresh grapes from vineyard A with respect to the previous harvest points of the same year. This behavior is probably influenced by the drought of the year, which could have induced a situation of slight dehydration of the grapes on the plant.[31,32]

3.2.2. Technological parameters of withered grapes

The average percentages of grape weight loss (WL%) for the three years and two vineyards at the end of the withering process were 19 ± 5, 16 ± 4, and 12 ± 4% for EL, MM, and LS, respectively. These differences detected in WL% are consistent with the expectations. Indeed, the harvest time of each binomial resulted in a step decrease of about 10 days in terms of withering length between EL and LS trials.

Table 1. Standard parameters of fresh grapes.

Harvest year	Ripeness parameter	Vineyard A				Vineyard B			
		EL	MM	LS	Sign	EL	MM	LS	Sign
2019	Reducing sugars (g/L)	230 ± 1 c	245 ± 2 b	258 ± 8 a	**	230 ± 3	240 ± 3	239 ± 5	ns
	pH	3.04 ± 0.01 c	3.12 ± 0.01 b	3.23 ± 0.01 a	***	3.09 ± 0.05	3.07 ± 0.02	3.09 ± 0.02	ns
	TA (g/L tartaric acid)	10.20 ± 0.00 a	8.27 ± 0.21 b	8.23 ± 0.12 b	***	9.67 ± 0.50	9.23 ± 0.15	8.93 ± 0.31	ns
	Citric acid (g/L)	0.22 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	ns	0.23 ± 0.01 a	0.19 ± 0.01 b	0.23 ± 0.01 a	**
	Tartaric acid (g/L)	8.34 ± 0.16 a	7.83 ± 0.09 b	7.11 ± 0.20 c	***	8.08 ± 0.01	8.26 ± 0.20	8.07 ± 0.03	ns
	Malic acid (g/L)	3.79 ± 0.04 a	2.94 ± 0.26 b	3.99 ± 0.26 a	**	3.54 ± 0.22	3.59 ± 0.26	3.25 ± 0.29	ns
	G/F ratio	1.00 ± 0.00 a	0.98 ± 0.00 b	0.98 ± 0.00 ab	*	0.97 ± 0.00	0.97 ± 0.00	0.98 ± 0.01	ns
2020	Reducing sugars (g/L)	216 ± 2 b	234 ± 5 a	224 ± 1 b	**	220 ± 4	226 ± 7	227 ± 3	ns
	pH	3.16 ± 0.02 b	3.18 ± 0.01 b	3.32 ± 0.02 a	***	3.19 ± 0.01	3.18 ± 0.02	3.17 ± 0.04	ns
	TA (g/L tartaric acid)	8.76 ± 0.23 a	7.81 ± 0.08 b	6.44 ± 0.02 c	***	7.51 ± 0.13 a	7.64 ± 0.10 a	7.05 ± 0.26 b	*
	Citric acid (g/L)	0.21 ± 0.01 a	0.19 ± 0.01 b	0.14 ± 0.00 c	***	0.16 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	ns
	Tartaric acid (g/L)	7.29 ± 0.14 a	7.59 ± 0.09 a	7.05 ± 0.20 b	**	7.79 ± 0.09	7.69 ± 0.09	7.42 ± 0.28	ns
	Malic acid (g/L)	2.76 ± 0.08 a	2.47 ± 0.30 a	1.46 ± 0.08 b	***	1.79 ± 0.13	1.79 ± 0.09	1.67 ± 0.08	ns
	G/F ratio	1.00 ± 0.00 a	0.99 ± 0.01 a	0.94 ± 0.00 b	***	0.98 ± 0.01 a	0.96 ± 0.00 ab	0.95 ± 0.01 b	**
2021	Reducing sugars (g/L)	208 ± 2 c	226 ± 2 b	237 ± 6 a	***	217 ± 3 b	227 ± 5 a	231 ± 2 a	**
	pH	3.11 ± 0.02 b	3.06 ± 0.01 b	3.18 ± 0.02 a	**	3.10 ± 0.02 b	3.15 ± 0.02 ab	3.16 ± 0.03 a	*
	TA (g/L tartaric acid)	9.95 ± 0.34 a	9.09 ± 0.34 b	8.55 ± 0.20 b	**	10.18 ± 0.04 a	8.78 ± 0.44 b	8.54 ± 0.06 b	***
	Citric acid (g/L)	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	ns	0.21 ± 0.00 a	0.17 ± 0.01 c	0.19 ± 0.01 b	***
	Tartaric acid (g/L)	7.20 ± 0.08 b	7.12 ± 0.13 b	7.45 ± 0.07 a	*	7.97 ± 0.23 a	7.53 ± 0.12 b	7.91 ± 0.09 ab	*
	Malic acid (g/L)	4.11 ± 0.20 a	3.60 ± 0.21 b	3.45 ± 0.03 b	**	3.78 ± 0.08 a	3.00 ± 0.29 b	3.01 ± 0.09 b	**
	G/F ratio	1.02 ± 0.00 a	1.00 ± 0.00 b	0.98 ± 0.00 c	***	1.00 ± 0.00 a	0.98 ± 0.00 b	0.96 ± 0.00 b	***

All data are expressed as average value ± standard deviation (n = 3). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively. Different Latin letters indicate significant differences among the three binomials tested for each vineyard studied according to Tukey-b test ($p < 0.05$). A: upper-valley vineyard, B: lower-valley vineyard, EL: early harvest/long withering, MM: medium-term harvest/medium withering, LS: late harvest/short withering. TA: total acidity, G/F ratio: glucose/fructose ratio.

Technological parameters of withered grapes for the years 2019, 2020, and 2021 are shown in Table 2. As regards sugar content in withered grapes, the longer was the withering period, the greater was the percentage increase of sugars in withered grapes with respect to fresh ones due to concentration effect, leading the EL thesis to be the richest in sugar content at the end of the process (247-292 g/L, with a mean difference of +9.8 g/L with respect to LS samples). For each binomial studied, dehydrated grapes showed a decreased glucose/fructose proportion with respect to fresh ones from a range 0.94-1.02 (fresh) to 0.91-0.96 (dehydrated grapes), coherently with previous studies.[33, 34] The observed movement of the ratio in favor of fructose suggested that, during the withering, glucose may have been used for the respiration or to feed other metabolic pathways.[35, 36]

As regards total acidity, the concentration effect due to dehydration opposed the metabolic losses of acidity detected in withered grapes compared to the fresh ones. Consequently, at the end of the process, the EL thesis showed higher total acidity values (+1.21 g/L and +0.85 g/L, respectively, on average with respect to LS and MM) and the lower pH values, confirming that the management of harvest time plays a central role in the achievement of a good sugar-to-acid ratio in withered grapes, as previously hypothesized by Failla et al.[37]

The content of individual organic acids in withered grape juice also changed. Indeed, at the end of the withering process, the EL thesis showed the highest contents of malic and citric acids with respect to the other binomials studied for each year (except for 2020 vineyard B). Interestingly, the contents of citric and

Table 2. Standard parameters of withered grapes.

Harvest year	Ripeness parameter	Vineyard A				Vineyard B			
		EL	MM	LS	Sign	EL	MM	LS	Sign
2019	Reducing sugars (g/L)	290 ± 5 a	289 ± 1 a	277 ± 6b	*	292 ± 2 a	278 ± 7 b	279 ± 2 b	*
	pH	3.14 ± 0.02 c	3.22 ± 0.02b	3.29 ± 0.02a	***	3.14 ± 0.03 b	3.21 ± 0.03 a	3.23 ± 0.01 a	*
	TA (g/L tartaric acid)	9.50 ± 0.60 a	7.73 ± 0.15b	8.07 ± 0.15b	**	9.13 ± 0.25 a	8.93 ± 0.15 a	8.10 ± 0.36 b	**
	Citric acid (g/L)	0.33 ± 0.03	0.26 ± 0.04	0.29 ± 0.06	ns	0.36 ± 0.04	0.29 ± 0.03	0.29 ± 0.06	ns
	Tartaric acid (g/L)	7.75 ± 0.78	7.13 ±0.43	7.05 ± 0.09	ns	6.75 ± 0.24 b	7.81 ± 0.14 a	7.43 ± 0.09 a	***
	Malic acid (g/L)	4.07 ± 0.18 a	3.18 ± 0.19 b	3.84 ± 0.18 a	**	3.95 ± 0.11 a	3.33 ± 0.09 b	3.18 ± 0.21 b	**
	G/F	0.95 ± 0.00 a	0.94 ± 0.00 b	0.95 ± 0.00 a	***	0.95 ± 0.00 a	0.93 ± 0.00 b	0.94 ± 0.01 b	*
	Glycerol (g/L)	1.98 ± 1.03	1.58 ± 0.63	1.12 ± 0.09	ns	2.14 ± 0.97	2.11 ± 1.10	1.36 ± 0.52	ns
2020	Reducing sugars (g/L)	278 ± 4 a	280 ± 3 a	265 ± 3 b	**	275 ± 8	273 ± 1	267 ± 15	ns
	pH	3.25 ± 0.01 c	3.36 ± 0.03 b	3.58 ± 0.01 a	**	3.35 ± 0.05	3.35 ± 0.02	3.36 ± 0.02	ns
	TA (g/L tartaric acid)	8.43 ± 0.08 a	7.33 ± 0.50 b	5.81 ± 0.24 c	***	7.46 ± 0.23	7.49 ± 0.14	7.03 ± 0.24	ns
	Citric acid (g/L)	0.32 ± 0.02	0.28 ± 0.00	0.31 ± 0.04	ns	0.26 ± 0.01 a	0.25 ± 0.02 a	0.20 ± 0.01 b	**
	Tartaric acid (g/L)	6.94 ± 0.29 a	5.91 ± 0.14 ab	5.00 ± 0.56 b	**	6.67 ± 0.12	6.72 ± 0.17	6.72 ± 0.49	ns
	Malic acid (g/L)	2.95 ± 0.06 a	2.70 ± 0.04 b	2.01 ± 0.05 c	***	2.20 ± 0.07	2.31 ± 0.06	2.15 ± 0.11	ns
	G/F	0.94 ± 0.00 a	0.94 ± 0.00 a	0.92 ± 0.00 b	***	0.92 ± 0.00 a	0.92 ± 0.00 ab	0.91 ± 0.00 b	*
	Glycerol (g/L)	0.37 ± 0.13	0.76 ± 0.11	1.00 ± 0.34	ns	0.36 ± 0.09	0.45 ± 0.30	0.20 ± 0.16	ns
2021	Reducing sugars (g/L)	247 ± 2 b	257 ± 2 a	250 ± 6 ab	*	264 ± 3 a	270 ± 5 a	249 ± 2 b	***
	pH	3.19 ± 0.01 a	3.13 ± 0.02 b	3.21 ± 0.01 a	***	3.24 ± 0.02 b	3.30 ± 0.02 a	3.26 ± 0.00 b	**
	TA (g/L tartaric acid)	10.20 ± 0.15 a	9.44 ± 0.24 b	9.62 ± 0.04 b	**	9.49 ± 0.07 a	8.22 ± 0.12 b	8.28 ± 0.11 b	***
	Citric acid (g/L)	0.28 ± 0.00 a	0.23 ± 0.01 b	0.24 ± 0.00 b	*	0.27 ± 0.01 a	0.21 ± 0.03 b	0.18 ± 0.02 b	**
	Tartaric acid (g/L)	6.84 ± 0.07	7.00 ± 0.16	6.70 ± 0.27	ns	7.36 ± 0.13	7.27 ± 0.10	7.37 ± 0.19	ns
	Malic acid (g/L)	4.31 ± 0.12 a	3.77 ± 0.08 b	4.26 ± 0.06 a	***	4.15 ± 0.08 a	3.25 ± 0.02 b	2.96 ± 0.08 c	***
	G/F	0.96 ± 0.00	0.96 ± 0.00	0.96 ± 0.01	ns	0.94 ± 0.01	0.94 ± 0.01	0.93 ± 0.00	ns
	Glycerol (g/L)	0.14 ± 0.03	0.14 ± 0.07	0.05 ± 0.02	ns	0.47 ± 0.15	0.61 ± 0.37	0.27 ± 0.23	ns

All data are expressed as average value ± standard deviation (n = 3). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively. Different Latin letters indicate significant differences among the three binomials tested for each vineyard studied according to Tukey-b test ($p < 0.05$). A: upper-valley vineyard, B: lower-valley vineyard, EL: early harvest/long withering, MM: medium-term harvest/medium withering, LS: late harvest/short withering. TA: total acidity, G/F ratio: glucose/fructose ratio.

malic acids progressively increased from fresh to withered grapes, presumably due to a positive balance between catabolism and concentration effect (T-test *p*-value between fresh and withered citric acid values < 0.01, while the difference was not statistically significant for malic acid). To the contrary, a decreasing trend in the concentration of tartaric acid was observed from fresh to withered grapes. Rösti et al. [38] explained the drop in tartaric acid observed during Merlot and Syrah winegrapes dehydration as consequence of precipitations occurred already inside the berries, probably due to a loss of compartmentation over the process.

A small amount of glycerol has been detected only in withered grapes (from 0.05 to 2.14 g/L), more prominently in the grapes from 2019 vintage. The increase of the glycerol content due to the withering process has often been observed in literature.[39, 40] Indeed, during dehydration, grape cells under hyper-osmotic stress for the increasing sugar concentration seem to react to stress by increasing the intracellular glycerol.[41] Nevertheless, the differences in glycerol contents from EL to LS observed after withering were not statistically significant.

3.3. Grape skin mechanical properties

The instrumental texture parameters of fresh and withered grape skins determined in the three consecutive harvest years are shown in Table 3. Berry skin hardness (F_{sk}) and thickness (Sp_{sk}) are important qualitative indexes used in oenology as predictors of anthocyanin extractability.[42] The possibility to estimate the extractability of phenolic compounds during the maceration phase is particularly interesting for Nebbiolo winegrapes, which are rich in di-substituted anthocyanins, the easiest extractable and oxidable ones.[43, 44]

Table 3. Fresh and withered grape skins mechanical properties.

Harvest year		2019						2020						2021					
Vineyard		A			B			A			B			A			B		
		fresh	withered	Sign[b]	fresh	withered	Sign[b]	fresh	withered	Sign[b]	fresh	withered	Sign[b]	fresh	withered	Sign[b]	fresh	withered	Sign[b]
Fsk (N)	EL	0.69 ± 0.19	0.70 ± 0.13	ns	0.63 ± 0.13	0.69 ± 0.17	ns	0.68 ± 0.17	0.65 ± 0.14	ns	0.60 ± 0.14	0.61 ± 0.15	ns	0.64 ± 0.10 b	0.68 ± 0.17 b	ns	0.72 ± 0.15 a	0.71 ± 0.17	ns
	MM	0.69 ± 0.13	0.75 ± 0.13	ns	0.62 ± 0.11	0.61 ± 0.16	ns	0.62 ± 0.15	0.68 ± 0.12	ns	0.60 ± 0.14	0.60 ± 0.13	ns	0.68 ± 0.13 ab	0.83 ± 0.13 a	***	0.55 ± 0.11 b	0.71 ± 0.20	***
	LS	0.66 ± 0.18	0.67 ± 0.14	ns	0.61 ± 0.13	0.61 ± 0.14	ns	0.60 ± 0.09	0.67 ± 0.10	ns	0.59 ± 0.14	0.54 ± 0.11	ns	0.74 ± 0.16 a	0.75 ± 0.26 ab	ns	0.67 ± 0.15 a	0.70 ± 0.17	ns
	Sign[a]	ns	ns		ns	ns		ns	ns		ns	ns		*	*		***	ns	
Wsk (mJ)	EL	0.71 ± 0.19	0.85 ± 0.18 a	**	0.64 ± 0.19	0.95 ± 0.31 a	ns	0.70 ± 0.23 a	0.86 ± 0.26	*	0.62 ± 0.20	0.77 ± 0.21 a	**	0.60 ± 0.16 b	0.79 ± 0.28 b	**	0.70 ± 0.21 a	0.97 ± 0.31	***
	MM	0.74 ± 0.21	0.88 ± 0.25 a	*	0.67 ± 0.18	0.66 ± 0.23 b	ns	0.59 ± 0.20 a	0.82 ± 0.23	***	0.59 ± 0.21	0.74 ± 0.20 a	**	0.68 ± 0.19 ab	1.00 ± 0.26 a	***	0.52 ± 0.16 b	0.87 ± 0.30	***
	LS	0.64 ± 0.24	0.73 ± 0.17 b	ns	0.65 ± 0.21	0.73 ± 0.24 b	ns	0.59 ± 0.13 a	0.79 ± 0.17	***	0.58 ± 0.20	0.59 ± 0.21 b	ns	0.80 ± 0.25 a	0.88 ± 0.40 ab	ns	0.70 ± 0.24 a	0.81 ± 0.29	ns
	Sign[a]	ns	*		ns	***		*	ns		ns	**		*	*		***	ns	
Esk (N/mm)	EL	0.30 ± 0.04 ab	0.21 ± 0.05 b	***	0.27 ± 0.05	0.19 ± 0.04 b	***	0.28 ± 0.06 a	0.18 ± 0.04 b	***	0.25 ± 0.04	0.18 ± 0.05 a	***	0.31 ± 0.05	0.22 ± 0.03 b	***	0.33 ± 0.05 a	0.20 ± 0.04 b	***
	MM	0.28 ± 0.05 b	0.24 ± 0.04 a	**	0.25 ± 0.05	0.22 ± 0.05 a	**	0.29 ± 0.05 a	0.21 ± 0.04 a	***	0.27 ± 0.03	0.19 ± 0.04 a	***	0.30 ± 0.04	0.26 ± 0.03 a	***	0.26 ± 0.03 b	0.22 ± 0.04 b	***
	LS	0.31 ± 0.06 a	0.24 ± 0.05 a	***	0.31 ± 0.06	0.20 ± 0.03 ab	***	0.26 ± 0.02 a	0.21 ± 0.02 a	***	0.26 ± 0.04	0.20 ± 0.03 a	***	0.29 ± 0.03	0.25 ± 0.05 a	***	0.28 ± 0.03 b	0.24 ± 0.04 a	***
	Sign[a]	*	*		ns	*		*	***		ns	*		ns	**		***	***	
Spsk (µm)	EL	197 ± 41 b	260 ± 42 ab	***	215 ± 45	289 ± 48	**	218 ± 41 b	261 ± 43 b	***	237 ± 39 b	285 ± 50	***	217 ± 39 b	236 ± 47 b	ns	215 ± 34 b	264 ± 41	***
	MM	244 ± 34 a	265 ± 47 a	ns	233 ± 40	279 ± 40	*	262 ± 43 a	271 ± 49 ab	ns	256 ± 42 a	294 ± 45	**	248 ± 49 a	269 ± 46 a	ns	235 ± 45 ab	266 ± 47	*
	LS	220 ± 37 b	235 ± 51 b	ns	221 ± 34	289 ± 50	***	237 ± 26 b	292 ± 48 a	***	261 ± 33 a	290 ± 34	***	232 ± 51 ab	274 ± 49 a	**	252 ± 35 a	270 ± 48	ns
	Sign[a]	***	*		ns	ns		***	*		**	ns		*	**		**	ns	

All data are expressed as average value ± standard deviation (n = 30). Sign: ***, **, * and ns indicate significance at $p < 0.001, 0.01, 0.05$ and not significant differences, respectively, for the differences among the three binomials studied ([a]) and between fresh and withered grapes ([b]) according to ANOVA and T-tests. Different Latin letters in the same column indicate significant differences ([a]) according to Tukey-b test ($p < 0.05$). A = upper-valley vineyard, B = lower-valley vineyard, EL = early harvest/long withering, MM = medium-term harvest/medium withering, LS = late harvest/short withering. Fsk = berry skins break force; Wsk= berry skins break energy; Esk= berry skin resistance against deformation; Spsk = berry skin thickness.

Moreover, it has been demonstrated that the berry skin hardness at harvest affects the dehydration kinetics.[45]

The F_{sk} values detected in fresh grapes in the present study were slightly higher (0.55-0.74 N) than the ranges present in literature on Nebbiolo grapes from Piedmont region (around the range of 0.23-0.55 N), probably for the influence of the Valtellina mountainous growing area, as previously found for Carema mountainous growing area when compared with La Morra and Barbaresco hill areas.[46] Indeed, several studies demonstrated that grape mechanical properties, particularly F_{sk}, are influenced by many variables such as variety, clonal differences, grape-growing location, and environmental conditions.[47, 48] However, among the three harvest points (early, medium, late), no significant differences were found in fresh grapes F_{sk} for vintages 2019 and 2020, confirming that high variability in the skin break force is found in grapes regardless of the changes in soluble solids happening in the advanced phases of grape.[23, 49] However, in 2021 the late harvest points presented a higher F_{sk} value for both the vineyards studied (0.74 and 0.67 N for vineyard A and B, respectively). This trend could be imputable to the dry season, which characterized the year 2021 until the heavy rain event that happened in the first days of October, therefore before A-MM, A-LS, and B-LS sampling points (Figure 2). Indeed, water availability seems to influence the grape skin physical features, especially in the period before the harvest.[48] After the withering process, F_{sk} tended to increase, even if the differences among treatments resulted statistically significant only in

a few cases. However, this phenomenon was more evident in berry skin break energy (W_{sk}), confirming what was previously reported in literature.[50]

The berry skin resistance against deformation (Young's modulus; E_{sk}) decreased significantly from fresh to withered grapes in all tested cases (vintage, vineyard, binomials assessed combinations) (- 24%). However, the length of withering seemed to influence this parameter more than harvest date: at the end of the whole process, EL samples showed generally lower values of E_{sk} than MM and LS berries, resulting in lower skin stiffness.[51] This information could be useful in programming the manipulation activities of grapes especially during the first wine maceration phase, such as the frequency of pumping-over, punching down and *délestage* pomace cap management operations.

As regards berry skin thickness (Sp_{sk}), as already observed for F_{sk}, the values detected on fresh skins in the three years of experiments (197-262 μm) were generally higher than those present in literature for Nebbiolo grapes from other regions. [45,46,52]As expected, Sp_{sk} had an increasing trend from early to late-harvested samples (+10%) and increased (significantly in 12 cases out of 18) from fresh to withered grapes (+17%), as already demonstrated by Rolle et al.[33]. However, the different lengths of the withering process and the high variability of this parameter balanced these differences, often resulting in no significant differences among the binomials at the end of the process.

Considering the different locations, vineyard A presented slightly lower Sp_{sk} values than vineyard B, meanwhile F_{sk} showed the opposite trend (*p*-value of the T-tests < 0.001 for both the parameters). In 2021 this tendency has been less

remarkable than the previous vintages probably for the higher variability of grape samples (*p*-value T-test = 0.09 and 0.308 for F_{sk} and Sp_{sk}, respectively). These mechanical properties may have influenced the extractable phenolic profile, as they are related with the extractability of these compounds, particularly for anthocyanins.[48] Indeed, lower values of Sp_{sk} and higher values of F_{sk} are linked to an easier diffusion of anthocyanins in wine during the maceration phase.[42] The results of phenolic compounds presented in the next section confirm this hypothesis.

3.4. Extractable phenolic composition of fresh and withered grapes

In this section, the results obtained from the analysis of seed and skin extracts using wine-like solution for both fresh and dehydrated samples will be presented and discussed considering the concentration effect in withered grapes and simulating winemaking conditions.

3.4.1. Grape seed extractable phenolics

The content of extractable total polyphenols (TPI), flavonoids (TF), and condensed tannins (MCP) in seeds seemed to show a decreasing trend by leaving the grapes on the plant longer by an averaged value of the three years, respectively, of -15/26%, -27/23%, and -20/28% for vineyards A/B (Fig. 3), in accordance with previous studies about the evolution of phenolic profile along ripening.[53, 54] In seeds, the main phenolic compounds are represented by flavanol monomers and their condensed forms, therefore the trends emerging from the different analysis carried out (TPI, TF, MCP) are generally in accordance

(correlation coefficients of 0.99, 0.88, and 0.89 for TPI-TF, TPI-MCP, and TF-MCP, respectively). The decrease in phenolic compounds observed in this study from early to late harvested samples was previously attributed by Kennedy et al.[55] to oxidation reactions, and it is also probably strongly related to the conjugation with other molecules, which reduced their extractability such as proteins and grape cell wall polysaccharides.[56, 57] This tendency has been observed in all the three vintages considered, but the differences were not always statistically significant due to the sample variability.

The impact of the grape's ripeness degree observed in fresh grapes increased considerably after withering, due to the concentration effect, with percentage amounts comprised from +30 to +109% for TPI, from +21 to +118% for FT, and from +7 to +94% for MCP. Therefore, at the end of the withering process, the greatest phenolic contents extracted from seeds were mainly found for EL and MM grape samples. The highest contents of seeds polyphenols, particularly for condensed tannins (MCP), detected in EL and MM samples after 7 days of maceration make the earlier harvested/longer withered grapes more suitable than the other binomials to produce wines destined to long ageing periods, as it is the case of Sforzato di Valtellina DOCG wines.

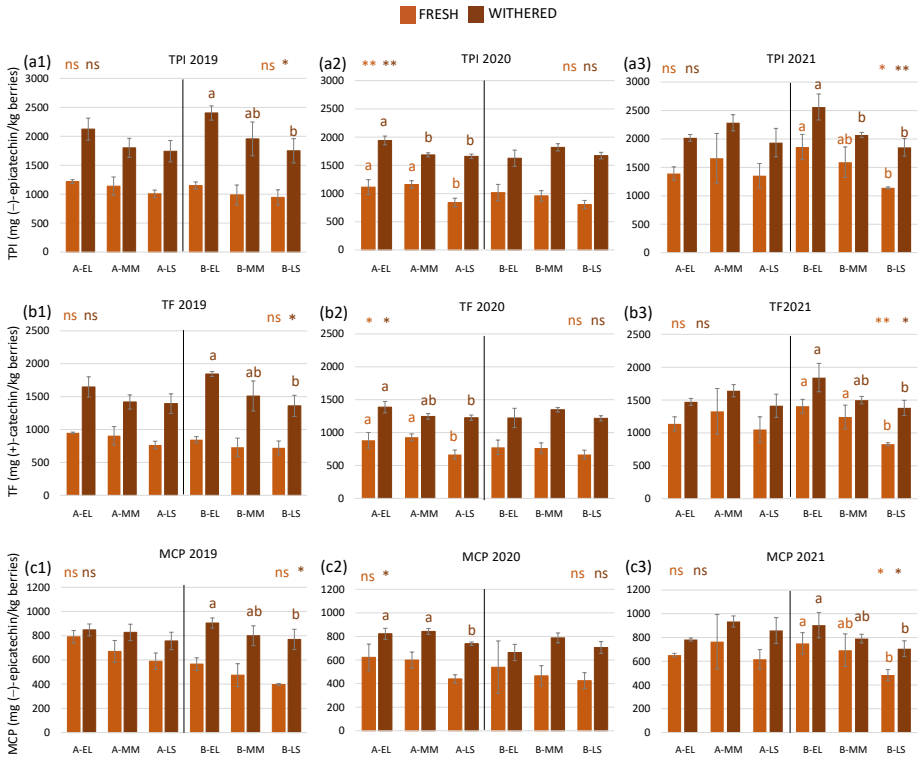

Figure 3. Phenolic profile of fresh and withered grape seeds. TPI: extractable total phenolic compounds (graphs a1, a2, a3 for vintages 2019, 2020 and 2021, respectively); TF: extractable total flavonoid compounds (graphs b1, b2, b3 for vintages 2019, 2020 and 2021, respectively); MCP: extractable condensed tannins determined by methyl cellulose assay (c1, c2, c3 for vintages 2019, 2020, 2021, respectively). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively. Different Latin letters indicate significant differences among the three binomials tested for each vineyard studied according to Tukey-b test ($p < 0.05$). A: upper-valley vineyard, B: lower-valley vineyard, EL: early harvest/long withering, MM: medium-term harvest/medium withering, LS: late harvest/short withering.

3.4.2. Grape skin extractable phenolics

The grape skin extractable total phenolic compounds (TPI) and total flavonoids (TF) appeared less influenced than those of seeds by the harvest period, but their contents expressed on grape weight generally increased after withering, due to the result of a balance between concentration and degradation effects (Figure 4: a1-3 and b1-3).

Extractable anthocyanins (TA) expressed as malvidin-3-O-glucoside equivalent on berries weight (mg/kg; Fig. 4: d1-3) showed no consistent differences among the three harvest points in fresh grape skins of vineyard A in any year under evaluation; meanwhile, a significant increase from early to late harvest was observed in vineyard B during 2020 vintage (from 245 to 310 mg/kg berries), as well in 2021 from early to medium harvest (from 312 to 329 mg/kg berries). The withering process affected the final concentrations of skins TA, without changing the trends observed in the corresponding fresh grapes. Therefore, for these compounds, the harvest time appears to have a higher impact than the withering length on the final extractable content, although the trend was no stable during the vintages or common for the two vineyards evaluated. Hence, considering the risks involved (climate, loss of product, etc.) when leaving the grapes on the vine longer, the wait does not seem to be justified by a real gain in anthocyanin compounds.

Figure 4. Phenolic profile of fresh and withered grape skins. TPI: extractable total phenolic compounds (graphs a1, a2, a3 for vintages 2019, 2020 and 2021, respectively); TF: extractable total flavonoid compounds (graphs b1, b2, b3 for vintages 2019, 2020 and 2021, respectively); MCP: extractable condensed tannins determined by methyl cellulose assay (graphs c1, c2, c3 for vintages 2019, 2020 and 2021, respectively); TA: extractable total anthocyanins (d1, d2, d3 for vintages 2019, 2020, 2021, respectively). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively. Different Latin letters indicate significant differences among the three binomials tested for each vineyard studied according to Tukey-b test ($p < 0.05$). A: upper-valley vineyard, B: lower-valley vineyard, EL: early harvest/long withering, MM: medium-term harvest/medium withering, LS: late harvest/short withering

Moreover, during the first two years of experiments, their content from fresh to withered grape skins experienced a distinct trend for the two vineyards assessed: their concentration increased in withered samples from the vineyard A (upper-valley; from +1% to +22%) and slightly decreased or remained almost constant in those from the vineyard B (lower-valley; from −14% to −1%) except for vintage 2020 B-MM (+6%). The grapes mechanical properties, particularly the higher F_{sk} and the lower Sp_{sk} values found after withering in the vineyard A compared to B, may have promoted enhanced extractable anthocyanin contents in withered grapes, as previously discussed (section 3.3). Instead, in 2021, the high variability likely induced by the dry season of the vintage makes difficult to highlight the trend observed in the previous years. It confirms the greater variability in the grape composition observed in dry years.[58] Therefore, among the compounds analyzed, anthocyanins resulted the most affected by the vineyard and by the vintage effect.

As regards the amounts of extractable condensed tannins determined by methyl cellulose precipitation assay (MCP, Fig. 4: c1-3), the differences among the three harvest dates resulted statistically significant in fresh grapes only in the case of vineyard B in vintage 2021, presenting a slight increase from EL (580 mg/kg berries) to MM (650 mg/kg berries), as observed by Ó-Marques et al.[54] on Cabernet sauvignon and Tinta Roriz varieties with the progress of maturation, but followed by a decrease in LS point to 580 mg/kg berries. However, for MCP the withering length seemed to have a greater influence on the extractable grape skin tannins than the harvest time. Indeed, at the end of the process, EL and MM

binomials often showed the highest concentrations of condensed tannins, as already observed for seeds (with an enhance among EL and LS comprised between +29 and +114 mg/kg of grapes), although not always significant, evidencing an important impact of the concentration effect over degradation during withering. Condensed tannin content has been previously proved to be less affected by dehydration than other phenolic compounds on a dry weight basis.[59] Their main changes are connected with structural modification, as observed previously in dehydrated Nebbiolo grapes at 10% and 20% weight loss,[60] which agrees with this study.

4. Conclusions

In this research, the combined effect of ripeness degree and withering process length has been evaluated on the mechanical proprieties, basic physico-chemical parameters, and extractable phenolic compounds of dehydrated red grapes cv. Nebbiolo. The results obtained show that these two variables can be modulated according to the desired oenological objective, searching for the valorization of grape potentialities.

During the three consecutive vintages, all the analyzed parameters were affected by the close interaction between the harvest time and the withering length. On one hand, the grapes harvested earlier had lower contents of sugar and higher acidity; on the other hand, at the end of withering, EL thesis usually showed the highest values of both sugars and acidity. These observations confirm the great importance of ripeness degree for grapes destined to dehydration, but also the

importance of considering the withering process as the result of a complex balance between concentration and loss of compounds.

Mechanical properties were affected by the combined effect of the studied variables. In particular, the skin stiffness (E_{sk}) resulted generally lower in EL than in MM and LS withered grapes. Skin break force (F_{sk}) and thickness (Sp_{sk}) increased in withered grapes with respect to their fresh counterpart, but the different withering rates tended to compensate the effect of grape ripeness degree, resulting in no consistent differences among the three binomials at the end of the process. To the contrary, the same parameters showed some different trends between the two vineyards tested, influencing anthocyanin extractability. Indeed, among the compounds analyzed, anthocyanins were the most affected by the vineyard position and by the vintage effect, and less by the dehydration in *Sfursat* usual withering conditions.

The content of seed extractable polyphenols showed a decreasing trend by leaving the grapes on the plant longer, whereas the concentration effect enhanced considerably this impact after withering. The extracted skin phenolic compounds were less influenced by harvest period, but their potential impact increased after dehydration. In most cases, EL and MM trials gave withered grapes characterized by higher amounts of extractable phenolic compounds, particularly for seeds and skins condensed tannins. Hence, the choice to anticipate the harvest time for Nebbiolo grapes destined to withering could be preferred in view of a long wine aging, as well as for grape health reasons to avoid possible adverse climate and pests.

The great weather differences among the three vintages studied allowed to highlight the common trends in very different situations, although also the vineyard location influenced the grape features. However, for the same reason, further studies are needed to better clarify the impact of the climate conditions of the year on the combined effect of the two variables studied, or even verify if these results can be extended for other wines made also from partially dehydrated grapes.

CRediT authorship contribution statement

Giulia Scalzini: Formal analysis; Investigation; Data curation; Visualization; Writing - original draft. Simone Giacosa: Conceptualization; Investigation; Methodology; Project administration; Writing - review & editing. Maria Alessandra Paissoni: Conceptualization; Formal analysis; Investigation; Methodology; Writing - review & editing. Susana Río Segade: Conceptualization; Investigation; Supervision; Writing - review & editing. Camilla De Paolis: Formal analysis; Data curation; Writing - review & editing. Domen Škrab: Formal analysis; Data curation; Writing - review & editing. Andrea Zava: Formal analysis; Data curation; Writing - review & editing. Giulia Motta: Formal analysis; Data curation; Writing - review & editing. Lorenzo Ferrero: Formal analysis; Data curation; Writing - review & editing. Sofia Beria D'Argentina: Formal analysis; Data curation; Writing - review & editing. Vincenzo Gerbi: Conceptualization; Supervision; Funding acquisition; Project

administration; Writing - review & editing. Luca Rolle: Conceptualization; Supervision; Funding acquisition; Writing - review & editing.

Conflict of Interest Statement

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

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Chapter III

Modifications of mechanical properties, phenolic extractability, and cell wall polysaccharides in Aleatico winegrapes under different off-vine withering conditions

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ABSTRACT

Aleatico is an Italian red winegrape variety traditionally used to produce Passito wines. The aim of this research is to assess the effect of different withering conditions (natural sun-exposed: SUN, and controlled temperature and humidity conditions: CTR) on grapes physicochemical modifications, focusing on mechanical properties, polyphenol extractions, and cell-wall polysaccharides composition. After withering, CTR skins were significantly more rigid than FRESH and less than SUN ($<E_{sk}$), coherently with pectins modifications. Indeed, after partial dehydration, the phenol extractability decreased in skins and increased in seeds, in both cases greatly for SUN than CTR. Skins extractable phenolic profiles resulted affected by the combination of their loss/concentration balance, together with the modified possibility to extract them, likely due to cell-wall gel network changes for the rearrangement of skins polysaccharides, strongly influenced by the withering conditions. Seeds polyphenols, instead, did not experienced chemical modifications, but their profiles were strongly affected by their modified extractability. These findings provide new awareness on the extractability of phenolic compounds from grapes subjected to different withering conditions.

Keywords: Grape dehydration, postharvest withering, Aleatico, Polysaccharides, Phenolic extractability

1. Introduction

The postharvest withering of winegrapes is an important practice in winemaking which consists in a partial dehydration of the grapes after the harvest inducing metabolic changes that modify their physical and chemical features (Mencarelli and Tonutti, 2013). The special sweet wines produced with withered grapes have generally unique characteristics, which improve their territorial identity and commercial values (Sanmartin et al., 2021).

Among wines produced with withered grapes, sweet red wines are very complex since they are rich in phenolic compounds, which strongly influence the sensory features of wines (Marquez et al., 2014a). In particular, the main classes of phenolic compounds including anthocyanins, which are present in the skins and determine the color traits of wine, monomeric, oligomeric, and polymeric tannins from both skins and seeds, responsible for bitterness and astringency, and other compounds such as phenolic acids which are involved in many crucial reactions (Chira et al., 2009; Scalzini et al., 2021).

During the withering process, the loss of water in the berries occurs, leading to grape weight loss (from relatively low values of 20-30%, reached for most wines, to the highest 40-50% such as for Passito di Pantelleria wines or up to 50% for certain Pedro Ximenez sweet Sherry wines) and causes the concentration of primary and secondary metabolites (Corona et al., 2020; Sanmartin et al., 2021). At the same time, catabolism and oxidation also take place because of the natural senescence of grape berry tissues and of the stress caused by water evaporation

which enhances continuously the concentration gradient (Mencarelli and Bellincontro, 2013). Therefore, the final concentration of grape quality markers, such as sugars, acidity, and phenolic compounds in the berries is the result of a composite balance between (i) concentration and synthesis of metabolites and (ii) loss of compounds caused by oxidation and catabolism (Bonghi et al., 2012; De Rosso et al., 2016). The dehydration kinetics and berry metabolism modifications strongly depend on the withering conditions (i.e. temperature, relative humidity, and air flow speed) and genetic features of grape variety, such as the skin thickness and the attitude to activate different genetic responses (Zenoni et al., 2016; Rolle et al., 2009). Moreover, under the same environmental conditions, specific classes of phenolic compounds are not affected in the same manner by the process (Toffali et al., 2011).

In addition, the compounds present in grapes are not all extracted in the same way. The extractability of phenolic compounds is strongly influenced by the cell wall structure. Indeed, it is influenced by cell wall porosity, as well as the binding of polyphenols with cell wall proteins and polysaccharides, and by the modifications of mechanical features induced by their rearrangements (Bindon et al., 2014; Halin et al., 2010; Rio Segade et al., 2019a). Therefore, in addition to the challenge of studying the withering complex metabolic modifications, there is the added difficulty of the changeset of extractability due to the adaptation of grape berry tissues to dehydration. Many studies have focused on the chemical modification of phenolic compounds during dehydration (Figueiredo-Gonzales et al., 2013), but very few were specifically dedicated to the effect of the modified extractability from the different parts of the grape berry, especially for seeds (Rio

Segade et al., 2016). Fasoli et al. (2019) highlighted a detailed understanding of cell wall biochemical changes and polysaccharide rearrangements during withering in the skins of cultivar (cv.) Corvina. Nevertheless, Zoccatelli et al. (2013) pointed out that postharvest withering affects pectin metabolism in a cultivar-specific way related to the kinetic of the water loss. Moreover, no study has been performed until now on the influence of different withering conditions on the polysaccharide composition of dehydrated grapes, and a complete understanding is still missing.

In the present study, two withering systems were assessed on Aleatico grapes: sun-withering practice and withering technique in controlled conditions. Aleatico (*Vitis vinifera* L.) was chosen, as a typical Italian winegrape variety with peculiar polyphenolic features, which are strongly affected by the withering technique. Indeed, Aleatico is an aromatic red variety traditionally used to produce high-quality sweet wines with postharvest withered grapes (Mencarelli et al., 2010, Frangipane et al., 2012). It is mainly cultivated in the warm area of central and southern Italy, but it is also present with a few hectares in France (Corse), Malta, California, Chile, Australia, and in the central Asian republics of Kazakhstan and Uzbekistan, due to its tolerance to poor soils and water stress conditions (Anderson et al., 2013; Robinson et al. 2015; Tuccio et al., 2011). It is rich in phenolic compounds, with low anthocyanin content, characterized by a majority of tri-substituted forms in the anthocyanin profile (Bellincontro et al.,2006; Tuccio et al., 2011). Traditionally on the Elba Island, one of the regions where Aleatico is most widespread and historically produced, the withering

occurred under the sun with overnight cover to protect them from the dew. More recently, withering under controlled conditions has spread (Frangipane et al., 2012).

The aim of the research was to examine the effect of two very different withering modalities on the potential and extractable phenolic profile of Aleatico winegrapes. The expected contribution is to provide new insights on the understanding of the modified extractability of phenolic compounds from the distinct parts of grapes subjected to very different withering conditions in order to add new awareness to the comprehension of the withering complexity both from a chemical and a practical winemaking point of view.

2. Materials and methods

2.1. Grape materials and withering process conditions

In 2021, about 100 kg of Aleatico red winegrapes (*Vitis vinifera* L.) were harvested at normal technological maturity (historically 24-25 °Brix, pH 3.25-3.35, 5.5-6.0 g/L of titratable acidity expressed as tartaric acid) by ten-years-old grapevines from a commercial vineyard managed with organic cultural practices located in Castagneto Carducci (Tuscany, Italy, latitude 43°09.936' and longitude 10°37.845', and 200 m altitude above sea level). Once harvested on 15th September 2021, a grape sample of 5 kg was randomly collected for the analysis of fresh material before the withering (fresh sample, FRESH). Then, the remaining 100 kg of grape bunches were placed in 40 single-layer IP/4615/UVA perforated plastic crates (about 2.5 kg of whole bunch clusters each, 400x600x15h mm, Plastic Boxes Srl, Castagnaro (VR), Italy). 20 perforated

boxes were placed in a controlled dehydration room at about 22°C, 60% relative humidity (RH) and 1.5 m/s air flow speed (controlled withering, CTR). At the same time, the other 20 boxes were exposed to the sun and covered with plastic panels during the night to protect them from dew during the withering period (sun withering, SUN). Thermo-hygrometers withering conditions were measured using a Temperature and Humidity Smart Sensor IBS-TH1 (-40-100 °C ± 0.5 °C, 0-100% ± 6% RH, Inkbird Tech. C.L., Shenzen, Hong Kong, China). Air flow speed in the controlled dehydration room was measured with the aid of a Mini Anemometer UT363BT (0-30 m/s ± 0.1 m/s, Uni-Trend Technology, China). The weight loss percentage (WL%) was calculated as [1−(net weight of withered grapes in kg/net weight of fresh grapes in kg)] using the weight data measured with a ODECA ACS-30Z precision electronic balance with a maximum weight capacity of 30 kg ± 1 g (Odeca Srl, Varese, Italy), by comparing the initial weight of eight sample boxes marked with a code for each withering method with the weight periodically measured for the same crates. The CTR and SUN samples were withered until reaching the 30% WL for both the two different systems.

2.2. Sample preparation and grape must technological parameters

For each sample (FRESH, SUN, and CTR at 30% WL), the berries were carefully separated from the stalk, cutting them near to the pedicel, and visually inspected to eliminate the damaged ones. Then, three replicates of 150 g of berries were randomly collected and carefully manually crushed for the technological analyses. The musts obtained were centrifuged at 3000 rpm for 15 minutes a 20 °C in a Hettich 32R centrifuge (Tuttlingen, Germany). An aliquot of supernatant

has been used to evaluate pH by potentiometry with an InoLab 730 pHmeter (WTW, Weilhelm, Germany), titratable acidity (as g/L of tartaric acid) by titration with sodium hydroxide 0,1 N, according to OIV method (OIV, 2016) and total soluble solids (°Brix) using a refractometer Atago Palette 0-32 with automatic temperature compensation (Atago Corporation, Tokyo, Japan).

2.3. Evaluation of berry mechanical properties

Two sets of 40 berries were randomly collected for each test on fresh and withered samples and individually subjected to the texture tests (i.e. puncture, compression, and traction test). Grape mechanical properties were evaluated using a TAxT2i Universal Texture Machine (Stable Micro Systems, Godalming, Surrey, United Kingdom) equipped with a 5 kg load cell and an HPD/90 platform. Stable Micro Systems probes have been mounted on the instrument, according to the test to be performed. In particular, a SMS P/2 N needle probe (Ø 2 mm) was used for the puncture test, a flat probe (Ø 2 mm) for the compression test, and an A/PS probe modified with a rigid arm for the traction test, in order to evaluate the skin hardness (F_{sk}, N; W_{sk}, mJ), thickness (Sp_{sk}, μm) and the peduncle detachment resistance (F_{ped}, N), respectively. The conditions applied were the same as described by Giacosa et al. (2019). The data were acquired using the software Texture Expert Exceed, version 2.54 (Stable Micro Systems, Godalming, UK).

2.4. Extraction of potential and extractable phenolic compounds

To evaluate the extractability of phenolic compounds, two different types of extractions were performed.

A simulated maceration was conducted separately for skins and seeds to evaluate the extractable phenolic profile of the two components in wine-like conditions. To carry out this goal, a wine-like buffer solution without ethanol was prepared (pH 3.5, 5 g/L tartaric acid, 100 mg/L sodium metabisulphite). For each sample, three replicates of 80 g of berries were weighted and manually peeled with the aid of a laboratory spatula. Skins and seeds were carefully separated, cleaned from the flesh, and each component was immediately immersed in 100 mL of buffer solution, in accordance with the liquid-to-solid ratio estimated by Mattivi et al. (2002). To simulate the wine fermentative maceration, skins and seeds were extracted separately throughout 10 days at 25 °C with stepwise addition of absolute ethanol at 24, 48, 96, 144 and 192 h of maceration to reach at the final point an ethanol concentration of 15% v/v (+2, +2, +4, +4, +3% v/v of ethanol addition, respectively). Every day, the extracts were homogenized twice with a magnetic stirring bar (20x6 mm) contained in each flask. Before each addition, an equal aliquot of extract sample was taken to maintain constant the volume of the macerating solution. The aliquots were used to study the extraction kinetics of anthocyanins, color, and total polyphenols. At the end of the simulated macerations (10 days), the whole liquid extracts were collected for more in depth analysis to investigate thoroughly their phenolic profile.The results were expressed as mg/g of skins or seeds using the respective weight of skins and seeds measured for each replicate, or as mg/kg of berries using the relative grape weight for each sample.

To extract the whole phenolic compounds potentially present in the grapes, three sets of 30 berries for each sample were randomly selected and

weighted following the method described by Abi-Habib et al. (2021). Frozen skins, seeds and flesh were gently ground to a fine powder in a liquid nitrogen mill with the aid of a Pulvérisette 2 mortar grinder (10-20 µm final finesses, 6 – 8 mm feed size, FRITSCH, Idar-Oberstein, Germany). 150 mg of frozen skins powder or 100 mg of frozen seeds powder were treated with 750 µL of methanol, then added with 5.25 mL of acetone/water/formic acid extraction solvent (60:40:1 v/v). In the case of the flesh, 100 mg of flesh powder were added with 600 µL of methanol and 3.4 mL of the extraction solvent. 10 marbles (Ø 11 µm) were added in each tube to stir the solution during the extraction-homogenization phase, which was performed at 20°C using a Precellys 24 orbital shaker (Bertin Technologies, Saint-Aubin, France) using the program 5000-3*40-20. Then, the extracts were centrifuged at 4 °C (3000 rpm for 5 min for skins, 5000 rpm for 5 min for the flesh, and 6000 rpm for 15 min for seeds, respectively) in a Hettich 4-16 KS centrifuge (Tuttlingen, Germany). The supernatant of each sample was redistributed in 1 mL aliquots and dried under vacuum at 35 °C for 2h in an EZ-2 plus Genevac rotatory evaporator (Warminster, USA). For every replicate of each sample, the extraction was performed in three replicates, to avoid adding variability due to the extraction phase. The dried extracts were redissolved in a wine-like solution (15% v/v, 5 g/L tartaric acid, pH 3.5, and 100 mg/L sodium metabisulphite) for UV-visible spectrophotometry analysis, a water/methanol/formic acid solution (50:50:1 v/v) for HPLC, or dimethyl formamide for High Performance Size Exclusion Chromatography (HPSEC) analysis.

2.5. Phenolic compounds analysis

Total anthocyanins (TA), total phenolic index (TPI), condensed tannins, also called proanthocyanidins, (MCP) and low molecular weight tannins as Flavanols Reactive to Vanillin (FRV) were determined by UV-visible spectrophotometry methods (Petrozziello et al., 2018) with a UV-1800 spectrophotometer (Shimazdu Corp., Kyoto, Japan). TA (mg of malvidin-3-O-glucoside/kg of grape berries) and TPI (mg of (-)-epicatechin/kg of grape berries) were evaluated at 536-540 and 280 nm, by diluting the sample with an ethanol/water/37% hydrochloric acid (70:30:1 v/v) and water, respectively. Condensed tannins (MCP, mg of (-)-epicatechin/kg of grapes) were quantified by methyl cellulose precipitation assay according to Sarnekis et al. (2006) method. FRV (mg of (+)-catechin/kg of grape berries) were determined by Torchio et al., 2010 method.

Anthocyanin profile and phenolic acids were analyzed by HPLC using a Waters HPLC-DAD system and a reversed-phase dC18 column Atlantis T3 (2,1x250 mm i.d.) with a guard column of the same material (Waters Corporation, Milford, USA). The samples were directly injected (5 µL), with a flow rate of 0.25 mL /min at 30 °C. The mobile phase consisted of A = formic acid/water (5:95 v/v) and B = acetonitrile/water/formic acid (80:15:5 v/v). Proportions of solvent B work in gradient mode from 0% for 5 min, increased up to 10% in 30 min, to 20% in 30 min, and to 100% 5 min (Fournand et al., 2006). Individual anthocyanins were quantified at 520 nm (malvidin-3-O-glucoside equivalents) and phenolic acids at 320 nm (caftaric and coutaric acid equivalents).

The size distribution of polymeric tannins was determined by high pressure size exclusion chromatography (HPSEC) using an Agilent 1260 HPLC system,

equipped with 3 PLgel columns (Phenomenex) composed of highly cross-linked polystyrene-divinylbenzene (300x7.8 mm, 5 µm, 50 and 100 Å pore size, respectively) connected in series with a guard column of the same material, according to the method described by Vernhet et al. (2020). The mobile phase (DMF) was composed of dimethylformamide, 1% v/v glacial acetic acid, 5% v/v water, and 0.15 M lithium chloride. The flow rate was 0.8 mL/min and the column temperature 60 °C. The columns were calibrated using commercial standards (ellagic acid, catechin, epicatechin, dimer B2 and trimer C1) as well as tannins fractions of different DP (degree of polymerization, the number of flavanol units in a compound), and fractions of anthocyanins purified in the laboratory (Gomez et al., 2009; Vernhet et al., 2014). These injections allow to perform an epicatechin equivalent quantification and DP estimation on the different samples using the calibration curve to evaluate the molar mass of tannins as a function of the retention time by following the relationship log (Mw)=f(Rt).

2.6. Color features determination

The study of the color of the skin extracts was carried out following the OIV methods (2016). In particular, the visible spectra (380-780 nm) of the undiluted samples were acquired at each maceration sampling point and at the end of the simulated maceration, using 1 mm optical path cuvettes. CIEL*a*b parameters were calculated as lightness (L*), red/green color coordinate (a*), and yellow/blue color coordinate (b*) were calculated following the OIV-MA-AS2-11 method. The color difference between control and threated samples (ΔE*) was calculated following the relationship ΔE* = [(ΔL*)2 + (Δa*)2 + (Δb*)2]1/2.

Color intensity (A420 nm + A520 nm + A620 nm on an optical path of 10 mm) and tonality (A420 nm/A520 nm) were evaluated according to the OIV-MA-AS2-07B method.

2.7. Preparation of skin alcohol-insoluble cell wall material

The alcohol insoluble solids (AISs) were isolated from the frozen skins and flesh powders according to the procedure described by Apollinar-Valiente et al. (2010), applying the modifications adopted by Abi-Habib et al. (2021). For each sample replicate, 5 g of skins frozen powder of 10 g of flesh frozen powder were suspended in 15 mL of boiling water for 5 min and homogenized with a magnetic stirrer to inactivate the enzymes. One part of the homogenized material was mixed with two parts of 96% ethanol for 30 min at 40 °C in a Polystat I 33194 220V heating circulator (Fisher Bioblock Scientific s.a., Illkirch, France). The AISs were centrifuged (8500 rpm, 10 min, 20 °C), the supernatant liquid was discarded and the washed sludge was again extracted with 70% ethanol for 30 min at 40 °C. This operation was repeated until no more sugar was detected in the liquid phase with the sulphuric phenol method (Dubois et al., 1956). The numbers of consecutive extractions needed to eliminate all sugars were in total 5 for skins, 6 for fresh flesh, and 11 for withered flesh. Then, AISs were further washed twice with 96% ethanol and once with acetone. After drying under air flux overnight to evaporate the acetone, the purified AISs powders obtained were used for the analysis of polysaccharides composition.

2.8. Polysaccharides composition of AISs determined by Gas chromatography methods

The carbohydrate composition of the skins and flesh AISs was estimated by three gas chromatography (GC) methods with a Shimadzu GCMS-QP2010SE gas chromatograph equipped with a capillary split/plitless inlet and a flame ionization detector (FID, Shimadzu, Kyoto, Japan) using a DB225 capillary column (30 m x 0.25 mm i.d., 0.25 μm film) or DB-1 capillary columns (30 m _ 0.25 mm i.d., 0.25 mm film) and hydrogen 5.6 B50 as the carrier gas.

To study the molar percentage composition of neutral and acidic glycosyl-residue of AISs polysaccharides, the per-*O*-trimethylsylated methyl glucoside derivatives (TMS) method was applied. Methanolysis for 16h at 80°C and trimethylsilylation were performed following the method detailed by Doco et al. (2001).

To quantify the neutral glycosyl-residue content of pectic and hemicellulose polysaccharides, the conversion of neutral sugars into volatile alditol acetate derivatives was performed after polysaccharide hydrolysis with trifluoroacetic acid (TFA, 120 °C, 75 min), as described by Apolinar-Valiente et al. (2015). 100 μL of 1 mg/mL inositol and allose solutions were used as internal standards. In combination with the alditol acetates procedure, a further hydrolysis with 72% sulphuric acid was applied to quantify the cellulose present in AISs carbohydrates, according to the method proposed by Seaman et al. (1945).

2.9. Comprehensive Microarray Polymer Profiling analyses (CoMPP)

For the purification phase, 10 g of flesh frozen powder or 2 g of skins frozen powder were mixed with 30 mL of absolute ethanol and immediately transfer to 80 °C water-bath for 15 min to inactivate the enzymes. Cooled to room temperature, each of the three replicates for every sample was centrifuged (3000 rpm, 4 °C, 10 min) in a Hettich 4-16 KS centrifuge (Tuttlingen, Germany) and the supernatant was discarded. The remaining pellet was subjected to a series of solvent washes using methanol, chloroform, and acetone according to the method described by Gao et al. (2015). The liquid-to-solid ratio was 3:1 (v/v) for each of the six washing-step. Then, the pellet was dried for 20 min in the fume hood and re-suspended in ice-cold milliQ water and freeze-dried to generate the alcohol insoluble residue (AIR) for the Comprehensive Microarray Polymer Profiling analysis (CoMPP).Once freeze-dried, 10.00 mg of AIR for each replicate were precisely weighted in 1.2 mL polypropylene Collection Microtubes (QIAGEN, Hilden, Germany) and extracted in 300 µL of cyclohexane-diamino-tetraacetic acid (CDTA, 50 mM, pH 7.5). Each biological replicate was used to generate 6 replicates. A glass bead was added to each tube and the extraction solution have been homogenized at a frequency of 30 Hz for 2 min and then 6 Hz for 2 h. The soluble pectin-rich fraction (CDTA-fraction) obtained were collected after centrifugation by taking the supernatant. The remaining pellet was re-extracted using 300 µL of NaOH (4 M + 0.1% $NaBH_4$) following the same agitation procedure, obtaining a hemicellulose-rich fraction (NaOH-fraction). Pectin and hemicellulose-rich fractions (CDTA-fraction and NaOH-fraction respectively) were pipetted into 384-microwell plates and printed into nitrocellulose membrane (0.45 mm pore size, Whatman, Maidstone, UK) using a microarray robot (Sprint,

Arrayjet, Roslin, UK). The printed arrays were incubated with several monoclonal Antibodies (mAbs) and Carbohydrate Binding Modules (CBMs) listed in Table S1. The arrays were scanned at 2400 dots/inch with a CanoScan 880F, Soborg, Denmark). The software Array-Pro Analyzer 6.3 was used to quantify probe signals (Media Cybernetics, Rockville, Maryland, USA), as described by Gao et al. (2021). The raw data were normalized and converted into a heatmap for visualization. The relative abundances of different polymers epitopes are displayed on a scale of 0–100. The values are means from three biological repeats and four dilutions.

2.10. Statistical Analysis

Statistical analysis was carried out using R statistic software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). The homoscedasticity and normality of the data were tested by using Levene's and Shapiro Wilk's tests. One-way analysis of variance (ANOVA) using the Tukey HSD post-hoc test was used to evaluate significant differences among treatments. Differences were considered statistically significant at p-value < 0.05. T-test was used to discriminate significant differences between the two different withering conditions. Pearson's correlation coefficients were calculated to determine significant relationship between seeds phenolic composition and extraction yields, and tannin composition quantified by SEC and spectrophotometric analyses. Principal component analysis (PCA) was performed on the raw data set of CoMPP results of the AISs skins and flesh cell walls to better understand the differences among treatments.

3. Results and discussion

Figure 1 shows the environmental conditions recorded during the withering period under sun uncontrolled withering (SUN, a) and controlled withering in the "*fruttaio"* dehydration room (CTR, b). The graphs reported the daily minimum, maximum and average values of temperature (°C) and relative humidity (%). The grapes withered outside under the sun were subjected to very variable conditions ranging between 14 and 40 °C of temperature (although only for short periods up to 35 °C) and 38-98 % relative humidity. For SUN the withering lasted 15 days in total until reaching the 30% WL foreseen by the experimental plan. Instead, the controlled environmental conditions of CTR had a lower range of variation (22 ± 1 °C temperature range and 61-70% relative humidity) and the achieving of 30% WL took 17 days in total.

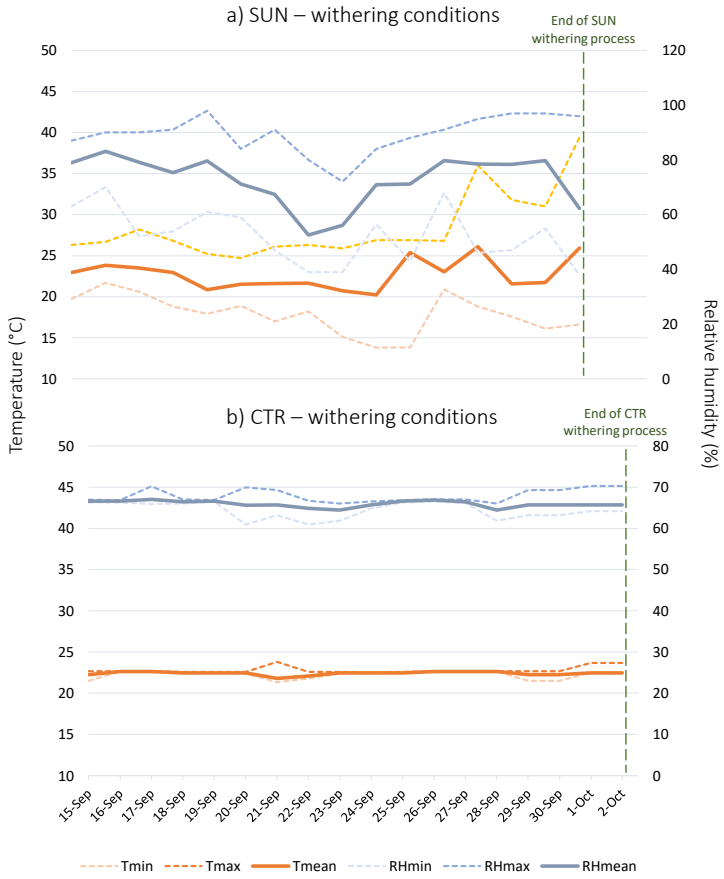

Figure 1 – Climatic conditions of uncontrolled-sun (SUN, a) and controlled-*fruttaio* (CTR, b) withering. Tmin: minimum daily temperature; Tmax: maximum daily temperature; Tmean: average daily temperature; RHmin: minimum daily relative humidity, RHmax: maximum daily relative humidity; RHmean: average daily relative humidity values.

3.1. Grape must composition and Texture analysis

Grape must technological parameters and mechanical properties performed before the withering (FRESH) and after the dehydration process in the two modalities (SUN and CTR) are shown in Table 1. As expected, significantly higher total soluble solids (°Brix) contents were found in withered grapes with

respect to the fresh ($p<0.01$), moving from 24.7 to 38.9 °Brix as the maximum value in grape SUN. Considering the same weight loss of 30% achieved from the grape at the end of the process, different dehydration techniques did not determine statistically significant differences at the same (p-value of the T-test SUN vs CTR = 0.253).

The pH was significantly higher in SUN samples than CTR (T-test p-value < 0.01). Its value increased significantly in SUN-withered grapes (+0.32 and +0.25 pH units compared to FRESH and CTR grapes, respectively), whereas CTR pH experienced a slight increase (+0.07) but did not significantly change compared to the FRESH. The increase in pH during withering has already been observed in previous studies (Bellincontro et al., 2016) on Corvina, Corvinone, and Rondinella, and is probably due to a greater release of cations (mainly K^+) from the cell's vacuoles of flesh and skins, which try to compensate for water loss to maintain the pressure of cellular turgor (Riberau-Gayon et al., 2006; Zoccatelli et al., 2013). Indeed, berry pH is a function of the levels of organic acids and cations (Boulton, 1980) and the climate features (i.e. higher temperature and sun exposure, different relative humidity values) affect indirectly the pH via the levels of acids and cations uptake during ripening in the vineyard (Barnuud et al. 2014). Thus, possibly, in the same way, temperature and sun-exposure may have affected the sun-withered grapes during post-harvest dehydration. This aspect could explain the differences between the two withering modes studied in the present experiment.

Also, titratable acidity was affected differently by the two withering modalities (T-test p-value<0.01): in CTR grapes it was preserved and concentrated (moving

from 5.79 of the FRESH to 6.14 g/L of the CTR expressed as tartaric acid), while SUN samples experienced a loss in titratable acidity after the sun-withering with respect to the FRESH (from 5.79 of FRESH to 5.08 g/L tartaric acid of SUN grapes). In fact, as demonstrated in other studies, titratable acidity may increase or decrease depending on the temperature of dehydration (Chkaiban et al., 2007; Constantinou et al., 2017).

Tab. 1 – Standard chemical parameters and mechanical properties of Aleatico grapes at harvest (FRESH) and after withering at 30% WL under sun natural withering (SUN) and controlled conditions in *fruttaio* (CTR).

Compound	*fresh grapes*	*withered grapes 30 % WL*		Sign.[a] ANOVA	Sign.[b] T-test
	FRESH	SUN	CTR		
Grape must technological parameters					
Total soluble solids (°Brix)	24.7 ± 0.4 b	38.9 ± 1.5 a	37.4 ± 1.2 a	***	ns
pH	3.31 ± 0.02 b	3.63 ± 0.04 a	3.38 ± 0.05 b	***	**
Titratable acidity (g/L tartaric acid)	5.79 ± 0.11 b	5.08 ± 0.13 c	6.14 ± 0.06 a	***	**
Mechanical properties					
F_{sk} (N)	0.63 ± 0.12	0.73 ± 0.20	0.64 ± 0.17	ns	ns
W_{sk} (mJ)	0.66 ± 0.22 b	1.01 ± 0.40 a	0.97 ± 0.41 a	***	ns
E_{sk} (N/mm)	0.27 ± 0.05 a	0.20 ± 0.04 b	0.16 ± 0.03 c	***	**
Sp_{sk} (µm)	232 ± 47 b	304 ± 56 a	290 ± 55 a	***	ns
F_{ped} (N)	2.24 ± 0.57 a	1.36 ± 0.50 b	1.29 ± 0.47 b	***	ns
W_{ped} (mJ)	1.31 ± 0.60	1.38 ± 0.58	1.44 ± 0.85	ns	ns

All data are expressed as average value ± standard deviation ($n = 3$ and n=40 for Grape must technological parameters and Mechanical properties, respectively). Sign: ***, **, and ns indicate significance at $p < 0.001$, 0.01, and not significant differences, respectively, for the differences among treatments (FESH, SUN and CTR) and between the two withering modalities (SUN, CTR) according to ANOVA([a]) and T-tests ([b]). Different Latin letters among the same raw indicate significant differences ([a]) according to Tukey-b test ($p < 0.05$) for ANOVA. F_{sk}: berry skin maximum break force; W_{sk}: berry skin break energy; E_{sk}: material resistance against deformation; Sp_{sk}: berry skin thickness; F_{ped}: force of peduncle detachment; W_{ped}: energy needed for peduncle detachment.

Regarding mechanical properties, no significant differences among samples were found for the maximum break force values (F_{sk}), whereas the break energy (W_{sk}) was significantly higher for withered grapes with respect to the fresh (ΔW_{sk} = 0.33-0.31 mJ), while not statistically differences between the two dehydration modalities were found (T-test p-value = 0.725), probably due to the high variability among the berries. Similarly, Sp_{sk} showed increased values in withered grapes with respect to the fresh material (+31 and +25% for SUN and CTR, respectively) coherently with the previous observations of several authors

on Mondeuse, Nebbiolo and Barbera on-vine and off-vine dehydrated grapes (Rolle et al., 2009; Rio Segade et al., 2019a), but no significant differences were found between SUN and CTR at the same WL% (T-test p-value = 0.356). FRESH Aleatico featured skin hardness, assessed by F_{sk} and W_{sk} parameters) and thickness (Sp_{sk}) values very similar to cv. Moscato white grapes (Giacosa et al., 2019), of which Aleatico is genetically descendant (D'Onofrio et al., 2021).

The skins resistance to the axial deformation during the puncture test (E_{sk}), instead, experienced significant differences among the withering modalities studied. Indeed, Aleatico E_{sk} decreased significantly after dehydration for both the methods, particularly for CTR (-27 and -41% from fresh to SUN and CTR, respectively). Therefore, the withering increased skins elasticity in Aleatico berries. Interestingly, grape skins withered under controlled conditions were significantly less rigid than sun-withered samples (T-test p-value < 0.01). These differences could be due to the reorganization of skins cells and to the modifications of cell wall polysaccharides. Indeed, Fasoli et al. (2019), studying the morphological alterations in skins sections of cv. Corvina during withering, evidenced a gradual cell disorganization from fresh to different postharvest whitening phases which could explain the differences showed between fresh and withered samples. Moreover, the differences observed between the two withering techniques may be explained by the modification of alcohol insoluble cell wall solids (AISs) polysaccharides. A possible explication may be imputable to the higher de-methylation of pectins observed in SUN with respect to CTR (see Figure 4). Indeed, it has been proven that homogalacturonans de-esterification releases COO^- functions, which allows the formation of egg-boxes through Ca^{2+}

bridges. This plays acrucial role in the structural features of the cell walls, increasing their rigidity (Basak et al., 2014).

The peduncle detachment force-time curves obtained by traction (tension) test showed a strong decrease of pedicel detachment force (F_{ped}) after grape dehydration in both the two modalities (-39 and -42% for SUN and CTR, respectively), with no statistically significant differences between them (T-test p-value = 0.5333). The average values measured at 30% WL for both SUN (1.36 N) and CTR (1.29 N) treatments suggested that, regardless of the withering modality, Aleatico berries had a great propensity to fall throughout the process if subjected to vertical bunch placement such as hanged clusters system or on-vine postharvest ripening (Rolle et al. 2010). Thus, it would be more suitable to place Aleatico grapes in horizontal bunches post-harvest withering systems such as single-layer plastic boxes or reed mats, independently of the withering conditions chosen.

3.3. Potential and extractable phenolic profiles

The different fractions of skins and seeds phenolic potential and extractable profiles at the end of the wine-like macerations were studied (Table 2). Skins and seeds extraction yields (%) and phenolic acids flesh composition were also evaluated.

With the aim of showing changes from an oenological applicative point of view, the data of Figure 2 (a, c) were expressed in mg/kg of berries. Therefore, these data refer to the winemaking conditions, considering the loss of juice inside the

dehydrated berries which allowed the concentration of some compounds in the final wine, such as already observed for sugars (Table 1). The extraction yields (%) shown in Table 2 were calculated using the data expressed as mg/kg of berries as well, to provide oenologists practical tools in the choice of the maceration strategy for withered grapes.

However, it needs to be emphasized that the final content of phenolic compounds in withered grapes is the result of a balance between concentration and metabolic losses of compounds occurring within the berry during the withering process (De Rosso et al., 2016). For this reason, the data of the detailed potential and extractable phenolic profiles at the end of the simulated maceration were expressed in Table 2 per grape-portions weight (mg/g of skins, seeds, or flesh), to avoid the influence of the whole berry weight loss and consider the chemical modifications occurring within the different berry's portions due to metabolism separately from the concentration effect.

Skins anthocyanins are the clearest example of the modifications described above. In fact, when the TA potential are expressed as mg/g skins (Table 2), sun-withered grapes showed the lowest TA level, which was statistically different with respect to the FRESH, and slightly to CTR (ANOVA p-value<0.05; T-test SUN vs CTR p-value=0.049). TA (mg/g skins) decreased after withering also in CTR sample, but to a lesser extent than in SUN one. There was then not statistically different from FRESH. In fact, as demonstrated by Mencarelli et al. (2010), the withering temperature plays a central role in the anthocyanin losses after dehydration, which are probably more strongly degraded at higher temperatures due to the oxidative activity of enzymes such as polyphenol oxidase

(PPO) or peroxidase, and maybe also to a decrease of their possible synthesis as the gene expression of phenylalanine ammonia lyase (PAL) was downregulated. Interestingly, when expressed in mg/kg of berries (Table S3), potential TA tended to slightly increase by an average of +108 mg/kg for CTR (average withering temperature 22.5 °C) and to decrease for SUN (average withering temperature 27°C) by -67 mg/kg berries with respect to the FRESH. This suggests that the concentration effect compensated for the slight losses of TA (mg/g skins) in CTR, but it was not enough to offset the greater loss of anthocyanins in SUN.

Tab. 2 – Potential and extractable phenolic composition at the end of the simulated maceration in a wine-like solution of fresh (FRESH), and withered Aleatico grapes under the sun (SUN) or in *fruttaio*-controlled conditions (CTR).

Compound	Potential/Extractable	*fresh grapes*	*withered grapes*		Sign.[a] ANOVA	Sign.[b] T-test
		FRESH	SUN	CTR		
Skins						
	Potential	41 ± 3 a	30 ± 3 b	35 ± 3 ab	*	*
TPI (mg EC/g skins)	Extractable	28 ± 3 a	11 ± 0 c	18 ± 1 b	***	***
	Extraction yield (%)[#]	60 ± 5 a	40 ± 6 c	49 ± 1 b	***	***
	Potential	6 ± 1 a	4 ± 0- b	5 ± 1 ab	**	*
TA (mg Mv-3-G/g skins)	Extractable	5 ± 0 a	2 ± 0 c	3 ± 0 b	***	***
	Extraction yield (%)[#]	72 ± 8 a	46 ± 9 b	55 ± 4 b	***	*
	Potential	17 ± 2	14 ± 1	14 ± 1	ns	ns
MCP (mg EC/g skins)	Extractable	8 ± 0 a	3 ± 0 c	5 ± 0 b	***	***
	Extraction yield (%)[#]	41 ± 4 a	29 ± 4 b	33 ± 1 b	***	ns
	Potential	7 ± 0 a	3 ± 0 c	5 ± 1 b	***	*
FRV (mg C/g skins)	Extractable	5 ± 0 a	1 ± 0 c	3 ± 0 b	***	***
	Extraction yield (%)[#]	57 ± 3	58 ± 7	60 ± 14	ns	ns
FRV/MCP	Potential	0.42± 0.06 a	0.21± 0.02 b	0.32± 0.08 ab	*	ns
	Extractable	0.58 ± 0.02 a	0.43 ± 0.02 b	0.58 ± 0.01 a	***	***
Anthocyanin profile (mg Mv-3-G/g skins)						
Dp-3-G	Potential	0.25 ± 0.04 a	0.08 ± 0.01 b	0.13 ± 0.02 b	**	*
Cy-3-G		0.04 ± 0.01 a	0.02 ± 0.00 b	0.02 ± 0.00 b	*	ns
Pt-3-G		0.28 ± 0.04 a	0.11 ± 0.02 b	0.16 ± 0.03 b	**	ns
Pn-3-G		0.26 ± 0.05 a	0.13 ± 0.02 b	0.13 ± 0.03 b	*	ns
Mv-3-G		2.95 ± 0.52 a	1.40 ± 0.19 b	1.97 ± 0.31b	**	ns
∑ Acetyl-G		0.62 ± 0.11 a	0.23 ± 0.03 b	0.33 ± 0.07 b	**	ns
∑ Cinnamoyl-G		1.21 ± 0.97 a	0.60 ± 0.10 b	0.91 ± 0.13 ab	**	*
Dp-3-G	Extractable	0.08 ± 0.02 a	0.01 ± 0.00 b	0.02 ± 0.00 b	***	ns
Cy-3-G		0.02 ± 0.01 a	0.00 ± 0.00 c	0.01 ± 0.00 b	***	**
Pt-3-G		0.13 ± 0.02 a	0.03 ± 0.00 c	0.05 ± 0.00 b	***	**
Pn-3-G		0.16 ± 0.02 a	0.03 ± 0.01 b	0.05 ± 0.01 b	***	*
Mv-3-G		2.16 ± 0.23 a	0.58 ± 0.04 c	1.06 ± 0.13 b	***	**
∑ Acetyl-G		0.38 ± 0.04 a	0.08 ± 0.00 c	0.16 ± 0.02 b	***	**
∑ Cinnamoyl-G		0.42 ± 0.08 a	0.11 ± 0.06 b	0.24 ± 0.03 b	***	**
∑ -G	Extraction yield (%)	69 ± 12 a	35 ± 9 c	50 ± 9 b	***	**
∑ Acetyl-G	Extraction yield (%)	61 ± 12 a	35 ± 4 c	48 ±12 b	***	**
∑ Cinnamoyl-G	Extraction yield (%)	35 ± 8 a	18 ± 4 c	26 ± 5 b	***	**
Phenolic acids (mg/g skins)						
Caftaric acid	Potential	0.19 ± 0.01 a	0.05 ± 0.02 c	0.09 ± 0.01 b	***	*
	Extractable	0.10 ± 0.01 a	0.01 ± 0.00 b	0.02 ± 0.00 b	***	*
	Extraction yield (%)[#]	30 ± 3 a	9 ± 4 c	16 ± 2 b	***	***
Coutaric acid	Total	2.09 ± 0.15 a	0.31 ± 0.13 b	0.65 ± 0.19 b	***	ns
	Extractable	0.76 ± 0.20 a	0.04 ± 0.01 b	0.06 ± 0.02 b	***	ns
	Extraction yield (%)[#]	21 ± 4 1 a	12 ± 6 b	6 ± 2 c	***	*
Flesh						
Phenolic acids (mg/g flesh)						
Caftaric acid	Potential	0.10 ± 0.01 ab	0.07 ± 0.02 b	0.11 ± 0.01 a	*	*
Coutaric acid	Potential	0.05 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	ns	ns
Seeds						
TPI (mg EC/g seeds)	Potential	149 ± 8	149 ± 4	155 ± 2	ns	ns
	Extractable	54 ± 5 b	77 ± 2 a	57 ± 9 b	**	*
	Extraction yield (%)[#]	30 ± 3 b	41 ± 6 a	34 ± 6 b	*	*
MCP (mg EC/g seeds)	Potential	90 ± 6	89 ± 9	85 ± 2	ns	ns
	Extractable	22 ± 9	41 ± 12	22 ± 7	ns	ns
	Extraction yield (%)[#]	21 ± 7 b	38 ± 11 a	25 ± 7 ab	***	**
	Potential	94 ± 10	85 ± 6	89 ± 6	ns	ns
FRV (mg C/g seeds)	Extractable	31 ± 5 b	46 ± 2 a	33 ± 6 b	*	*
	Extraction yield (%)[#]	28 ± 4 c	45 ± 2 a	34 ± 7 b	***	***
FRV/MCP	Potential	1.04 ± 0.10	0.95 ± 0.04	1.05 ± 0.09	ns	ns
	Extractable	1.58 ± 0.75	1.20 ± 0.33	1.50 ± 0.59	ns	ns

All data are expressed as average value ± standard deviation ($n = 3$). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively, for the differences among treatments (FRESH, SUN and CTR) and between the two different withering modalities (SUN, CTR) according to ANOVA([a]) and T-tests ([b]). Different Latin letters among the same raw indicate significant differences ([a]) according to Tukey-b test ($p < 0.05$) for ANOVA. TPI: total phenolic index (A280); TA: total anthocyanins; MCP: condensed tannins by methyl cellulose assay; FRV: flavanols reactive to vanillin; Dp-3-G: delphinidin-3-glucoside; Cy-3-G: cyanidin-3-Glucoside; Pt-3-G: petunidin-3-glucoside; Pn-3-G: peonidin-3-glucoside; Mv-3-G: malvidin-3-glucoside; -G: simple glucosides; Acetyl-G: acetylated glucosides; Cinnamoyl-G: cinnamoylated glucosided; EC: epicatechin; C: catechin; [#]: extraction yields (%) are calculated using the data expressed as mg/kg berries.

The anthocyanin profiles (Table 2) confirmed that a greater oxidation occurred within the skins of sun-exposed grapes for all the anthocyanin forms.

Moreover, only a part of these compounds is extracted in wine-like conditions (Abi-Habib et al., 2021), reason why studying both the potential and extractable components provide very useful information. Indeed, anthocyanins extraction yields (%) were very different among the three conditions studied: the final TA extraction yield in wine-like solution for FRESH (72%) was higher than CTR one (55%), which was itself higher than SUN one (46%). Thus, in this experiment, the effects changes in TA extractability due to the reorganization of cell wall composition (see Figure 4) were added to the degradation that already occurred within the berry during withering. Therefore, the combination of these two aspects resulted in a lower content of extractable anthocyanins in withered grapes with a greater extent for sun-dehydrated grapes (p-value of the T-test SUN vs CTR <0.001). Moreover, glycosylated anthocyanins were slightly more extracted than acetylated, which were much more extracted than the cinnamoylated forms at the end of the simulated macerations (Table 2), in accordance with previous findings (Abi-Habib et al., 2021). The extractions depended both on the structure of the anthocyanin and on the withering modality. Indeed, the extraction yields of the different anthocyanin forms followed the same trend of TA (FRESH>CTR>SUN). The lower extractions of anthocyanins and flavanols observed in sun-withered grapes represent a disadvantage also for the long-term color stability, since the formation of pigmented polymers is very important to this purpose (Cheynier et al., 2006).

Skin potential condensed tannins (MCP), expressed in mg/g skins, were not significantly affected by the different withering methods (Tables 2, S3). In line with anthocyanins, the skins extraction yields (%) decreased significantly

from fresh to withered grapes, with a greater extent in SUN also for TPI and MCP parameters (TPI: -20 and -11%; MCP: -12 and -8% for SUN and CTR with respect to FRESH, respectively).

A strong decrease in skin potential monomers and low-molecular weight flavanol (FRV) was observed for both the withering methods with respect to FRESH. This decrease was observed when data are expressed in mg/g skins and in mg/kg berries (ANOVA p-value<0.001). The loss of FRV detected in withered skins was higher for SUN, possibly due to polymerization phenomena rather than to degradation, but further analyses are needed to confirm this hypothesis. Moreover, the extraction yields of FRV were not significantly different among FRESH, SUN, and CTR skins (57-58%). Although flavan-3-ols and low-molecular tannins can access more sites, their interactions with skins cell walls are thought to be negligible, explaining that they are more easily desorbed (Bindon et al., 2014) and not affected by the skins cell wall polysaccharide modifications observed among treatments (Figure 4), unlike other phenolic compounds. Consequently, the FRV/MCP ratio of SUN skins was lower than those of FRESH and CTR, indicating a potential softening effect in the mouth-feel perception of the wine due to the lower proportion of low-molecular weight tannins, which are held responsible for bitterness and “green taste” astringency sensations (Busse-Valverde et al., 2011).

Therefore, at the end of the 10 days of simulated maceration of skins in wine-like solutions, the extractable profile of the sun-withered grape skins showed the lowest contents of polyphenols, anthocyanins, phenolic acids, and tannins, but also a lower percentage of low-polymerized forms on the totality of the

condensed tannins (both in mg/kg and mg/g). Instead, the CTR phenolic profile was not significantly different from the profile of FRESH grape skins.

The potential contents of hydroxycinnamic acids (HCAs) esters detected in skins and flesh (mg/g skins and flesh, Table 2) confirmed the greater loss of compounds of sun-withered Aleatico grapes, whereas in controlled conditions these compounds were better preserved, in accordance with Frangipane et al. (2012). Indeed, the oxidation of coumaric and caftaric acid by PPO plays a crucial role in the enzymatic related oxidation phenomena of the other phenolic compounds (Cheynier et al., 1989). The lower amounts of HCAs skins esters found in wine-like extracts of withered grapes, particularly for SUN, were possibly due to the implication of these compounds in other reactions during the maceration, as supposed by Marquez et al. (2012). However, the ratio (%) of sum of cinnamoylated forms in the anthocyanin profiles resulted not significantly affected by the treatment (Table S3), suggesting a more probable involvement of HCAs in oxidation rather than in the formation of p-coumaroilated anthocyanins. The higher proportion of yellow color component (A_{420}) with respect to the red (A_{520}) found in SUN (Table S2) could be related to the browning of the extracts due to oxidation, or to the formation of anthocyanin-derived pigments such as pinotins (Scalzini et al., 2021).

Regarding seeds, for all the parameters considered of the phenolic profile potentially present in grapes (TPI, MCP, FRV), no significant differences were found among FRESH, SUN and CTR in mg/g seeds (Table 2), indicating that potential oxidation did not occur, or that oxidation was balanced to a possible synthesis of seeds polyphenols potentially happen within seeds during

dehydration, as supposed by Centioni et al. (2014) for grape seeds of cv. Cesanese. Moreover, for the increased potential profile as expressed in mg/kg berries basis, the concentration effect observed is attributable of the loss of juice in the whole berry after dehydration, as it was demonstrated that during withering the seed weight remained almost constant until reaching 60% WL (Río Segade et al., 2016).

Concerning the contents of extractable seed polyphenols in wine-like conditions, both TPI, MCP, and FRV increased on mg/g and mg/kg basis moving from fresh to withered grapes (Tab. 2 and S3), following the same trend described in Figure 2c for TPI (mg/kg of berries): SUN > CTR > FRESH (Figure 2c). Some other authors demonstrated an increase of proanthocyanidins and flavanol oligomers in the seeds of dehydrated grapes compared with fresh, in accordance with these findings (Moreno et al., 2008; Rolle et al., 2013).

In this experiment, the differences observed among the three modalities studied are clearly related to the modified extraction yields of these compounds, particularly for MCP and FRV (Pearson's correlation factors of the extractable contents vs extractability: TPI = 0.74, MCP = 0.96, and FRV = 0.94, p(f)-values<0.05). Indeed, on the contrary to what was observed for the skins (for which the extractability decreased with the withering particularly for SUN), the extractability of phenolic compounds in seeds increased after withering process, particularly for SUN. Indeed, SUN % yields were higher than FRESH and CTR for all the parameters studied, comprising FRV (IPT: +11 and +4%; MCP: +17 and +4%; FRV: +17 and +6% with respect to FRESH for SUN and CTR, respectively). Also, the extractability of CTR increased compared with the

FRESH, but the yield was weaker than SUN (+4, +4, and +6% for TPI, MCP and FRV, respectively). FRV/MCP ratio was always lower for SUN samples as observed for skins, although in seeds the differences were not statistically significant.

The increased extraction yields (%) for withered grape seeds are in accordance with the few studies present in the literature concerning the effect of grape dehydration on the extractability of phenolic compounds from seeds (Centioni et al., 2014; Río Segade et al., 2016). Moreover, for the first time, the greater extractability of polyphenols, proanthocyanidins and low-molecular weight tannins in grape seeds of sun-dried grapes with respect to seeds of grapes subjected to withering controlled conditions has been shown. Rolle et al. (2009) highlighted a tendential decrease of seeds hardness after the on-vine dehydration process, on the contrary to the hardening of the seeds which occurs throughout the ripening. The softening of seed tissues induced by dehydration could explain the modifications observed in withered grapes compared to the fresh. It is possible that the high temperatures to which the sun-dried grapes were subjected may have intensified the softening of seeds, leading to the higher extraction yields of the SUN compared to CTR, but there are not proofs of causalities. Indeed, as there are no studies on the anatomical and histological changes in seeds during grape dehydration, and due to the presence of the secondary cell walls in seeds (Hanlin et al., 2010), more precise hypotheses require further targeted studies.

The differences observed among treatments in the extractability of FRV found in seeds and not in skins are probably due to the presence of secondary cell walls in seeds, which contain lignin and more cellulose than skins (Hanlin et al., 2010).

3.2. Extraction kinetics and color implications

Figure 2 shows the gradual extraction of anthocyanins compounds from Aleatico FRESH, SUN and CTR skins (a), the parallel evolution of the visual color (b), and the extraction of total polyphenols (TPI, A280) from the seeds (c) during the macerations in wine-like solutions.

The anthocyanin levels found during the simulated fermentative maceration are in line with the values detected in real winemaking conditions (Cotarella et al., 2013). Throughout the simulated process, the progressive solubilisation of anthocyanins showed significant differences among the samples studied. As can be seen in the graph (Figure 2a), the anthocyanins diffusion in wine-like solutions was higher and faster in fresh grapes with respect to the withered ones. For FRESH, the data showed a rapid rise of TA content, which reached a maximum at 24h (523 mg/kg of berries expressed as malvidin-3-O-glucoside chloride), followed by a normal decreasing trend of these compounds to 413 mg/kg of berries after 10 days of maceration. This trend was probably due to oxidation and polymerization reactions and confirms the importance of the early maceration steps in the color traits of the final wine (Paissoni et al., 2020) also for Aleatico grapes, although it is a variety rich in stable forms (malvidin-3-glucoside and acylated glucosides which account for more than 50% and 20% of the profile, respectively). Nevertheless, at the end of the process, FRESH still had the highest content of extractable TA in wine-like conditions (+43% and +0.08% than SUN and CTR final TA contents, respectively). For withered samples, instead, the skins diffusions were slower, especially for SUN samples with a maximum of TA reached after 48h (24h more than control).

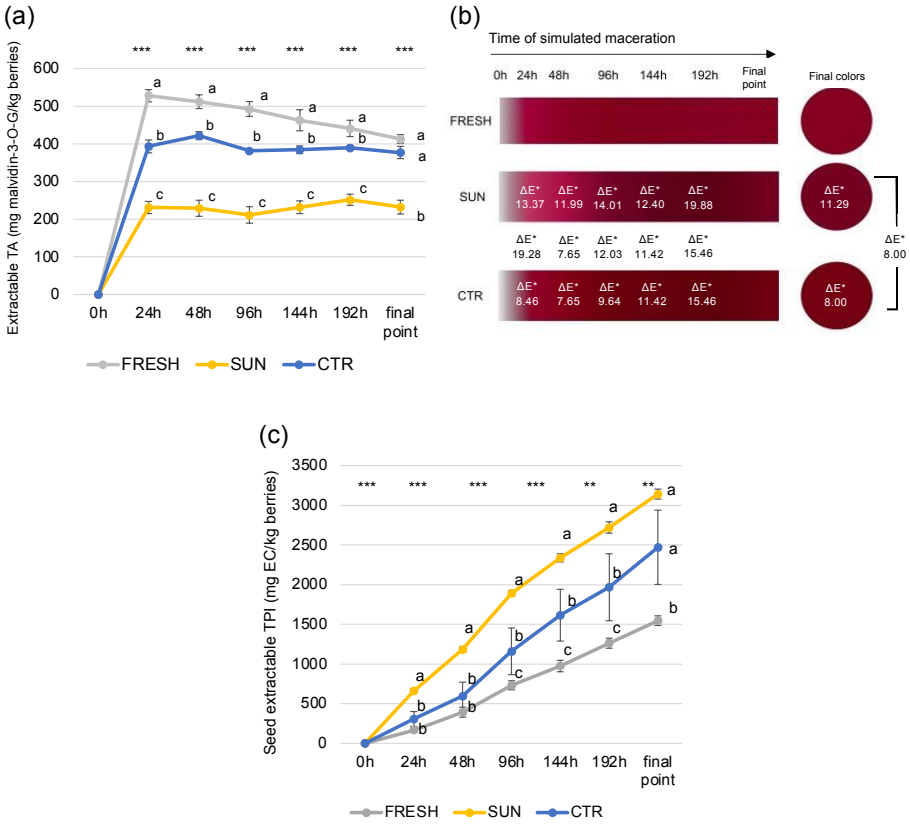

Figure 2 – Evolution of different parameters during the simulated macerations in wine-like solutions of skins and seeds. a: skins extractable anthocyanins (TA); b: visual color of the skins wine-like extracts (CIEL*a*b* coordinates converted to RGB 24-bit color values); c: seeds extractable polyphenols (TPI). Malvidin-3-O-G: malvidin-3-O-glucoside chloride; EC: (-)-epicatechin. All data are expressed as average value ± standard deviation (n=3). Sign: *** and ** indicate significance at $p<0.001$, and 0.01, respectively, for the differences among fresh material before the withering (FRESH), sun-withered grapes (SUN) and withered grapes in controlled conditions (CTR) at each maceration time. ΔE* values between FRESH and withered grapes (SUN or CTR) during macerations are reported in white inside the colored bars at the correspondent points, while ΔE* values between SUN and CTR are reported in black between the correspondent bars; ΔE* values among treatments at the end of the process are shown inside the circle corresponding to the final visual color.

This delay is probably related to the higher Sp_{sk} of withered grape skins (Table 2), as previously postulated (Rolle et al., 2008; Río Segade et al., 2011), as well as the modifications in cell walls composition, which will be discussed in the next section (see Figures 4 and 5). However, the red pigments content after 48h of skins simulated extraction remained more stable in withered samples than in FRESH, probably because the delay in extraction allowed to better preserve the more easily extractable and degradable anthocyanin forms (i.e. cyanidin-3-O-glucoside and peonidin-3-O-glucoside; González-Neves et al., 2008). Also, they have already been partially degraded/modified during the withering process, as the total anthocyanin profile shows, especially for SUN (Table 3). Indeed, although the kinetics of the two different withering methods were similar, CTR had on average +41% higher amounts of anthocyanins than SUN throughout the entire process (mg/kg of berries as malvidin-3-O-glucoside chloride).

The differences detected in the content of extractable anthocyanins in wine-like conditions influenced the visually perceived color of extracts during the simulated macerations. Figure 2b shows the visual colors obtained by the conversion of the CIEL*a*b* coordinates (Table S2) in the corresponding color on RGB scale, which is comparable to the shades perceived by the human biological processing visual system (Cheng et al., 2001; Río Segade et al., 2019b; Scalzini et al., 2020). The progressive solubilisation of anthocyanins reflects color features of the macerating extracts particularly for SUN, which experienced a major color change after 96h of simulated process. However, the different shades perceived progressively during the SUN wine-like maceration seemed to be related only to the color intensity and not to the hue, which did not show

statistically significant differences among the six SUN sampling points. Since the beginning of the simulated process, SUN extracts showed significantly higher values of hue with respect to the others extracts, reaching at the final point +0.148 and +0.092 hue units than FRESH and CTR, respectively. This indicated a higher yellow color component (A_{420}) with respect to the red (A_{520}). At the end of the 10 days of maceration, the color of the three extracts showed differences perceptible from the human eye (Figure 2b), with a ΔE* of 11.29 and 8.00 for SUN and CTR by comparison to FRESH, respectively, and a ΔE* of 8.00 between CTR and SUN samples (Martínez et al., 2001).

Extraction kinetics obtained with seeds are shown Figure 2c. The amounts of extractable polyphenols (TPI, mg/kg of berries, expressed as (-)-epicatechin) increased progressively with the increasing maceration time for the three samples studied with a greater extent for seeds of withered grapes, especially for SUN. In accordance with the literature, in fact, the higher is the ethanol concentration, the greater is the diffusion of polyphenols from seeds (Canals et al., 2005). Under the same ethanol conditions, this trend was more remarkable for seeds of withered grapes, particularly for sun-withered samples. Indeed, for the whole maceration time, the TPI of SUN resulted higher than CTR (with an average of +65%), and CTR was higher than FRESH (on average +62%). However, even if throughout the maceration the differences in TPI between the two dehydration modalities studied were statistically significant at each sampling point until 192h, at the end no significant differences were found in polyphenols from a quantitative point of view (p-value T-test = 0.128), probably because after 96h the diffusion rate from the SUN seeds slowed down slightly, while that for

CTR thinly increased. Considering the high tannic content of Aleatico seeds, particularly for low-molecular weight tannins, the duration of the maceration phase should be handled with caution for Aleatico withered grapes, especially in the case of sun-dehydration, preferring short macerations, in order to avoid unbalanced wines due to excess bitterness or astringency.

3.4. SEC Profile

The HPSEC method was performed to study the proanthocyanidin fractions mass distribution from potential and extractable skins and seeds of FRESH, SUN and CTR samples (Figure 3). According to the SEC principle, to which molecules of different sizes elute at different rates, the larger tannin molecules eluted earlier (Kennedy and Taylor, 2003). This technique allows to also analyse the oxidized compounds, which cannot be revealed by the phloroglucinolysis or thiolysis reactions, which is often utilized to determine the degree of polymerization (DP) of grape tannins (Poncet-Legrand et al., 2010; Bautista-Ortin et al., 2014).

The HPSEC chromatograms showed three main populations: “Peak 1” related to polymeric tannins (DP > 5) at a retention time from 20.7 to 24.9 min; “Peak 2” related to oligomers (DP > 2), eluting from 26.0 and 26.4 min; and “Other compounds” related to other phenolic compounds, such as anthocyanins and phenolic acids, at retention times greater than 26.5 min (Abi-Habib et al., 2021; Abi-Habib et al., 2022a).

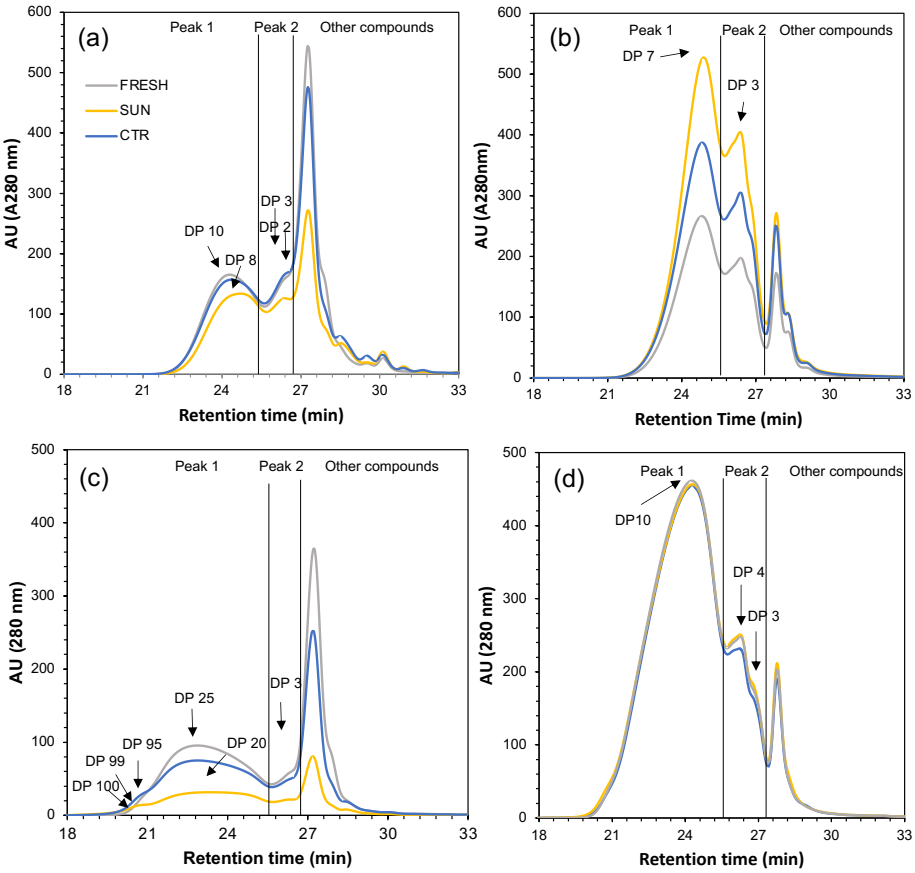

Figure 3 – Size distribution of polymeric tannins (Peak1), oligomeric tannins (Peak2) and other polyphenol compounds such as anthocyanins or phenolic acids (Other compounds) determined by high-pressure size exclusion chromatography (HPSEC) at 280 nm. Extractables (a: skins; b: seeds) and totals (c: skins; d: seeds) HPSEC phenolic profiles for fresh (FRESH) and withered grapes under the sun (SUN) or in controlled conditions (CTR) are shown. Potential profiles of skins (c) and seeds (d) are referred to the analysis of potentially maximum extracted solutions: 150 (skins) or 100 mg (seeds) of frozen powder in 6 mL of solvent (methanol + acetone/water/formic acid); Extractable profiles are referred to the analysis of the wine-like solutions at the end of the 10 days simulated macerations of skins (a) and seeds (b). DP: degree of polymerization calculated with the calibration curve. The concentrations of potential and extractable polymers, oligomers and other compounds are shown in Table S4.

The HPSEC profiles were different for skins and seeds, as already observed by Abi-Habib et al. (2022a). In particular, in accordance with these authors, the profiles showed in the seeds a majority of polymeric tannins ("Peak 1"), associated with higher content in oligomers than in skins (Table S4). However, the average DPs of the high-molecular-weight fraction were higher for skins than for seeds (Figure 3). Indeed, HPSEC profiles of potential skins (Figure 3c) showed two sub-peaks within the polymeric tannin population: the first, identified at a retention time above 21 min was characterized by a DP at the top of 95, 100 and 99 for FRESH, SUN and CTR, respectively; the second, eluted at above 23 min, presented a DP of 25, 20 and 25 for the same samples. The first sub-peaks of larger polymers were too polymerized to be extracted in wine-like conditions, probably due to their skin cell walls interactions (Bindon et al., 2014; Abi-Habib et al., 2022a). Consequently, the HPSEC profiles of extractable skins in wine-like solutions (Figure 3a) showed only the first peak eluted at about 24 min within the polymeric tannin population, presenting lower DPs than those estimated for the potential profile (10, 8, 10 for FRESH, SUN and CTR, respectively). For seeds, the potential profiles (Figure 3d) showed an average DP of the polymeric tannins population of 10 for all the samples studied, while the DP of the corresponding fraction in the extractable profiles (Figure 3b) in wine-like conditions were 7 for all the solutions, indicating that the molecular size is an important factor also for seeds, but to a lesser extent than for skins. This is probably due to the lower interactions with cell walls constituents and to the presence of the secondary cell walls in seeds (Hanlin et al., 2010; Boulet et al., 2023). Oligomeric tannins population (average DP = 3 for all the samples both in

potential and extractable skins profiles and for extractable seeds; DP= 4-3 for potential seeds), as well as polymeric population, followed the same trends described for spectrophotometric analyses: FRESH > CTR > SUN, for both potential and extractable skins profiles; FRESH=SUN=CTR for potential seeds profile; SUN > CTR > FRESH, for extractable seeds profile. Indeed, in the present work, Pearson's correlation factors higher than 0.9 were obtained between the HPSEC estimated contents of condensed tannins (polymers + oligomers fractions) expressed in in mg/g in skins or mg/kg of berries as (-)-epicatechin equivalents (Table S4) and MCP correspondent values, and between oligomers fraction and FRV corresponding values only in for seeds ($p(t)$-values< 0.05 for skins and seeds MCP and $p<0.01$ for seeds FRV). As FRV included also monomeric flavanols, which are not comprised in HPSEC oligomer fractions eluting with "other phenolic compounds" from which they are indistinguishable, the FRV/MCP ratios were not well correlated with the [oligomers/ (oligomers + polymers)] ratios quantified by HPSEC (Pearson's correlation factors = 0.75 with $p=0.08$ for skins, and 0.82 with $p<0.05$ for seeds).

Interestingly, in line with the previous section, the comparison of the potential and extractable profiles showed substantial differences between skins and seeds: for skins (Figure 3 a, c) the extractable profiles appeared to be the results of the combination between chemical modifications that occurred inside the berry skins during dehydration in different conditions (as demonstrated by the differences among treatments showed by potential profiles) and the modified extractability; instead, seeds potential profiles were not quantitatively and qualitatively different among treatments, demonstrating that the differences shown by the extractable

profiles in wine-like conditions were due to the modified extractability due to the different withering conditions and not to metabolic modifications which involved tannins structure or their quantity occurred during the withering process studied. This consideration about seeds is important, since to the best of our knowledge it has never been proven. Kennedy et al. (2000) pointed out that the seeds coat permeability is related to the content of phenolic compounds and to their level of oxidation, which leads to a decline in their extractability. Nevertheless, as no differences were found in the present study at these levels, the observed changes in the extractability of these compounds were likely due to anatomical and histochemical tissue changes. Considering the difficulties to study seeds structure, as they are strongly lignified, very few studies are available in the literature (Cadot et al., 2006) and no one has been dedicated to seeds of withered grapes. Then, further specific studies need to be carried out on the potential impact of seeds histochemistry modifications due to the withering process in different conditions.

3.5. Polysaccharides of skins and pulps insoluble materials using CoMPP method

Pectin, cellulose, and hemicellulose are the three major components of the primary cell wall network (Jones-Moore et al., 2021). The polysaccharides composition of skins and flesh cell walls was analyzed using the CoMPP method (Gao et al., 2015). The results were reported separately for the two fractions resulting from sequential extraction of the AIR material: (i) the CDTA fraction is

rich in pectic polysaccharides [homogalaturonans (HG), rhamnogalaturonans I (RGI), and side chains (i.e. arabinans, galactans and arabinogalactans)], arabinogalactan-proteins (AGPs) and extensins; (ii) the NaOH fraction is rich in hemicellulosic polysaccharides (glucans/xyloglucans, mannans, xylans) and cellulose (Figure 4). The monoclonal Antibodies (mAbs) and Carbohydrate Binding Modules (CBMs) used are listed in Table S1. No mAb or CBMs is available to study rhamnogalaturonans of type II (RGII), while only one is available for cellulose (Abi-Habib et al., 2021).

The signals reported in Figure 4a indicates the epitope abundance which is accessible to the antibodies. In accordance with the literature (Abi-Habib et al. 2022b; Gao et al., 2019), mAbs JIM7 and LM20 showed the highest signals in the pectin-rich fraction (CDTA) both in skins and pulps for all the samples studied, confirming that grape pectins are highly methyl-esterified. By examining the JIM7/JIM5 ratio (methyl de-esterified/esterified HG), among treatments SUN showed the lowest values both for skins and flesh (skins JIM7/JIM5 ratios: 3.1, 2.3, 2.8; flesh JIM7/JIM5 ratios: 5.6, 3.6, 4.15 for FRESH, SUN, and CTR, respectively), meaning a higher level of de-methylation. These results seem to be perfectly in line with the trend of skins extractability of phenolic compounds (FRESH>CTR>SUN) found in this experiment.

Indeed, HG de-methylation releases COO^- functions allowing the formation of egg-boxes through Ca^{2+} bridges, which play an important role in the structural features of the cell walls, forming a very rigid gel, which can hinder the release of compounds, modifying the cell wall porosity, and the strength of intracellular adhesion (Basak et al., 2014; O'Neil and York, 2003).

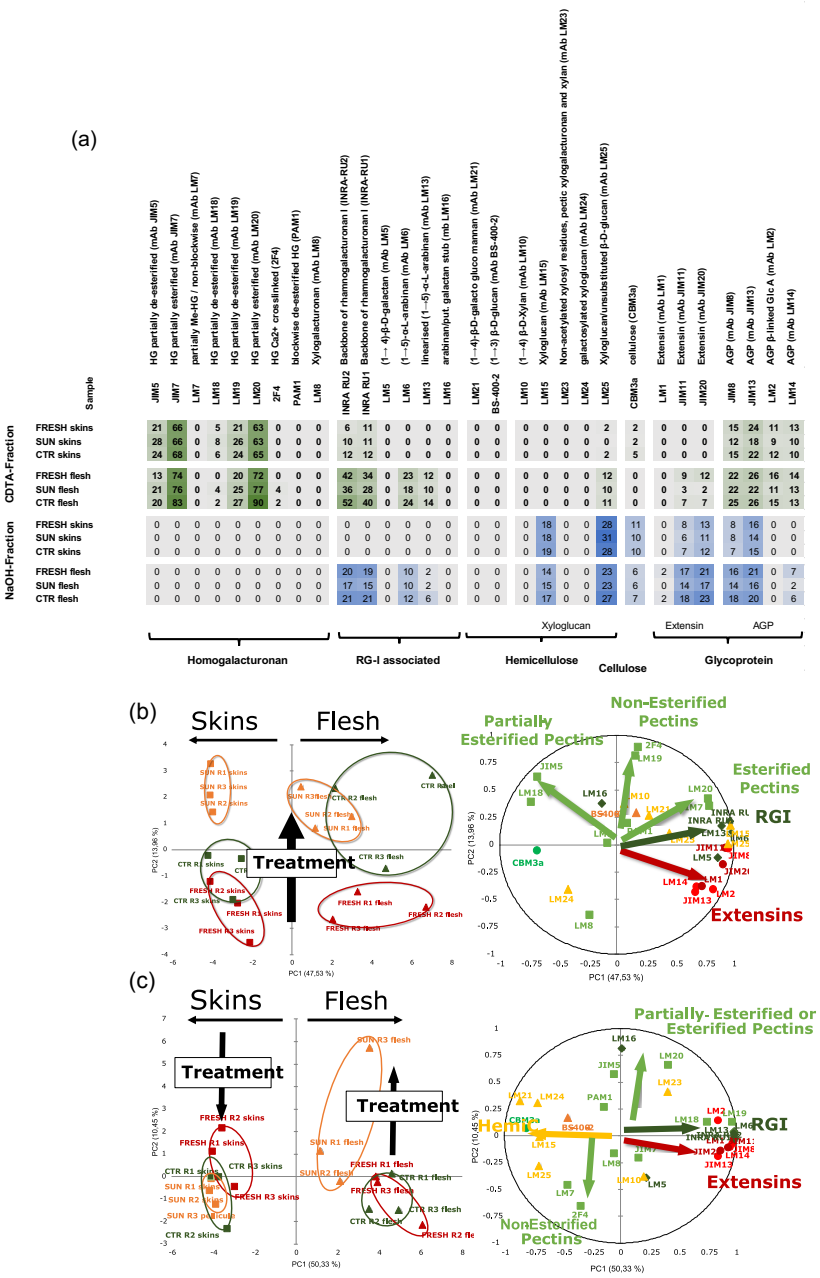

Figure 4 – Comprehensive microarray polymer profiling (CoMPP) heatmap (a) representing cell wall polysaccharides and glycoproteins relative abundance of fresh

grapes (FRESH), sun-withered (SUN) or withered grapes in controlled conditions (CTR). The antibodies signal intensities were read on the cyclohexane-diamino-tetraacetic acid (CDTA, pectin-rich) and NaOH (hemicellulose-rich) fractions extracted from the alcohol insoluble residue (AIR) of skins and flesh of each sample. Principal component analysis (PCA) of the variables and individual distribution regarding CDTA-fraction (b) and NaOH-fraction (c). On the PCAs, treatments (b1 and c1) are colored in red, orange, and dark green for FRESH, SUN, and CTR, respectively; the antibodies (b2, c2) are coloured as follows: in green those for pectin polysaccharides (HG: homogalacturonans, RG: rhamnogalacturonans and side chains: arabinans, galactans and arabinogalactans); in yellow the antibodies for hemicellulosic polysaccharides (mannans, glucan/xyloglucans, and xylans); in red those for glycoproteins (AGP: arabinogalactan-Proteins, and extensins). The antibodies used and their functions are described in detail in Table S1.

The changes in the cell wall structure by de-esterification and de-polymerization, together with the formation of new cross-linking bridges could reduce tannin extractability due to their encapsulation in the modified gel network (Hanlin et al., 2010). This pattern is probably a metabolic expedient to reduce the loss of water from grape berries during dehydration. It is noticeable that in the present study, the 2F4 signal for calcium egg-boxes is higher for withered grapes (SUN>CTR) only in pulps. Nevertheless, the absence of these signals in the skins could be due to the extraction fractions (CDTA and NaOH) which are not strong enough to reveal them (Gao et al., 2015), as the polysaccharides are more strongly held in the skins than in the pulp cell walls matrix (Vidal et al., 2001). The higher de-methylation of withered samples compared to FRESH observed in the present study was in accordance with the increased activity of pectin methyl esterase (PME) found in literature during dehydration (Vincenzi et al., 2012; Zoccatelli et al., 2013). The higher temperatures of SUN withering than CTR conditions may

have increased the PME activity, explaining the great difference between the two dehydration modalities (Coletta et al., 2019). Skins resulted higher de-esterified than flesh, in accordance with the findings of Fasoli et al. (2019), who highlighted a higher level of de-esterification in the external layers of the skins compared with the internals. Moreover, Zoccatelli et al. (2013) demonstrated that the coordination of PME and polygalacturonate (PG) activity is variety-dependent: they led to the degradation of skins pectins only for one of the three varieties studied (Corvina), whereas in cv. Oseleta and Sangiovese the activity of the two enzymes was uncoupled throughout dehydration and it was supposed to serve a different role. Indeed, the increase of pH and the higher availability of Ca^{2+} ions due to the intense de-methylation of withered grapes may have inhibited the PG activity in withered grapes, to a greater extent for SUN samples (Botondi et al., 2011), making significant only the effect of the increased de-methylation, which probably determined the lowest extractability of anthocyanins and polyphenols from SUN skins. The high amounts of HG, RGI, and AGPs detected in the CDTA fractions could confirm the supposed low activity of PG, as Kuhlman et al. (2022) demonstrated a complete or significant reduction of these signals after its action. Instead, the NaOH extraction (hemicellulose-rich fraction) showed strong signals of xyloglucans (mAbs LM15 and LM25), cellulose (CBM3a), as well as glycoproteins (mAbs JIM11 and JIM20 as extensins, and JIM8 and JIM13 as AGPs). In SUN withered grapes, a decline in signals of extensins epitopes were found (mAbs LM1 for pulps, and JIM 11, JIM20 for both skins and pulps), together with a decline in AGPs signals (mAbs JIM8, JIM13 for skins and pulps, and LM14 only for the pulps). These findings agree with the observations of Gao

et al. (2021) on Syrah overripe grapes. These authors attributed the degradation of glycoproteins in the NaOH fraction to a degradation of the pectin extension network due to heat and water deficit stress.

To better understand the differences among treatments and tissues, a principal component analysis (PCA) was performed separately for each fraction (Figure 4 b, c). The PCA graphs highlight the distribution distances between profiles, which translate their degree of similarity. Thus, the positions of the samples on the graph gave the structure of the relationships between them. For both CDTA and NaOH, the horizontal axis reproduced the type of tissue studied (skins or flesh), while the vertical axis (PC2) the differences among treatments. Regarding CDTA-fraction (Figure 4b), principal component 1 (PC1) accounted for 47.53% of the explained variance, whereas principal component 2 (PC2) explained 13.96%, with a total explained variance by the first two components of 61.49%. Skins and pulps were separated on PC1 axis for the higher presence of RGI hairy regions and extensins/AGPs in the Aleatico flesh than in skins for all the treatments studied. In accordance with these findings, Vidal et al. (2001), studying the composition of polysaccharides from different tissues (skins and flesh) of grapes cv. Grenache blanc, pointed out that 80% of grape AGPs come from the flesh, whereas, differently from what observed for Aleatico, RGI were present in both tissues in comparable amounts. Indeed, Ortega-Regules et al. (2008) demonstrated that the composition and total sugar content of cell walls are variety-dependent. Comparing treatments, CTR replicates were more similar to FRESH in the skins and to SUN in the pulps. PC2 separated FRESH, SUN, and CTR treatments based on their changes in pectin HG zones. In particular, the

withering modalities studied (SUN and CTR) differed for the HG zones of partially esterified pectins (JIM5 and LM18) for the skins, and with a lesser extent of esterified pectins for the flesh (JIM7 and LM20). Similarly, the NaOH fraction, the PCA showed a total explained variance of 60.78% (50.33 and 10.45 for PC1 and PC2, respectively). Skins and flesh were separated on the axis PC1 because the higher hemicellulose detected in the skins, whereas the pulps were richer in pectins RGI hairy regions and extensins/AGPs. On the contrary to what was observed in the CDTA-PCA, CTR resulted more like SUN in the skins and to FRESH in the pulps. Among treatments (PG2 vertical axis), FRESH showed lower de-esterified HG (LM7 and 2F4) than withered samples for the skins, while for the pulps SUN presented higher contents of HG partially esterified (JIM5) or totally esterified (LM20) than other samples.

3.6. Neutral sugar composition determined by GC analyses

To complete the study of the cell wall polysaccharides modifications in Aleatico grapes subjected to different withering conditions, the chemical composition of polymers was also analyzed with the classical chemical analyses, which are based on the determination of their monosaccharide constitutive units after depolymerization steps. Indeed, some studies have recently been oriented towards the combination of the chemical composition determined by chemical analyses coupled with the CoMPP immunochemical analysis, which can provide new awareness on the conditions of cell walls polymers (Boulet et al., 2023). TMS was used to assess the qualitative profile of neutral and acidic sugars, providing the molar ratio of the monosaccharides composition of skins and flesh

AISs cell wall material (Figure 5). Instead, the quantification (mg/g AIS) of single monosaccharides was performed by GC of alditol acetates (pectins and hemicelluloses) and alditol acetates after Seaman hydrolysis (pectins; hemicelluloses and cellulose). The results are reported in the supplementary material (Table S5) However, from the comparison between the glucose detected by alditol acetates and Seaman methods, emerged that the Seaman worked for pulps and fresh skins, but failed to completely hydrolyse the cellulose in the withered skins as alditols acetates showed higher glucose than Seaman for these samples. The reasons for this difficulty are probably due to morphological and chemical alterations that occurs during withering (Fasoli et al., 2019) and particularly to the reorganization of the gel network structures, as CoMPP results highlighted.

Galacturonic acid (from pectins) and glucose (from cellulose and hemicellulose) were the major sugars in skins (Figure 5a), accounting from 36 to 49% and 14-18%, respectively. Also, arabinose and galactose (from pectins rhamnogalacturonan side chains) showed high molar ratios (9-17% and 13-15%). Nevertheless, the significantly higher molar ratio of arabinose, rhamnose, and fucose highlighted in SUN skins were due to the most remarkable decrease of all the other sugars and not to their real increase in quantity, as shown in Table S5 by the results expressed in mg/g AIS obtained by GC of alditol acetates and by Seaman hydrolysis. On the contrary, significantly lower % of galacturonic acid and mannose (hemicellulose) were found in SUN with respect to other treatments. The only significant differences between FRESH and CTR grape skins molar ratio of neutral sugars was detected for glucuronic acid, which was significantly

higher for CTR than FRESH, whereas SUN was intermediate between the two values (2.3, 2.5 and 3.0% for FRESH, SUN and CTR).

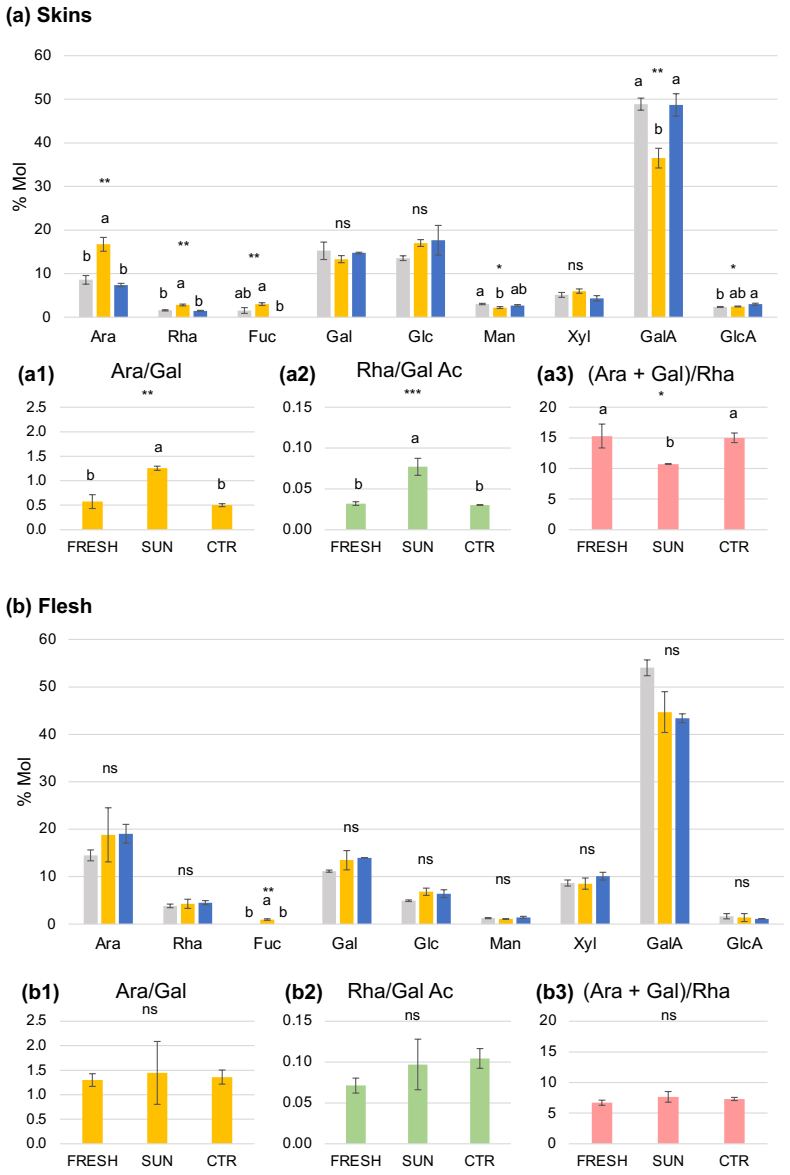

Figure 5 – Molar ratio of neutral glycosyl-residue of AISs polysaccharides of Aleatico skins (a) and pulps (b) of the three modalities studied (FRESH (grey): fresh grapes; SUN (yellow): sun-withered grapes; CTR (blue): withered grapes in controlled conditions)

obtained with the TMS analysis. Ara: arabinose; Rha: rhamnose; Fuc: fucose: Gal: galactose; Glc: glucose; Man: mannose; Xyl: xylose; GalA: galacturonic acid; GlcA: glucuronic acid.

The calculation of different specific ratios of neutral sugars (Figure 5 a1, a2, a3) allowed to estimate the relative importance of polysaccharides to better understand their structural modifications: arabinose/galactose (Ara/Gal), rhamnose/galacturonic acid (Rha/Gal), and (arabinose + galactose)/rhamnose [(Ara+Gal)/Rha] (Apolinar-Valiente et al., 2018). For all the specific ratios, SUN resulted significantly different compared with CTR and FRESH, whereas FRESH and CTR were not statistically different each other. In particular, the Ara/Gal ratio is characteristic of the PRAG-like structures (polysaccharides rich in arabinose and galactose). Its modification detected in AISs cell wall skins (0.57, 1.25, and 0.50 for FRESH, SUN and CTR, respectively) could be due to the degradation of AGPs observed in the CoMPP hemicellulose-rich fraction (Doco et al., 2007). Moreover, the higher rhamnose/galacturonic acid ratio of SUN skins (0.08 for SUN vs 0.03 for both CTR and FRESH) indicates that SUN cell walls insoluble pectins were characterised by higher relative amounts of HG than RG with respect to other samples, consistently to the CoMPP results (Fig. 4). In addition, since most of arabinose and galactose are associated with pectin hairy regions, the lower (arabinose + galactose)/rhamnose ratio calculated for SUN skins (15.30, 10.73 and 15.01 for FRESH, SUN, and CTR, respectively) indicates that they were characterized by RG-like backbone structures which carry fewer neutral chains than FRESH and CTR. Therefore, withering process strongly affected the neutral sugar composition of skins AISs cell walls, particularly when

the grapes were dehydrated in natural conditions under the sun. However, our finding regarding the relationship between extractability of phenolic compounds and skins cell wall composition seems to be in contrast with the correlations found by other authors, which recently studied fresh grapes extractability linked with polysaccharides composition (Abi-Habib et al., 2022a; Abi-Habib et al., 2022b; Boulet et al., 2023). It is possible that the supposed modifications of gel network structures in withered grapes physically influence the extractability of phenolic compounds from withered skins differently than fresh grapes. However, as Boulet et al. (2023) concluded, predicting the extraction of anthocyanins and tannins is wine from the grapes remains a challenge, and further studies aimed at a polysaccharides-polyphenols characterization could help in this direction.

As regards flesh (Figure 4b), they showed on average lower % of mannose and glucose than skins, confirming the lower presence of hemicellulose in pulps AISs cell wall of Aleatico with respect to the skins, in accordance with CoMPP results. Instead, galacturonic acid is highly represented (more than 40% in all the samples), indicating an important quantity of pectins in Aleatico pulps. Among treatments, however, the withering process did not significantly affect the composition of the pulps, regardless of the conditions applied (Figure 4b), except for fucose, which is a specific component of RGII (Doco et al., 2007).

In conclusion, the present study assessed the effect of different off-vine withering conditions on the physicochemical modifications of grape must composition and mechanical properties, with a particular focus on phenolic compounds and alcohol insoluble cell walls polysaccharides composition.

Sun-withered grapes showed the highest pH and lowest acidity, whereas these parameters were better preserved in controlled conditions. As regards mechanical properties, withered grapes had greater skin thickness (S_{psk}) and lower pedicel detachment force (F_{ped}), without significant differences between the dehydration modalities. Thus, while the first parameter affected the extraction kinetics of anthocyanins which was slower than fresh grapes for the two withering modalities, the second suggests that it would be more suitable to choose horizontal bunch placements for the withering of Aleatico grapes, independently of the withering conditions chosen. Moreover, CTR berries were significantly more rigid than FRESH and less rigid than SUN ($<E_{sk}$). These findings are consistent with the modifications of polysaccharide composition due to the formation of egg-boxes caused by higher de-esterification of pectins in SUN samples.

The extractability of phenolic compounds decreased in skins after withering, with a greater extent for sun-withered samples, except for the low-molecular tannins (FRV), which showed no significant differences in extractability among treatments, although they were involved in other chemical reactions (oxidation, polymerization).

The extractable phenolic profiles of skins in wine-like conditions were determined by different extraction yields probably caused by the cell walls modifications, combined with a loss of compounds by oxidation or other chemical reactions, which was overcome by the concentration effect for withering in controlled conditions, but not always for sun-dehydrated grapes. In fact, CTR skins phenolic profile was more like FRESH than SUN. Finally, SUN

skins macerating solutions experienced the lowest contents of anthocyanins and tannins, both for oligomers and polymers. SEC profiles highlighted a higher level of tannins polymerization in withered skins compared to FRESH, especially for SUN. However, high-polymerized tannins (DP>10) were too large to be extracted from the skins, leading to a lower DP of the high-polymerized fraction in SUN wine-like extracts compared with other samples.

As opposed to the skins, the extractability of phenolics and tannins from seeds increased after withering, again with a greater extent for SUN. For the first time, it was clearly demonstrated that seeds total profiles were not quantitatively and qualitatively affected by the withering. Therefore, the differences shown by the extractable profiles in wine-like conditions were due to a modified extractability due to the different withering conditions and not to chemical modifications of phenolic compounds occurred during the withering. Given the difficulty of studying seeds tissues, specific studies are necessary to understand the causes of the differences observed in extractability. Also, in the case of seeds, at the end of the 10 days of simulated macerations, the phenolic profile of CTR extracts were more similar to FRESH, than SUN. Consequently, concerning phenolic compounds the differences between the two withering conditions studied were substantial: sun-dehydrated grapes contained fewer polyphenols from the skins than fresh and CTR (including fewer anthocyanins responsible of the different color), and higher phenols from the seeds. These findings provide new awareness on the extractability of phenolic compounds from grapes subjected to different withering conditions, helping winemakers to choose the best maceration strategy to valorise the varietal characteristics of withered grapes.

A characterization of AISs cell walls polysaccharide composition through chemical and immunochemical approaches was also performed on pulps and skins AISs cell walls materials. This first characterization of the polysaccharide cell wall component of Aleatico grapes confirmed that this is a varietal-dependent feature. The results showed that withering process strongly affected the neutral sugar composition of skins AIS cell walls, particularly when the grapes were dehydrated in natural conditions under the sun, especially at the level of pectin chains and de-esterification. Instead, pulps polysaccharides undergo less marked changes.

SUPPLEMENTARY MATERIALS

Table S1 – Monoclonal Antibodies (mAb) and Carbohydrated Binding Modules (CBMs) used in this study for CoMPP analysis (Weiller et al., 2020).

Type	Code	Specificity	Group
mAb	JIM5	De-esterified partially methyl-esterified HG	HG
mAb	JIM7	Heavily methyl-esterified HG	
mAb	LM7	Partially methyl-esterified HG/ non-blockwise	
mAb	LM18	Partially de-esterified HG, higher affinity to shorter chain (DP <4)	
mAb	LM19	Partially de-esterified HG, higher affinity to longer chain (DP >4)	
mAb	LM20	Methyl-esterified HG	
mAb	2F4	HG Ca^{2+} crosslinked (methyl-esterified < 40)	
mAb	PAM1	Blockwise de-esterified HG	
mAb	LM8	Xylogalacturonan	
mAb	LM15	Xylogalacturonan (XXXG motif of xyloglucan)	
mAb	JIM8	Arabinogalactan protein	AGP
mAb	JIM13	Arabinogalactan protein	
mAb	LM2	Arabinogalactan protein β-linked Arabinose	
mAb	LM14	Arabinogalactan protein	
mAb	JIM11	Extensin	Extensins
mAb	JIM20	Extensin	
mAb	LM1	Extensin	
mAb	INRA-RU2	Backbone of rhamnogalacturonan I (need DP > 6)	RG-1 and side chains
mAb	INRA-RU1	Backbone of rhamnogalacturonan I (maximum binding to DP = 14)	
mAb	LM5	(1→ 4)-β-D-galactan	
mAb	LM6	(1→5)-α-L-arabinan	
mAb	LM16	Arabinan/put. galactan stub	
mAb	LM13	Linearised (1→5)-α-L-arabinan	
mAb	LM10	(1→4) β-D-Xylan	Xyloglucan
mAb	LM11	(1→4) β-D-xylan/arabinoxylan	
mAb	LM21	(1→4) β-D-galacto gluco mannan	
mAb	LM24	Galactosylated xyloglucan	
mAb	LM25	Xyloglucan/unsubstituted β-D-glucan	
mAb	BS-400-2	(1→3) β-D-glucan	
CBM	CBM3a	Crystalline cellulose	Cellulose

HG: homogalacturonan; AGP: arabinogalactan proteins; RG1: rhamnogalacturonan I

Tab. S2 – Color intensity, hue and CIEL*ab* coordinates measured during the wine-like simulated macerations of Aleatico skins at harvest (FRESH) and after withering in different conditions: SUN (sun-withered grapes) and CTR (withered grapes in controlled conditions).

Parameter	Treatment	24h	48h	96h	144h	192h	final point	Sign[b]
Color Intensity	FRESH	4.52 ± 0.452 b, β	5.740 ± 0.416 a, α	6.490 ± 0.600 b, α	6.380 ± 0.117 ab, α	5.980 ± 0.212 b, α	5.643 ± 0.194, α	***
	SUN	2.870 ± 0.209 c, γ	3.760 ± 0.567 b, βγ	5.090 ± 0.284 c, αβ	5.388 ± 1.101 a, αβ	6.204 ± 0.998 b, α	5.627 ± 0.871 αβ	***
	CTR	5.407 ± 0.263 a, β	6.673 ± 0.710 a, αβ	7.773 ± 0.788 a, α	7.613 ± 1.326 b, α	7.748 ± 0.543 a, α	6.543 ± 0.450 αβ	***
	Sign[a]	***	**	***	*	**	ns	
Hue	FRESH	0.399 ± 0.004 b, δ	0.390 ± 0.003 b, δ	0.391 ± 0.007 b, δ	0.414 ± 0.007 b, γ	0.442 ± 0.004 b, β	0.481 ± 0.007 b, α	***
	SUN	0.541 ± 0.024 a	0.491 ± 0.022 a	0.578 ± 0.0050 a	0.604 ± 0.114 a	0.630 ± 0.077 a	0.629 ± 0.055 a	ns
	CTR	0.415 ± 0.006 b, β	0.415 ± 0.005 b, β	0.433 ± 0.027 b, β	0.488 ± 0.027 b, α	0.513 ± 0.033 b, α	0.537 ± 0.013 b, α	***
	Sign[a]	***	***	***	**	**	**	
L*	FRESH	32.93 ± 2.68 b, α	27.27 ± 1.92 b, β	24.06 ± 2.87 β	24.37 ± 0.90 β	25.18 ± 1.00 β	24.98 ± 0.17 β	***
	SUN	43.85 ± 2.02 a, α	36.60 ± 3.99 a, αβ	22.96 ± 4.07 βγ	22.83 ± 11.76 βγ	16.15 ± 9.29 γ	20.15 ± 7.57 βγ	***
	CTR	27.87 ± 1.73 b, α	24.31 ± 1.98 b, αβ	18.13 ± 5.52 αβ	17.22 ± 8.12 αβ	15.39 ± 4.74 β	20.94 ± 2.74 αβ	*
	Sign[a]	***	**	ns	ns	ns	ns	
a*	FRESH	62.66 ± 2.16 a, α	58.97 ± 1.57 ab, αβ	55.72 ± 3.41 αβ	55.93 ± 1.43 αβ	56.21 ± 0.77 αβ	54.99 ± 0.73 αβ	***
	SUN	57.08 ± 2.12 b	60.52 ± 0.61 a	45.68 ± 6.66	45.98 ± 16.38	40.41 ± 12.97	45.10 ± 9.94	ns
	CTR	58.50 ± 1.67 ab	55.99 ± 1.59 b	48.19 ± 7.60	46.20 ± 11.16	44.26 ± 6.69	50.93 ± 3.53	ns
	Sign[a]	*	*	ns	ns	ns	ns	
b*	FRESH	13.70 ± 2.64 b, β	19.91 ± 1.41 b, α	22.31 ± 1.85 a, α	23.04 ± 1.85 α	21.17 ± 0.69 α	18.19 ± 2.98 αβ	***
	SUN	8.36 ± 0.94 c	12.54 ± 2.76 c	12.60 ± 3.08 b	15.81 ± 6.33	13.17 ± 6.21	15.66 ± 4.53	ns
	CTR	19.04 ± 0.85 a	26.30 ± 2.48 a	23.33 ± 5.55 a	21.01 ± 8.31	20.49 ± 5.76	23.77 ± 1.34	ns
	Sign[a]	***	***	**	ns	ns	ns	

All data are expressed as average value ± standard deviation (n = 3). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, for the differences among treatments for each maceration time ([a]) and among different maceration times for each treatment ([b]) according to ANOVA tests. Different Latin letters within the same column indicate significant differences ([a]) and different Greek letters within the same row indicate significant differences ([b]) according to Tukey HSD tests ($p < 0.05$ for ANOVA. L*: lightness; a*: red/green colour coordinate; b*: yellow/blue colour coordinate.

Tab S3 – Potential and extractable phenolic composition at the end of the simulated maceration in a wine-like solution of fresh (FRESH), and withered Aleatico grapes under the sun (SUN) or in *fruttaio*-controlled conditions (CTR) expressed as mg/kg berries.

Compound	Potential/Extractable	*fresh grapes*	*withered grapes*		Sign.
		FRESH	SUN	CTR	
Skins					
IPT (mg EC/kg berries)	Potential	3821 ± 286 b	4177 ± 719 ab	5036 ± 107 a	*
	Extractable	2296 ± 119 a	1629 ± 52 b	2446 ± 66 a	***
	Extraction yield (%)	60 ± 5 a	40 ± 6 c	49 ± 1 b	***
TA (mg Mv-3-G/kg berries)	Potential	580 ± 74	513 ± 101	688 ± 107	ns
	Extractable	413 ± 12 a	232 ± 18 b	377 ± 16 a	***
	Extraction yield (%)	72 ± 8 a	46 ± 9 b	55 ± 4 b	***
MTC (mg EC/kg berries)	Potential	1594 ± 154 a	1888 ± 261 ab	2104 ± 40 a	*
	Extractable	649 ± 12 a	533 ± 36 b	690 ± 14 a	***
	Extraction yield (%)	41 ± 4 a	29 ± 4 b	33 ± 1 b	***
FRV (mg EC/kg berries)	Potential	660 ± 34 a	397 ± 47 b	685 ± 190 a	*
	Extractable	379 ± 18 a	229 ± 16 b	397 ± 9 a	***
	Extraction yield (%)	57 ± 3	58 ± 7	60 ± 14	ns
FRV/MTC	Potential	0.42± 0.06 a	0.21± 0.02 b	0.32± 0.08 ab	*
	Extractable	0.58 ± 0.02 a	0.43 ± 0.02 b	0.58 ± 0.01 a	***
Anthocyanin profile					
Dp-3-G (%)	Potential	4.4 ± 0.1 a	3.3 ± 0.2 b	3.5 ± 0.1 ab	*
Cy-3-G (%)		0.7 ± 0.1 a	0.7 ± 0.2 a	0.4 ± 0.0 b	***
Pt-3-G (%)		5.1 ± 0.2	4.2 ± 0.5	4.4 ± 0.3	ns
Pn-3-G (%)		4.6 ± 0.7 ab	5.1 ± 0.3 a	3.7 ± 0.3 b	*
Mv-3-G (%)		52.7 ± 0.3	54.5 ± 1.3	54.0 ± 0.3	ns
∑ Acetyl-G (%)		11.1 ± 0.1 a	9.0 ± 0.3 b	9.0 ± 0.4 b	***
∑ Cinnamoyl-G (%)		21.5 ± 0.2 b	23.2 ± 1.5 ab	25.1 ± 0.5 a	**
Dp-3-G (%)	Extractable	2.2 ± 0.2	1.4 ± 0.4	1.2 ± 0.3	ns
Cy-3-G (%)		0.7 ± 0.1 a	0.3 ± 0.0 b	0.6 ± 0.1 ab	*
Pt-3-G (%)		4.0 ± 0.2 a	3.0 ± 0.3 b	2.9 ± 0.2 b	***
Pn-3-G (%)		4.7 ± 0.1 a	3.4 ± 0.8 b	3.1 ± 0.3 b	**
Mv-3-G (%)		64.5 ± 1.9 b	69.3 ± 1.1 a	67.1 ± 0.5 ab	*
∑ Acetyl-G (%)		11.5 ± 0.4 a	9.7 ± 0.6 b	10.3 ± 0.3 ab	*
∑ Cinnamoyl-G (%)		12.4 ± 1.3	12.9 ± 1.5	14.9 ± 0.6	ns
Phenolic acids (mg/kg berries)					
Caftaric acid	Potential	17.88 ± 1.65 a	7.09 ± 3.20 c	13.22 ± 1.71 a	**
	Extractable	5.33 ± 0.40 a	0.53 ± 0.14 c	2.10 ± 0.22 b	***
	Extraction yield (%)	30 ± 3 a	9 ± 4 c	16 ± 2 b	***
Coutaric acid	Potential	194.71 ± 25.76 a	44.76 ± 22.38 b	92.73 ± 27.47 a	**
	Extractable	40.57 ± 6.90 a	4.39 ± 1.24 b	4.98 ± 1.23 b	***
	Extraction yield (%)	21 ± 4 1 a	12 ± 6 b	6 ± 2 c	***
Flesh					
		Phenolic acids (mg/kg berries)			
Caftaric acid	Potential	53.54 ± 4.44 a	31.20 ± 5.37 b	53.04± 7.73 a	**
Coutaric acid	Potential	24.93 ± 6.23 a	12.07 ± 2.40 b	18.83 ± 4.60 ab	*
Seeds					
IPT (mg EC/kg berries)	Potential	5086 ± 514 b	7326 ± 700 a	7231 ± 276 a	**
	Extractable	1546 ± 60 b	3141 ± 63 a	2470 ± 467 a	**
	Extraction yield (%)	34 ± 9 b	41 ± 6 a	34 ± 6 ab	ns
MTC (mg EC/kg berries)	Potential	3080 ± 425 b	4370 ± 197 a	3955 ± 119 a	**
	Extractable	633 ± 239 b	1662 ± 553 a	970 ± 333 ab	*
	Extraction yield (%)	21 ± 7 b	38 ± 11 a	25 ± 7 ab	***
FRV (mg EC/kg berries)	Potential	3205 ± 425 b	4145 ± 194 a	4155 ± 119 a	**
	Extractable	880 ± 83 b	1870 ± 6 a	1427 ± 316 a	**
	Extraction yield (%)	28 ± 4 c	45 ± 2 a	34 ± 7 b	***
FRV/MTC	Potential	1.04 ± 0.10	0.95 ± 0.04	1.05 ± 0.09	ns
	Extractable	1.58 ± 0.75	1.20 ± 0.33	1.50 ± 0.59	ns

All data are expressed as average value ± standard deviation ($n = 3$). Sign: ***, **, * and ns indicate significance at $p < 0.001$, 0.01, 0.05 and not significant differences, respectively, for the differences among treatments (FRESH, SUN and CTR) and between the two different withering modalities (SUN, CTR) according to ANOVA([a]) and T-tests ([b]). Different Latin letters among the same raw indicate significant differences ([a]) according to Tukey-b test ($p < 0.05$) for ANOVA. TPI: total phenolic index (A280); TA: total anthocyanins; MCP: condensed tannins by methyl cellulose assay; FRV: flavanols reactive to vanillin; Dp-3-G: delphinidin-3-glucoside; Cy-3-G: cyanidin-3-Glucoside; Pt-3-G: petunidin-3-glucoside; Pn-3-G: peonidin-3-glucoside; Mv-3-G: malvidin-3-glucoside; EC: epicatechin; C: catechin; [#]: extraction yields (%) are calculated using the data expressed as mg/kg berries

Tab. S4 – Total area determined by SEC converted populations relative percentages (%) and in mg (-)-epicatechin/g of skins or seeds for Aleatico grape skins and seeds for fresh (FRESH) and 30% WL withered grapes naturally under the sun (SUN) and in *fruttaio* controlled conditions (CTR).

Skins/Seeds	Sample	SEC Percentages (%)			SEC equivalent quantifications (mg EC/g skins or seeds)		
		Polymers (%)	Oligomers (%)	Other compounds (%)	Polymers (mg/g)	Oligomers (mg/g)	Other compounds (mg/g)
Skins	*Potential*						
	FRESH	49.6 ± 1.1 c	3.1 ± 1.2 b	47.4 ± 2.3 a	16.4 ± 2.0 a	1.0 ± 0.4	15.6 ± 2.1 a
	SUN	59.4 ± 1.3 a	7.0 ± 0.4 a	33.6 ± 1.4 c	7.2 ± 0.4 b	0.9 ± 0.1	4.1 ± 0.5 c
	CTR	54.4 ± 1.2 b	4.3 ± 0.3 b	41.3 ± 0.9 b	15.0 ± 1.1 a	1.2 ± 0.1	11.4 ± 1.1 b
	Sign.	***	**	***	***	ns	**
	Extractable						
	FRESH	60.1 ± 1.4	20.8 ± 1.7	19.1 ± 0.3	7.0 ± 1.0 a	2.4 ± 0.2 a	2.2 ± 0.3 a
	SUN	58.6 ± 0.8	20.8 ± 1.9	20.6 ± 1.4	3.0 ± 0.3 b	1.1 ± 0.1 c	1.1 ± 0.1 b
	CTR	57.4 ± 1.3	22.6 ± 0.5	20.0 ± 1.0	4.0 ± 1.5 b	1.6 ± 0.2 b	1.4 ± 0.1 b
	Sign.	ns	ns	ns	***	***	**
Seeds	*Potential*						
	FRESH	76.9 ± 1.0	11.2 ± 0.2	11.8 ± 0.9	107.6 ± 7.3	15.7 ± 0.9	16.5 ± 0.5
	SUN	76.1 ± 1.7	11.5 ± 0.8	12.4 ± 0.9	104.9 ± 3.9	15.9 ± 1.0	17.1 ± 1.1
	CTR	76.4 ± 0.4	11.3 ± 0.6	12.3 ± 0.3	108.4 ± 6.7	16.1 ± 1.6	17.5 ± 1.3
	Sign.	ns	ns	ns	ns	ns	ns
	Extractable						
	FRESH	57.7 ± 0.4	19.2 ± 0.4 b	23.2 ± 0.2 a	28.2 ± 3.1 b	9.4 ± 1.2 b	11.3 ± 1.4
	SUN	57.9 ± 1.0	21.5 ± 0.8 a	20.7 ± 0.5 b	37.5 ± 2.1 a	13.9 ± 0.4 a	13.4 ± 0.3
	CTR	56.1 ± 1.3	20.7 ± 0.3 a	23.2 ± 1.1 a	26.7 ± 4.5 b	9.8 ± 1.5 b	11.0 ± 1.3
	Sign.	ns	**	**	*	**	ns

All data are expressed as average value ± standard deviation ($n = 3$). *Sign.*: ***, **, *, and ns indicate significance at $p < 0.001$, 0.01, 0.005, and not significant, respectively. Total profiles of skins (c) and seeds (d) are referred to the analysis of totally extracted solutions: 150 (skins) or 100 mg (seeds) of frozen powder in 6 mL of solvent (methanol+acetone/water formic acid); Extractable profiles are referred to the analysis of the wine-like solutions at the end of the 10 days simulated macerations of skins. EC= (-)-epicatechin

Tab. S5 – Quantitative composition (mg/g AIS) of skins and flesh cell walls glycosyl-residue (neutral sugars determined by GC analysis of alditol acetates and alditol acetates after Seaman hydrolysis).

	Sugars (mg/g AIS)	Alditol acetates				Seaman			
		FRESH	SUN	CTR	Sign.	FRESH	SUN	CTR	Sign.
Skins	Rha	8.03 ± 1.20	7.16 ± 0.86	6.80 ± 0.41	ns	1.46 ± 0.27 a	1.03 ± 0.08 ab	0.79 ± 0.01 b	*
	Fuc	3.03 ± 0.99	2.17 ± 0.23	1.98 ± 0.13	ns	1.01 ± 0.09 a	0.82 ± 0.04 b	0.67 ± 0.01 b	**
	2-O-MeXyl	0.22 ± 0.20	0.12 ± 0.10	0.06 ± 0.10	ns	0.20 ± 0.03	0.22 ± 0.05	0.18 ± 0.01	ns
	Rib	0.35 ± 0.31	0.43 ± 0.04	0.45 ± 0.02	ns	2.63 ± 0.49 a	2.22 ± 0.19 ab	1.55 ± 0.12 b	*
	Ara	29.08 ± 1.77 a	24.36 ± 1.49 b	23.53 ± 1.38 b	**	12.22 ± 1.60 a	9.30 ± 1.05 b	6.14 ± 0.76 b	**
	Xyl	14.06 ± 1.40	11.62 ± 0.51	11.21 ± 1.64	ns	0.76 ± 0.23	0.49 ± 0.29	0.16 ± 0.12	ns
	Mann	5.91 ± 0.68	5.71 ± 0.53	5.88 ± 1.78	ns	14.29 ± 3.06	8.74 ± 3.46	5.60 ± 1.07	ns
	Gal	18.41 ± 1.41 a	16.14 ± 0.89 b	15.38 ± 1.94 b	ns	6.60 ± 0.84 a	3.76 ± 1.07 b	2.29 ± 0.18 b	**
	Glc	8.02 ± 0.44 b	13.84 ± 0.92 a	9.99 ± 2.68 ab	*	11.71 ± 3.48 a	7.86 ± 3.98 ab	3.38 ± 0.70 b	ns
	Total sugars	87.09 ± 3.01 a	81.55 ± 4.08 ab	75.29 ± 9.28 b	ns	50.88 ± 7.70 a	34.44 ± 9.80 ab	20.68 ± 2.94 b	*
Flesh	Rha	15.05 ± 4.26	8.93 ± 4.08	9.99 ± 3.08	ns	8.3 ± 2.65	6.06 ± 0.58	6.15 ± 2.12	ns
	Fuc	3.59 ± 0.70 a	1.41 ± 0.13 b	2.00 ± 0.62 b	*	2.33 ± 0.56	1.64 ± 0.19	1.82 ± 0.60	ns
	2-O-MeXyl	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	ns	0.33 ± 0.10	0.21 ± 0.02	0.24 ± 0.06	ns
	Rib	1.39 ± 0.32	1.21 ± 0.88	0.45 ± 0.39	ns	2.41 ± 1.81	0.60 ± 0.05	0.96 ± 0.70	ns
	Ara	49.25 ± 11.85	28.06 ± 12.10	30.23 ± 8.74	ns	32.59 ± 6.54	22.21 ± 2.36	23.22 ± 7.02	ns
	Xyl	24.81 ± 2.18	17.32 ± 3.02	22.61 ± 9.39	ns	15.42 ± 5.84	16.28 ± 2.61	16.88 ± 6.43	ns
	Mann	3.67 ± 0.58	1.65 ± 2.85	1.73 ± 1.54	ns	13.92 ± 5.43	4.87 ± 1.53	7.68 ± 2.54	ns
	Gal	30.21 ± 7.39	19.58 ± 7.38	20.96 ± 5.35	ns	19.02 ± 6.49	17.56 ± 2.37	15.89 ± 4.74	ns
	Glc	6.14 ± 1.26	6.26 ± 2.98	4.94 ± 0.66	ns	84.36 ± 27.04	50.34 ± 37.06	68.71 ± 26.02	ns
	Total sugars	134.10 ± 27.79	89.24 ± 40.59	92.91 ± 25.16	ns	178.67 ± 45.52	119.79 ± 40.82	141.55 ± 47.21	ns

All data are expressed as average value ± standard deviation ($n = 3$). *Sign.*: **, *, and ns indicate significance at $p < 0.01$, 0.005, and not significant, respectively. Rha: rhamnose; Fuc: fucose: 2-O-MeXyl: 2-O-Me xylose; Rib: ribose; Ara: arabinose; Xyl: xylose: Man: mannose; Gal: galactose; Glc: glucose

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EXPERIMENTAL SESSION

Part B - Innovation in winemaking practices

Chapter IV

Can a Corn-Derived Biosurfactant Improve Colour Traits of Wine? First Insight on Its Application during Winegrape Skin Maceration versus Oenological Tannins

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ABSTRACT

In winemaking, oenological tannins are used to preserve wine colour by enhancing the antioxidant activity, taking part in copigmentation, and forming polymeric pigments with anthocyanins. As a novel processing aid, in this study, a biosurfactant extract was evaluated as a solubilizing and stabilizing agent of anthocyanins in red wine. The biosurfactant extract under evaluation was obtained from a fermented residual stream of the corn milling industry named corn steep liquor (CSL). Two red winegrape varieties (*Vitis vinifera* L. cv. Aglianico and Cabernet sauvignon) were studied for anthocyanin content and profile, and colour traits, during simulated skin maceration for 7 days at 25 °C, as well as polymerization and copigmentation at the end of maceration. A model wine solution was used as a control, which had either the CSL biosurfactant or with four different oenological tannins added (from grape skin, grape seed, quebracho, and acacia). The results showed that CSL biosurfactant addition improved the colour properties of skin extracts by the formation of more stable compounds mainly through copigmentation interactions. These preliminary results highlighted that the effectiveness of CSL biosurfactant is variety-dependent; however, there is no significant protection of individual anthocyanin compounds as observed for delphinidin and petunidin forms using quebracho tannin.

Keywords: wine grapes; biosurfactant; exogenous tannins; colour properties; anthocyanin composition; skin maceration; copigmentation; polymerization

1. Introduction

Perceived colour is an important attribute directly influencing the quality of red wine [1]. This feature can determine the product acceptability by consumers as it is related to wine 'healthy' and age. Compositionally, red wines are complex because a wide variety of compounds are extracted from grapes during the maceration process, metabolites are released by yeasts during alcoholic fermentation, and different chemical and enzymatic reactions occur [2–4]. Particularly, monomeric anthocyanins are located in the berry skin and they are responsible for the colour of red grapes and resulting wines [5]. These phenolic compounds are extracted in the first stages of maceration, even though their diffusion rate depends on the anthocyanin profile. It is well-known that disubstituted anthocyanins diffuse faster than trisubstituted forms [3]. Nevertheless, they can be easily oxidized, and thus, wine colour protection requires the formation of more stable anthocyanin-derived pigments.

The presence of other phenolic compounds can help to stabilize the colour of red wines by their interaction with anthocyanins. In young wines, non-covalent molecular associations through copigmentation can account for up to 30% of the observed colour [6]. Moreover, condensation reactions between anthocyanins and flavanols can occur either directly or mediated by acetaldehyde, leading to the formation of polymeric pigments [2]. These covalently formed adducts, which represent between 35% and 63% of the total wine colour, are resistant to oxidation and sulphur dioxide bleaching [6]. Some studies have highlighted that the content of different phenolic compounds in grape berries and their extractability into the must during maceration are interrelated [7]. At the same time, the concentration and release of both anthocyanins and flavanols are influenced by several factors such as variety, ripeness degree, berry skin mechanical properties, soil conditions, climate, vintage, and viticultural practices [8,9]. In addition, maceration strategies greatly impact the extractability of phenolic compounds during winemaking [9]. Bearing in mind all these aspects,

the anthocyanins/tannins ratio has been proposed as an indicator of polymeric pigment formation, wine colour, and overall wine quality [10].

Nowadays, the addition of exogenous tannins during maceration is an oenological practice used for multiple purposes, such as to promote the formation of anthocyanin-derived pigments and therefore preserving anthocyanins and stabilizing wine colour amongst others [11,12]. A wide range of commercial oenological tannins is available, which differ in phenolic composition, botanical origin, and tannic richness [13]. They usually consist of pure or mixed formulations of hydrolysable and condensed tannins. Hydrolysable tannins include gallotannins coming from gallnuts and tara, as well as ellagitannins from chestnut and oak. Condensed tannins, known as proanthocyanidins, are mainly extracted from grape seeds (procyanidins), from grape skins (prodelphinidins and procyanidins), from quebracho (profisetinidins), from mimosa (prorobinetinidins), and acacia (profisetinidins, prorobinetinidins, and prodelphinidins) [14–16].

A novel alternative for wine colour preservation has recently been proposed, which is based on the use of surface-active compounds. Particularly, the protection mechanism of a polysorbate-based chemical surfactant (Tween 20) for anthocyanins may be related to the solubilisation of these pigments within the micelles [17]. Nevertheless, the main disadvantage of using chemical surfactants in foods is their low degradability, being not yet admitted as oenological adjuvants by the International Organisation of Vine and Wine (OIV). Instead, biological surfactants, namely biosurfactants, are less toxic as well as more biodegradable and biocompatible than chemical surfactants and emulsifiers [18]. Biosurfactants are produced by microorganisms through biotechnological processes [19] and they are composed of biomolecules. In the food industry, biosurfactants have been used for different purposes, such as fat stabilization, antifoaming, increased solubility in instant drinks and soups, starch complexation, and protective coatings [20,21]. Among biosurfactants, the extract obtained from corn steep liquor (CSL), which is a spontaneously fermented agri-food residue, is cost-competitive and has an important antioxidant activity due to

the presence of phenolic compounds [22]. Additionally, its amphiphilic nature, derived from a hydrophobic tail composed of fatty acids [22,23] and a hydrophilic head containing nitrogen similar to lipopeptides [24], makes possible the solubilisation of a great diversity of compounds.

There is evidence that hydrogen bonds and hydrophobic interactions regulate the association between proanthocyanidins and cell wall material [25]. These interactions occur through hydroxyl groups as well as aromatic and glycosidic oxygen atoms contained in proteins and polysaccharides of cell walls [26]. In this regard, the presence of surfactants may also increase the solubility of these hydrophobic complexes and therefore may promote copigmentation and polymerization reactions. Polysorbates are often used in the food industry to solubilize hydrophobic compounds in water-based products [27].

To our knowledge, a biosurfactant has never been tested during grape skin maceration to improve the colour features of red wines. Therefore, the main aim of this study was to evaluate the effectiveness of the biosurfactant extract obtained from CSL to improve the release and stabilization of skin anthocyanins in the first steps of maceration. Furthermore, four different exogenous tannins extracted from grape seeds, grape skins, quebracho, and acacia were also evaluated because they are commonly used for this purpose during winemaking. For two red winegrape varieties (*Vitis vinifera* L. cv. Cabernet sauvignon and Aglianico), the berry skins were subjected to simulated macerations in presence of each exogenous tannin or CSL biosurfactant to reduce the side-reactions due to the complex wine matrix.

2. Materials and Methods

2.1. Chemicals and Standards

Solvents of HPLC-gradient grade, Folin–Ciocalteu reagent, bovine serum albumin, and standards of gallic acid, cyanidin chloride, (−)-epicatechin, and (+)-catechin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Malvidin-3-glucoside chloride standard was purchased from Extrasynthese (Genay, France).

The solutions were prepared in deionized water produced by a Milli-Q system (Merck Millipore, Darmstadt, Germany).

2.2. Grape Samples

In 2018, whole bunches of *Vitis vinifera* L. cv. Aglianico and Cabernet sauvignon red winegrapes were harvested at ripeness (about 24 Brix) from the CNR-IPSP ampelographic collection of Grinzane Cavour (Cuneo province, north-west Italy, 44.651 N, 7.995 E). Once in the laboratory, ten kilograms of berries were manually separated from the stalks by cutting the pedicel of each single berry in the proximity of the receptacle. For each grape variety, a set of 200 berries was randomly sampled ("unsorted" samples) for the determination of the grape must standard compositional parameters. For the simulated maceration tests, the remaining berries were density sorted by flotation in different saline solutions (from 130 to 190 g/L NaCl corresponding to densities between 1087 and 1125 kg/m^3) as described by Fournand et al. [28]. Only the berries belonging to the most representative density class for each variety were selected, corresponding to 1106 kg/m^3 for Aglianico and 1100 kg/m^3 for Cabernet sauvignon. Sorted berries were washed with water and visually inspected before analysis, those with damaged skins were discarded. The use of density-sorted berries minimizes the differences in grape berry ripeness within the vineyard. For the chosen density class, two subsamples of 200 berries each were randomly taken to determine the grape must standard compositional parameters and whole grape phenolic ripeness indices. Additionally, three sets of 10 sorted berries were randomly selected to determine total skin phenolic composition, and other eighteen sets of 20 sorted berries were used for skin simulated maceration tests.

2.3. Standard Chemical Parameters

The compositional parameters of grape must, which are usually used to define technological ripeness, were determined for each variety in unsorted and sorted samples. Two replicates of about 100 grape berries were manually crushed and the liquid must was centrifuged at 3000 × g for 15 min at 20 °C, using a

Hettich 32R centrifuge (Tuttlingen, Germany). The supernatant obtained was used for analysis. Total soluble solids (°Brix) were evaluated using an Atago Palette 0–32 Brix refractometer with automatic temperature compensation (Atago Corporation, Tokyo, Japan). Titratable acidity (expressed as g/L of tartaric acid) and pH determinations were conducted using OIV methods [29] by titrimetry and potentiometry with an InoLab 730 calibrated pHmeter (WTW, Weilheim, Germany), respectively. Reducing sugars (glucose and fructose) were quantified (g/L) using an HPLC (Agilent Technologies, Santa Clara, USA) equipped with a refractive index detector [30].

The two phenolic ripeness indices, cell maturity index (EA%) and seed maturity index (Mp%), were assessed on two replicates of 100 berries, for which grapes were homogenized by grinding according to the method proposed by Saint-Cricq et al. [31] with slight modifications [5].

2.4. Total Extraction of Phenolic Compounds from Berry Skins

For each variety, three replicates of ten sorted berries were randomly selected. For each replicate, the berries were weighed, and the skins were manually separated from the pulp, weighed, and quickly immersed into 25 mL of a buffer solution at pH 3.40 containing 14% v/v of ethanol, 5 g/L of tartaric acid, and 2 g/L of sodium metabisulphite [9]. The extract was obtained by homogenization with an Ultra-Turrax T25 high-speed homogenizer (IKA Labortechnik, Staufen, Germany) for 1 min at 8000 rpm, and subsequent centrifugation at 3000 × g for 15 min at 20 °C in the Hettich 32R centrifuge. The supernatant obtained was used for the analytical determination of the skin phenolic composition as indicated below.

2.5. Oenological Tannins and Biosurfactant

Four different condensed oenological tannins were considered in this study as representative of the various formulations on the market: i) two proanthocyanidin preparations extracted from grapes, procyanidins from grape seeds and procyanidins/prodelphinidins from grape skins; ii) other two from

exotic woods, prorobinetinidins from acacia (*Mimosaceae sp.*) and profisetinidins from quebracho (*Schinopsis* spp.). All these oenological tannin formulations were characterized as follows. Total phenolic content was determined using the Folin–Ciocalteu assay in a wine-like solution at pH 3.5 (12% v/v of ethanol and 4 g/L of tartaric acid) containing 1 g/L of tannin [13]. The tannin richness, expressed as g of gallic acid/100 g of commercial formulation, was 62.1 ± 1.8 for grape seed-derived tannins, 56.6 ± 1.9 for grape skins, 51.5 ± 3.2 for acacia, and 55.8 ± 6.9 for quebracho (mean ± standard deviation of three replicates).

As a novel alternative, a biosurfactant was also evaluated for its surface-active and antioxidant properties [22]. The biosurfactant under evaluation comes from a corn steep liquor (CSL), which is a residual stream produced by the corn wet-milling industry, spontaneously fermented by lactic acid bacteria. *Lactobacillus* strains are defined by the US Food and Drug Administration (FDA) as "Generally Recognized As Safe" (GRAS) [32]. The biosurfactant extract was obtained by liquid–liquid extraction with ethyl acetate (CSL solution:ethyl acetate, 1:3 v/v), at room temperature for 60 min, followed by subsequent evaporation of the organic phase. In addition to lipopeptides, different phenolic compounds have been identified in the CSL biosurfactant extract, including protocatechuic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, epicatechin, and quercetin, which are directly related to the antioxidant activity [22].

2.6. Skin Simulated Maceration

For each winegrape variety, three replicates of 20 sorted berries were randomly selected for each of the six skin simulated maceration tests conducted (control, four oenological tannins, and CSL biosurfactant) and then treated following the procedure reported by Paissoni et al. [12]. The berries were weighed, manually peeled, and the resulting skins were carefully separated from the pulp, weighed, and quickly immersed into 100 mL of a buffer solution at pH 3.40 containing 5 g/L of tartaric acid (control), in which an established dose of

tannin formulation (grape seeds, grape skins, quebracho, or acacia derived) or CSL biosurfactant was previously added as follows. Each tannin formulation was dissolved in 100 mL of warm (40 °C) buffer solution enriched with 2% v/v of ethanol to help solubilisation. Then, 10 mL of the tannin solution was added to 90 mL of the buffer solution (without ethanol) for each replicate**.** For this reason, the macerating buffer solution also contains 0.2% v/v of ethanol. The dose of each tannin formulation used in this experiment corresponds to the dosage commonly added during maceration in industrial winemaking, that is 4/5 of the maximum recommended dose (20, 25, 22, and 40 g/hL for grape seeds, grape skins, acacia, and quebracho, respectively). For the CSL biosurfactant, a dose of 100 g/hL was used for the trial, which is higher than the critical micellar concentration (about 200 mg/L) to ensure the formation of micelles [33].

To simulate the wine fermentative maceration process, berry skins were macerated for 7 days at 25 °C with progressive addition of 96% v/v ethanol at 6, 24, 48, 72, and 96 h of maceration. Just before each addition, an equal aliquot of sample was taken to maintain constant the volume of the macerating solution. In particular, the ethanol concentration was 2.50, 4.80, 7.10, 10.6, and 14.0% v/v after addition at 6, 24, 48, 72, and 96 h of maceration, respectively. Once the maceration was completed (168 h), the whole liquid extract was taken for a more complete analytical determination. The effect of adding oenological tannin or biosurfactant was evaluated on the colour traits and anthocyanin extraction yield throughout skin simulated maceration, as well as on the phenolic composition at the end of the process, as indicated below.

2.7. Phenolic Composition Determination

The phenolic composition was determined through spectrophotometric methods [34] using a UV-1800 spectrophotometer (Shimazdu Corp., Kyoto, Japan). Total anthocyanin (TA) and non-anthocyanin flavonoid (FNA) concentrations were quantified (mg of malvidin-3-glucoside chloride/kg of grape berries and mg of catechin/kg of grape berries, respectively) by diluting the sample with an ethanol:water: 37% hydrochloric acid (70:30:1, v/v) solution and

subsequent measurement of absorbance at 536−540 and 280 nm. Total phenolic index (IPT) was evaluated (mg of (−)-epicatechin/kg of grape berries) by measuring absorbance at 280 nm of the sample diluted in water. Total phenolic compounds were also determined (mg of gallic acid/kg of grape berries) through the Folin–Ciocalteu (FC) assay. For the determination of FC in the total skin extracts, since the buffer solution had a very high concentration of sulphur dioxide, the 20-diluted samples were submitted to solid-phase extraction (SPE) on C_{18} Sep-Pak cartridge (Waters Corporation, Milford, MA, USA). Proanthocyanidins (PRO) were quantified (mg of cyanidin chloride/kg of grape) according to the Bate–Smith reaction. Monomeric and oligomeric flavanols were determined (mg of (+)-catechin/kg of grape) as Flavanols Reactive to Vanillin (FRV) [35].

The determination of anthocyanin profile was performed with an Agilent 1260 HPLC-DAD system (Agilent Technologies, Santa Clara, CA, USA), using the chromatographic conditions previously reported by Río Segade et al. [9]. Each skin extract was diluted 1:1 with an HCl solution at pH 0.5, filtered through a 0.45 µm PTFE membrane filter, and then injected (50 µL). A LiChroCART analytical column (25 cm × 0.4 cm i.d.) was used, which was purchased from Merck (Darmstadt, Germany) and packed with LiChrospher 100 RP-18 (5 µm) particles supplied by Alltech (Deerfield, IL, USA). The mobile phase consisted of A = formic acid/water (10:90, v/v) and B = formic acid/methanol/water (10:50:40, v/v), working in gradient mode from 28% of solvent B, increased up to 45% of B in 15 min, to 70% in 20 min, and 90% in 10 min. Individual anthocyanins were quantified at 520 nm and expressed as a percentage, whereas the sum of all individual forms was expressed as mg of malvidin-3-glucoside chloride/kg of grape berries.

At the end of skin simulated maceration (168 h), the formation of polymeric pigments between anthocyanins and tannins was assessed following the method proposed by Harbertson et al. [36]. The combination of a protein precipitation assay (bovine serum albumin protein, BSA) and the traditional bisulphite bleaching was used to distinguish two classes of polymeric pigments: long

polymeric pigments (LPP) and small polymeric pigments (SPP), expressed as a percentage. To evaluate the possible non-covalent molecular associations between anthocyanins and other organic molecules, a copigmentation assay was performed following the Boulton method [37]. Copigmentation and free anthocyanins were estimated as a percentage.

2.8. Colour Characteristics Determination

At each maceration sampling point (6, 24, 48, 72, 96, and 168 h), the visible spectra (380−780 nm) of the undiluted samples were acquired using 1 mm optical path cuvettes. Colour intensity ($A_{420\ nm} + A_{520\ nm} + A_{620\ nm}$ on an optical path of 10 mm) and tonality ($A_{420\ nm}/A_{520\ nm}$) values were obtained according to the method OIV-MA-AS2-07B [29]. CIEL*a*b* parameters, namely lightness (L*), red/green colour coordinate (a*), and yellow/blue colour coordinate (b*), were calculated following the OIV-MA-AS2-11 method [29]. The ΔE* parameter defined as colour difference between control and treated samples was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ [29].

2.9. Statistical Analysis

Statistical analysis was carried out using R statistic software, version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). The normality and homoscedasticity of the data were tested for all parameters by using the Shapiro–Wilk's and Levene's tests, respectively. For each studied variable distributed normally and with homogeneity in variance, one-way analysis of variance (ANOVA) using the Tukey HSD post-hoc test was used to evaluate significant differences among treatments at the same maceration time or among different maceration times for the same treatment. When populations presented heterogeneity in variance or were not distributed normally, non-parametric tests were performed (Welch-one-way ANOVA test with Games–Howell post-hoc and Kruskal–Wallis test with Conover post-hoc, respectively). Differences were considered statistically significant at p-value < 0.05. Principal component analysis (PCA) using the R package 'factoextra' [38] was performed to compare

the effect of the different treatments conducted on the two varieties studied while minimizing the contribution of different values of chemical parameters by normalization as *z*-scores before multivariate analysis.

3. Results and Discussion

3.1. Grape Characterization

The average values of analytical parameters determined at harvest in unsorted samples for the two red winegrape varieties studied were the following: 24.65 Brix, pH 3.29, and 7.31 g/L as tartaric acid for titratable acidity in Aglianico; and 22.65 Brix, pH 3.44, and 6.08 g/L as tartaric acid for titratable acidity in Cabernet sauvignon. Nevertheless, the experiment was conducted on sorted berries to reduce the heterogeneity in the berry characteristics caused by the different ripening evolution in the vineyard [28,39]. For each variety, the berries belonging to the most representative density class were chosen. Table 1 shows the parameters defining the technological and phenolic ripeness. The metabolites that most influence the grape berry density are sugars and organic acids [40] and therefore Aglianico grapes (1106 kg/m^3 density) were richer in reducing sugars and acids than Cabernet sauvignon (1100 kg/m^3 density). Regarding phenolic ripeness, both EA% and Mp% indices were quite similar for the two varieties (43.66 and 39.96 for EA%, 75.67 and 69.85 for Mp% in Aglianico and Cabernet sauvignon, respectively).

Aglianico and Cabernet sauvignon red winegrape varieties were used for this study because of their different phenolic profile. Table 1 reports the phenolic composition and anthocyanin profile of berry skins for the two winegrape varieties at harvest. The richest variety in total skin phenolic compounds, anthocyanins, and flavanols was Cabernet sauvignon. Regarding the anthocyanin profile, Aglianico and Cabernet sauvignon were characterized by a high percentage of trisubstituted anthocyanins (70.48% and 61.72%, respectively, as a sum of delphinidin, petunidin, and malvidin glucosides), with a clear prevalence of malvidin-3-glucoside in both the varieties. However, compared to Aglianico,

Cabernet sauvignon had a significantly lower percentage of malvidin-3-glucoside but higher one of delphinidin-3-glucoside. Furthermore, there were significant differences in acylated anthocyanins, Aglianico being richer in cinnamoylated forms whereas Cabernet sauvignon is richer in acetylated derivatives. These results agree with those previously published for these varieties [41].

Table 1. Composition of sorted grape berries for Aglianico and Cabernet sauvignon winegrapes.

Compound	Unit	Grape Cultivar		Sign
		Aglianico	Cabernet Sauvignon	
Grape must [a]				
Reducing sugars	g/L	262 ± 4	234 ± 5	*
pH	-	3.30 ± 0.01	3.49 ± 0.00	*
Titratable acidity	g/L as tartaric acid	7.09 ± 0.05	5.68 ± 0.03	*
EA%	%	43.66 ± 1.55	39.96 ± 0.69	ns
Mp%	%	75.67 ± 0.01	69.85 ± 0.48	**
Grape skin phenolic composition [b]				
TA	mg malvidin-3-glucoside chloride/kg grapes	879 ± 15	1060 ± 41	**
IPT	mg (-)-epicatechin/kg grapes	3173 ± 180	3731 ± 178	*
FC	mg gallic acid/kg grapes	1871 ± 298	2671 ± 494	ns
PRO	mg cyanidin chloride/kg grapes	2561 ± 272	4270 ± 185	***
FRV	mg (+)-catechin/kg grapes	462 ± 43	642 ± 80	*
FRV/PRO	-	0.18 ± 0.02	0.15 ± 0.01	ns
Anthocyanin profile [b]				
Dp-3-G	%	5.37 ± 0.27	12.58 ± 0.97	***
Cy-3-G	%	0.30 ± 0.05	1.53 ± 0.32	**
Pt-3-G	%	6.53 ± 0.26	5.32 ± 0.04	**
Pn-3-G	%	2.55 ± 0.30	5.44 ± 0.82	**
Mv-3-G	%	58.58 ± 0.99	43.82 ± 1.38	***
∑ Acetyl	%	3.76 ± 0.13	22.21 ± 0.57	***
∑ Cinnamoyl	%	22.92 ± 1.66	9.10 ± 0.11	***

All data are expressed as average value ± standard deviation ([a] $n = 2$, [b] $n = 3$). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, according to ANOVA test. EA%: cell maturity index, Mp%: seed maturity index, TA: total anthocyanins, IPT: total phenolic index, FC: Folin–Ciocalteu index, PRO: proanthocyanidins, FRV: flavanols reactive to vanillin. Dp-3-G: delphinidin-3-glucoside, Cy-3-G: cyanidin-3-glucoside, Pt-3-G: petunidin-3-glucoside, Pn-3-G: peonidin-3-glucoside, Mv-3-G: malvidin-3-glucoside.

3.2. Colour Parameters Evolution During Skin Maceration

The effect of CSL biosurfactant and the four oenological tannins was assessed on the colour of the macerating solutions during the simulated process. The visible spectra acquired at each sampling point were used to calculate colour intensity as the sum of yellow ($A_{420\ nm}$), red ($A_{520\ nm}$), and blue ($A_{620\ nm}$) colour fractions, as well as tonality (the ratio between yellow and red colour fractions), indicating the contribution of the fractions composing the overall colour. The evolution of colour intensity and tonality during skin simulated maceration is shown in Table 2 for each product tested.

Throughout the maceration process, colour intensity showed a similar trend for all the treatments on Aglianico and Cabernet sauvignon varieties. The colour intensity of macerating solutions increased progressively until reaching a maximum value and then decreased in the latter stages of maceration. This maximum was achieved at 72 h for Aglianico and 48 h for Cabernet sauvignon, although the differences found in the colour intensity values between these two maceration times were not significant. In any case, colour intensity increased between 2.1 and 3.2 units in all the samples tested from 6 to 168 h of maceration. Table 2 also shows the different effects of adding biosurfactant and oenological tannins on colour intensity in the two varieties studied during maceration. For Aglianico winegrapes, the highest values of colour intensity found in the macerating solutions were generally found for quebracho-based tannin formulation, even though the increase observed was not always significant with respect to control. Regarding Cabernet sauvignon, the skin extracts had the most intense colour in the presence of CSL biosurfactant, followed by quebracho tannin, with very few exceptions. At the end of maceration (168 h), the two varieties showed a different influence of treatments on the colour intensity values. No significant differences were observed among the treatments tested for Aglianico, whereas the experiment conducted on Cabernet sauvignon highlighted significantly higher values of colour intensity for CSL biosurfactant treated samples when compared to control as well as to grape skin and seed proanthocyanidin tannins.

It should be also evidenced that, on average, colour intensity values relative to the extracts obtained from the maceration of Cabernet sauvignon skins were lower than those obtained from Aglianico skins, despite the higher concentration of total anthocyanins. It may be due to differences in the anthocyanin profiles of the two varieties during maceration [42].

Table 2. Colour intensity and tonality of skin extracts during maceration with tannins from different origins and corn steep liquor (CSL) biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Colour Index	Grape Cultivar	Treatment	6 h	24 h	48 h	72 h	96 h	168 h	Sign [b]
Colour intensity (A.U.)	Aglianico	control	1.823 ± 0.187 γ	4.110 ± 0.288 b, β	5.213 ± 0.313 a, α	5.540 ± 0.431 α	5.157 ± 0.341 a, α	4.847 ± 0.407 $\alpha\beta$	***
		grape seeds	2.077 ± 0.218 γ	4.423 ± 0.134 ab, β	5.367 ± 0.177 a, α	5.630 ± 0.177 α	5.337 ± 0.175 a, α	4.790 ± 0.248 β	***
		grape skins	2.203 ± 0.280 δ	4.587 ± 0.180 ab, γ	5.583 ± 0.050 a, α	5.890 ± 0.078 α	5.540 ± 0.151 a, $\alpha\beta$	5.053 ± 0.257 $\beta\gamma$	***
		acacia	2.137 ± 0.203 δ	4.243 ± 0.103 ab, γ	5.227 ± 0.119 a, α	5.433 ± 0.119 α	5.137 ± 0.125 a, α	4.637 ± 0.108 β	***
		quebracho	2.407 ± 0.222 ε	4.740 ± 0.131 a, δ	5.763 ± 0.120 a, $\alpha\beta$	6.103 ± 0.055 α	5.723 ± 0.049 a, β	5.190 ± 0.085 γ	***
		biosurfactant	2.150 ± 0.212 γ	4.473 ± 0.219 ab, β	5.560 ± 0.295 a, α	5.803 ± 0.351 α	5.548 ± 0.335 a, α	5.323 ± 0.494 $\alpha\beta$	***
		Sign [a]	ns	*	*	ns	*	ns	
	Cabernet sauvignon	control	1.477 ± 0.160 c, β	3.703 ± 0.349 b, α	4.620 ± 0.406 α	4.633 ± 0.421 α	4.120 ± 0.398 b, α	3.703 ± 0.223 c, α	***
		grape seeds	1.710 ± 0.100 abc, δ	4.003 ± 0.196 ab, $\beta\gamma$	4.670 ± 0.108 α	4.483 ± 0.333 $\alpha\beta$	4.233 ± 0.190 ab, $\alpha\beta\gamma$	3.857 ± 0.181 c, γ	***
		grape skins	1.540 ± 0.139 bc, γ	4.100 ± 0.249 ab, β	5.003 ± 0.310 α	4.510 ± 0.246 $\alpha\beta$	4.443 ± 0.280 ab, $\alpha\beta$	4.090 ± 0.274 bc, β	***
		acacia	1.757 ± 0.045 abc, δ	4.173 ± 0.110 ab, γ	5.097 ± 0.234 α	5.023 ± 0.280 α	4.657 ± 0.092 ab, $\alpha\beta$	4.257 ± 0.015 abc, $\beta\gamma$	***
		quebracho	1.887 ± 0.117 a, δ	4.360 ± 0.215 a, γ	5.090 ± 0.278 α	5.033 ± 0.301 $\alpha\beta$	4.873 ± 0.214 ab, $\alpha\beta\gamma$	4.450 ± 0.090 ab, $\beta\gamma$	***
		biosurfactant	1.807 ± 0.042 ab, γ	4.377 ± 0.012 a, $\beta\gamma$	5.490 ± 0.676 α	5.217 ± 0.621 $\alpha\beta$	4.987 ± 0.434 a, $\alpha\beta$	4.637 ± 0.360 a, $\alpha\beta\gamma$	*
		Sign [a]	**	*	ns	ns	*	**	
Tonality	Aglianico	control	0.454 ± 0.010 c, α	0.397 ± 0.009 b, β	0.398 ± 0.006 b, β	0.409 ± 0.005 b, β	0.435 ± 0.008 α	0.478 ± 0.020 α	***
		grape seeds	0.513 ± 0.005 a, α	0.431 ± 0.010 a, β	0.418 ± 0.007 a, β	0.428 ± 0.009 a, β	0.457 ± 0.008, β	0.488 ± 0.010 β	***
		grape skins	0.487 ± 0.005 b, α	0.416 ± 0.005 ab, γ	0.416 ± 0.002 ab, γ	0.420 ± 0.003 a, γ	0.453 ± 0.005 β	0.484 ± 0.007 α	***
		acacia	0.511 ± 0.007 ab, α	0.428 ± 0.002 a, β	0.423 ± 0.006 a, β	0.427 ± 0.006 a, β	0.460 ± 0.006 α	0.491 ± 0.005 α	***
		quebracho	0.486 ± 0.012 b, α	0.416 ± 0.003 ab, γ	0.414 ± 0.003 ab, γ	0.418 ± 0.000 a, γ	0.451 ± 0.002 β	0.476 ± 0.003 α	***
		biosurfactant	0.440 ± 0.008 c, β	0.396 ± 0.008 b, γ	0.397 ± 0.008 b, γ	0.408 ± 0.004 b, γ	0.433 ± 0.004, β	0.477 ± 0.003 α	***
		Sign [a]	***	***	***	*	ns	ns	
	Cabernet sauvignon	control	0.460 ± 0.017 c, $\beta\gamma$	0.413 ± 0.012 c, γ	0.427 ± 0.015 b, γ	0.463 ± 0.015 $\beta\gamma$	0.490 ± 0.020 β	0.570 ± 0.026 α	***
		grape seeds	0.537 ± 0.012 a, β	0.487 ± 0.025 a, $\gamma\delta$	0.483 ± 0.006 ab, δ	0.473 ± 0.015 δ	0.527 ± 0.006 $\beta\gamma$	0.607 ± 0.015 α	***
		grape skins	0.510 ± 0.017 ab, $\beta\gamma$	0.487 ± 0.021 a, $\gamma\delta$	0.487 ± 0.006 a, $\gamma\delta$	0.463 ± 0.012 δ	0.530 ± 0.010 β	0.593 ± 0.012 α	***
		acacia	0.517 ± 0.006 ab, β	0.460 ± 0.010 ab, γ	0.463 ± 0.012 ab, γ	0.460 ± 0.010 γ	0.507 ± 0.006 β	0.597 ± 0.006 α	***
		quebracho	0.530 ± 0.017 a, β	0.470 ± 0.000 ab, γ	0.457 ± 0.006 ab, γ	0.457 ± 0.006 γ	0.507 ± 0.006 β	0.587 ± 0.015 α	***
		biosurfactant	0.477 ± 0.015 bc, β	0.440 ± 0.017 bc, β	0.457 ± 0.047 ab, β	0.457 ± 0.038 β	0.510 ± 0.026 β	0.593 ± 0.015 α	***
		Sign [a]	***	*	*	ns	ns	ns	

All data are expressed as average value ± standard deviation ($n = 3$). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, for the differences among treatments for each maceration time ([a]) and among different maceration times for each treatment ([b]) according to ANOVA, Welch's ANOVA, or Kruskal–Wallis tests. Different Latin letters within the same column indicate significant differences ([a]) and different Greek letters within the same row indicate significant differences ([b]) according to Tukey HSD, Games–Howell, and Conover's tests ($p < 0.05$) for ANOVA, Welch's ANOVA, and Kruskal–Wallis tests, respectively.

Regarding tonality (Table 2), its evolution during maceration followed the same trend for both Aglianico and Cabernet sauvignon varieties, independently on the treatment. During the first 24 h of maceration, a decrease in the tonality value was observed, meaning a higher red colour component ($A_{520\ nm}$) with respect to the yellow component ($A_{420\ nm}$) and, therefore, the macerating solution shifted to a red hue. This value remained fairly constant until 72 h of maceration and then increased probably due to a loss of red colour component. An advantage of the CSL biosurfactant addition, differently from oenological tannins, is that no significant increase in tonality values was observed at any sampling time if compared with the control maceration. In any case, the differences were not significant among treatments and control for the two varieties from 96 h of skin maceration.

To better describe how the addition of the CSL biosurfactant and oenological tannins (grape seeds, grape skins, acacia, and quebracho) affected the visually perceived colour of skin macerating solutions compared to the control during the simulated maceration process, CIEL*a*b* coordinates were calculated (Table S1) and then converted in the corresponding colour on the RGB scale (Figure 1). In agreement with the significant changes observed in colour intensity and tonality (Table 2), CIEL*a*b* coordinates were strongly affected by the skin maceration and treatments tested. Since the RGB space corresponds to the biological processing of colour in the human visual system [43], this representation allows us to visualize the wine colour in a similar way to the real one [44]. For each variety and at each maceration time, objective comparisons were done by quantifying the colour differences found for each treatment for control using ΔE* values. The results for Aglianico and Cabernet sauvignon varieties are reported in Figure 1. A ΔE* threshold of about three units was established to correctly detect wine colour differences by the human eye [45] or of five units when considering that the colour observation is carried out through a wine taste glass [46].

Figure 1 shows that most of the ΔE* values were greater than 3.0 units, except for acacia tannin at 24, 48, 72, and 96 h of maceration as well as CSL

biosurfactant at 48 and 72 h only for Aglianico variety. Nevertheless, some trends can be evidenced. For Aglianico variety, the highest ΔE* values were found at the beginning of maceration (6 h), ranging from 5.64 for CSL biosurfactant to 10.36 for quebracho-based tannin formulation, and, after their decrease until 96 h, the ΔE* parameter increased at the end of maceration up to values between 4.31 for grape skin tannin and 5.88 for quebracho tannin. To understand these variations, the evolution of the three main CIEL*a*b* coordinates was analysed (Table S1). At 6 h of maceration, the differences observed in ΔE* with respect to control corresponded to the increase of b* values (yellow/blue colour coordinate) even though it was significant only for the different tannin formulations tested, evidencing a colour displacement towards yellow hue. Nevertheless, the high ΔE* values observed at 168 h were associated mainly with an increased b* parameter for quebracho tannin (+ 21%), with a higher value of L* and a* coordinates (lightness and red/green colour coordinate) for acacia tannin (+ 8 and +5%, respectively), but with a combined decrease of the three CIEL*a*b* coordinates (−11% of lightness, −1% of yellow/blue colour coordinate, and −6% of red/green colour coordinate) for CSL biosurfactant. Therefore, the use of this last product led to the darkest colour macerating solutions (Table S1). Table S2 shows that the colour differences are visible for the use of the CSL biosurfactant not only with respect to the control but also with respect to all the tannin formulations evaluated (ΔE* values from 5.02 to 9.85).

Regarding Cabernet sauvignon, the extracts from the addition of each tannin formulation tested and the CSL biosurfactant showed quantitative differences in the visually perceived colour, compared to the control, as can be observed from ΔE* values above 3.49 for any sampling time (Figure 1). The CSL biosurfactant showed an interesting trend because ΔE* data increased almost progressively during the skin simulated maceration process and reached the highest values at 96 and 168 h of maceration (9.32 and 11.53, respectively).

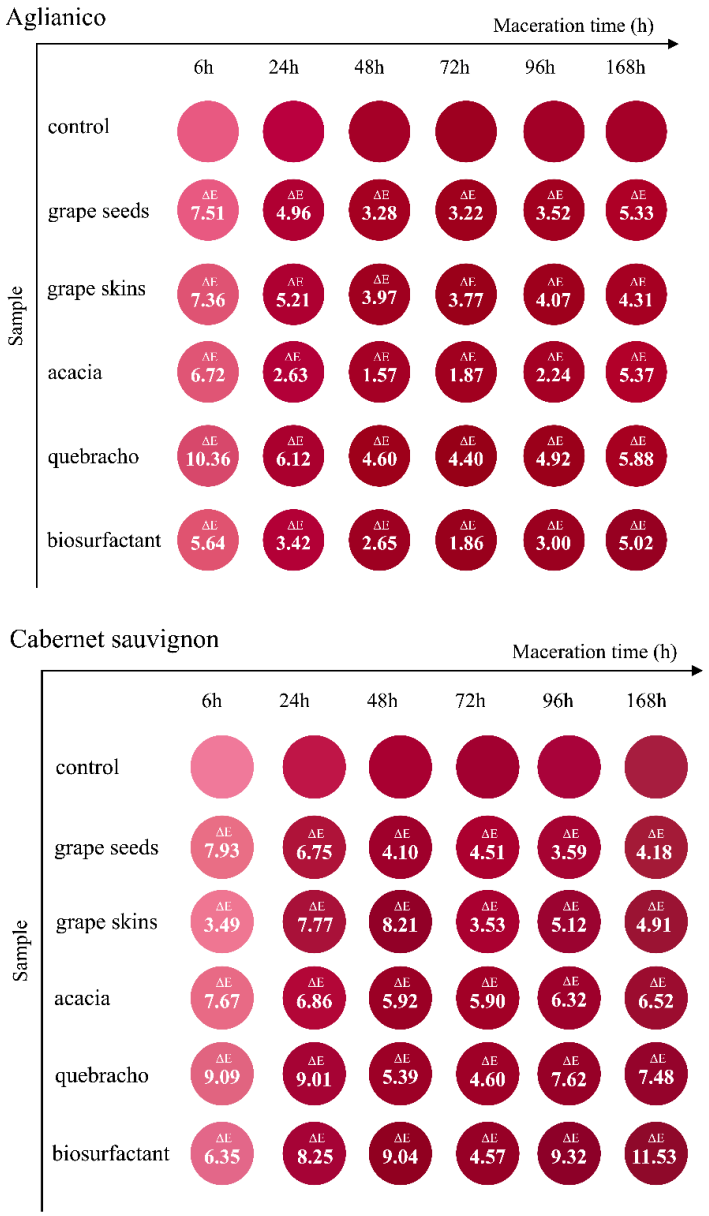

Figure 1. Evolution of the visual colour for different solutions from skin maceration: non-treated control and added with exogenous tannins from different origin (grape seeds, grape skins, acacia, and quebracho) and CSL biosurfactant. Each colour was acquired by spectrophotometry, expressed in CIEL*a*b* coordinates, and converted to RGB (24-bit colour) values. ΔE* values for prefermentative addition versus control are shown inside the circle corresponding to visual colour for every sampling point throughout maceration.

Particularly, these last two macerating solutions were the darkest despite the reduced red colour component (significantly lower values of L* and a* coordinates, respectively averaged −22% and −11% when compared to control) and had the lowest yellow hue among CSL biosurfactant and tannin added samples (no significant increase of b* colour coordinate with respect to control) (Table S1). The opposite trend was observed for grape seed and acacia tannins, showing a decrease of ΔE* values with the advance of maceration until 48 h and remaining then practically constant (Figure 1). At the end of maceration, the greatest increase in the visually perceived colour (ΔE* values) with respect to control corresponded to CSL biosurfactant, followed by quebracho and acacia tannins. A great increase in ΔE* values (7.66−11.05) for CSL biosurfactant was also observed with respect to all the tannins tested (Table S2). When compared to CSL biosurfactant, both quebracho and acacia tannins led to less dark extracts with more reddish and yellowish hue (Table S1). These results confirmed the differences found in colour intensity (Table 2).

The impact observed for oenological tannins on colour parameters agrees with previous studies on skin simulated maceration for Aglianico and Cabernet sauvignon winegrape varieties [12]. At 72 h of maceration, colour intensity values for Cabernet sauvignon skins increased with respect to control when quebracho tannin was added. In general, Aglianico tonality seems to be sharply influenced by tannin addition from the beginning of maceration, particularly grape seed tannin formulation led to the greatest increase in tonality values also at 72 h of skin maceration. Other studies have reported that the prefermentative addition of grape seed-derived exogenous tannin has no significant effect on colour intensity and CIEL*a*b* coordinates throughout the winemaking process of red Syrah grapes [47]. Nevertheless, wine colour properties can be diversely influenced by the prefermentative addition of oenological tannins, as found on Sangiovese depending on the initial phenolic concentration of grapes [48].

3.3. Anthocyanin Content and Profile During Skin Maceration

Figure 2 shows total anthocyanin extraction yield for control and tannin added samples throughout the maceration process for Aglianico and Cabernet sauvignon varieties, which was calculated as the ratio between the concentration extracted and that initially present in the berry skins.

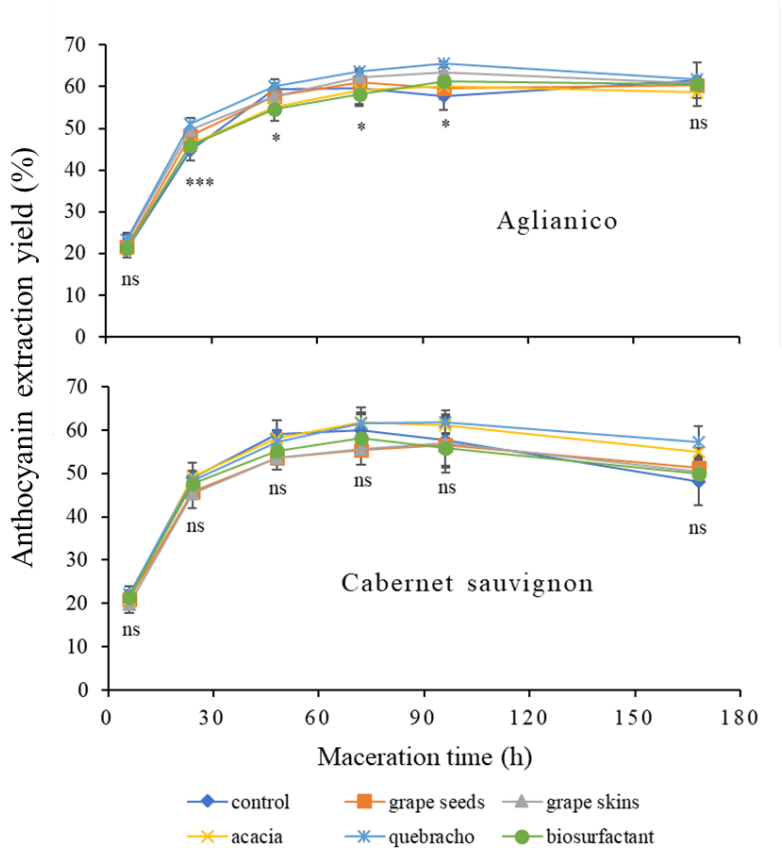

Figure 2. Effect of exogenous tannins and CSL biosurfactant addition on the extraction yield of total anthocyanins during skin simulated maceration. All data are expressed as average value ± standard deviation (n = 3). Sign: *, ***, and ns indicate significance at $p < 0.05$, 0.001, and not significant, respectively, for the differences among treatments at each maceration time.

Regarding control samples for the two varieties, the evolution of extraction yield was similar until 72 h of maceration. The maximum extraction of total

anthocyanins was reached at 72 h and then the extraction yield decreased for Cabernet sauvignon while it was kept practically constant for Aglianico. Although anthocyanins diffuse quickly from the beginning of fermentation as a consequence of their hydrophilic character, trisubstituted forms are released slower into the must than disubstituted forms [3], besides skin structural characteristics influencing the diffusion process [49,50]. For the Aglianico variety, being it richer in cinnamoylated anthocyanins (Table 1), the slower diffusion of these anthocyanin forms may have counterbalanced their possible decrease related to chemical reactions as commented below. The progressive extraction of anthocyanins from berry skins (Figure 2) can explain the higher colour intensity and lower tonality values observed for the extracts sampled between 24 and 72 h of maceration (Table 2). The subsequent decrease of the first parameter and the increase of the second one could be attributable to polymerization reactions rather than to the oxidation of phenolic compounds [12].

When the different treatments were compared with respect to control for Aglianico skins, the highest values of total anthocyanin extraction yield corresponded to quebracho tannin, followed by grape skin tannin. Although these differences were significant until 96 h of maceration, then the higher concentration of alcohol tends to minimize them [5]. As reported in Figure 2, once reached the extraction peak, the extraction yield remained practically constant for control and treated samples. For Cabernet sauvignon, it is important to highlight that the addition of quebracho and acacia tannins allowed to reduce slightly the decrease observed in the extraction yield after 72 h of skin maceration, although the differences were not significant. At the end of maceration (168 h), these two treatments increased the anthocyanin extraction yield between +9% and 7% with respect to control.

Table 3 shows the monomeric anthocyanin composition of the skin extracts at the beginning, half, and end of the simulated maceration process (6, 72, and 168 h, respectively) for all the treatments tested and the untreated control on Aglianico and Cabernet sauvignon varieties. Although the different treatments tested did not induce any difference in the total monomeric anthocyanin

concentration during skin maceration, with respect to control, quebracho tannin showed concentrations significantly higher than other treatments such as acacia at 72 h of maceration for Aglianico and both the grape-derived formulations at 72 and 168 h for Cabernet sauvignon (Table 3). Moreover, some significant differences were found in the anthocyanin profile of the macerating solutions. The two varieties are malvidin-3-glucoside prevalent, even if the percentage concentrations of the predominant individual forms in the macerating solutions were different from those found in berry skins (Table 1).

Regarding non-acylated anthocyanins for Aglianico variety, the first most abundant form was malvidin-3-glucoside with an average relative concentration of 73.31%, 68.63%, and 67.71% at 6, 72, and 168 h of maceration, respectively. An increase of +14.46%, +10.44%, and +8.89% in the concentration of this compound was found in the control macerating solutions at 6, 72, and 168 h, respectively, when compared to that of grapes. This increase can be attributable to the stability of malvidin-3-glucoside as a consequence of the presence of methoxylated groups in the B-ring [51]. In addition, decreased relative contents of some compounds, such as delphinidin-3-glucoside and petunidin-3-glucoside, occurred, although the addition of quebracho tannin reduced these losses, particularly at the beginning of maceration. In fact, significantly higher delphinidin-3-glucoside and petunidin-3-glucoside contents were found at 6 and 168 h of maceration with respect to the control when quebracho tannin was used (about +0.8% and +0.5%, respectively, for 6 and 168 h). The CSL biosurfactant also preserved delphinidin-3-glucoside and petunidin-3-glucoside but their relative abundances were not significantly different from those of control samples.

The percentages of cyanidin-3-glucoside and peonidin-3-glucoside decreased when maceration progressed at the same time that the malvidin/peonidin ratio increased. Disubstituted anthocyanins are the first diffused from the skins but they are also the most prone to oxidation because of their molecular conformation [3,9]. The same trend was observed for delphinidin-3-glucoside, probably due to its *o*-diphenolic structure, just like cyanidin-3-

glucoside [52]. No treatment tested was effective in preserving disubstituted anthocyanin compounds.

Table 3. Anthocyanin composition during maceration with tannins from different origins and corn steep liquor (CSL) biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Grape Cultivar	Time (h)	Treatment	Dp-3-G (%)	Cy-3-G (%)	Pt-3-G (%)	Pn-3-G (%)	Mv-3-G (%)	∑ Acetyl (%)	∑ Cinnamoyl (%)	Total (mg/kg grapes)
Aglianico	6	control	3.49 ± 0.47 b	0.38 ± 0.11	4.78 ± 0.45 b	3.63 ± 0.64	73.04 ± 1.72 α	4.45 ± 0.18 a, $\alpha\beta$	10.23 ± 1.35 β	260 ± 30 β
		grape seeds	3.90 ± 0.34 ab	0.38 ± 0.02 α	5.14 ± 0.28 ab	3.46 ± 0.37 α	73.11 ± 0.59 α	4.37 ± 0.24 a, α	10.10 ± 0.97 β	256 ± 26 β
		grape skins	3.81 ± 0.14 ab, $\alpha\beta$	0.36 ± 0.02 α	5.02 ± 0.12 ab, β	3.25 ± 0.10 α	73.36 ± 0.82 α	3.91 ± 0.62 ab	10.84 ± 1.60 β	277 ± 23 β
		acacia	3.73 ± 0.11 ab, α	0.36 ± 0.06 α	4.91 ± 0.14 ab, γ	3.40 ± 0.43	73.34 ± 1.30 α	3.82 ± 0.15 abc, β	10.44 ± 0.74 γ	261 ± 23 β
		quebracho	4.31 ± 0.18 a, α	0.39 ± 0.02 α	5.52 ± 0.20 a, β	3.44 ± 0.15 α	73.15 ± 0.25 α	3.35 ± 0.02 bc, γ	9.84 ± 0.48 γ	294 ± 21 γ
		biosurfactant	3.92 ± 0.11 ab, α	0.40 ± 0.04 α	5.20 ± 0.11 ab, β	3.71 ± 0.30 α	73.85 ± 0.74 α	3.03 ± 0.08 c, γ	9.89 ± 0.89 β	277 ± 23 β
		Sign [a]	*	ns	*	ns	ns	***	ns	ns
	72	control	4.14 ± 0.60 a	0.34 ± 0.08	6.01 ± 0.59 ab	2.90 ± 0.55	69.02 ± 1.91 $\alpha\beta$	3.52 ± 0.77 c, β	14.07 ± 1.94 $\alpha\beta$	694 ± 31 ab, α
		grape seeds	4.17 ± 0.51 a	0.26 ± 0.03 β	6.03 ± 0.44 ab	2.53 ± 0.13 β	68.65 ± 0.15 β	4.55 ± 0.18 a, α	13.80 ± 0.82 α	704 ± 26 ab, α
		grape skins	4.24 ± 0.26 a, α	0.27 ± 0.02 β	5.97 ± 0.21 ab, α	2.48 ± 0.13 β	68.02 ± 0.27 β	4.34 ± 0.15 ab	14.68 ± 0.25 α	707 ± 7 ab, α
		acacia	3.79 ± 0.07 a, α	0.26 ± 0.04 $\alpha\beta$	5.50 ± 0.12 b, α	2.59 ± 0.43	68.49 ± 0.39 β	4.33 ± 0.16 ab, α	15.05 ± 0.18 β	673 ± 13 b, α
		quebracho	4.52 ± 0.20 a, α	0.31 ± 0.01 β	6.35 ± 0.18 a, α	2.67 ± 0.10 β	68.22 ± 0.22 β	3.90 ± 0.04 bc, β	14.02 ± 0.45 β	745 ± 4 a, α
		biosurfactant	4.09 ± 0.15 a, α	0.32 ± 0.03 $\alpha\beta$	5.93 ± 0.12 ab, α	2.84 ± 0.24 β	69.35 ± 0.83 β	3.45 ± 0.06 c, β	14.01 ± 0.63 α	664 ± 27 b, α
		Sign [a]	*	ns	*	ns	ns	*	ns	**
	168	control	3.16 ± 0.80 b	0.30 ± 0.02	5.61 ± 0.91 b	2.65 ± 0.56	67.47 ± 1.55 β	4.88 ± 0.31 a, α	15.93 ± 2.08 α	644 ± 67 α
		grape seeds	3.41 ± 0.73 ab	0.25 ± 0.06 β	5.80 ± 0.83 ab	2.28 ± 0.19 β	68.73 ± 0.57 β	3.23 ± 0.61 b, β	16.33 ± 1.76 α	692 ± 50 α
		grape skins	3.50 ± 0.38 ab, β	0.27 ± 0.02 β	5.79 ± 0.51 ab, $\alpha\beta$	2.28 ± 0.10 β	68.28 ± 0.94 β	3.37 ± 1.14 ab	16.51 ± 0.71 α	665 ± 21 α
		acacia	2.96 ± 0.09 b, β	0.24 ± 0.03 β	5.20 ± 0.07 b, β	2.34 ± 0.42	67.15 ± 0.07 β	4.53 ± 0.17 ab, α	17.58 ± 0.38 α	634 ± 5 α
		quebracho	3.74 ± 0.08 a, β	0.27 ± 0.01 γ	6.02 ± 0.09 a, α	2.38 ± 0.08 γ	66.97 ± 0.20 γ	4.14 ± 0.07 ab, α	16.49 ± 0.20 α	685 ± 8 β
		biosurfactant	3.42 ± 0.28 ab, β	0.27 ± 0.02 β	5.87 ± 0.32 ab, α	2.41 ± 0.11 β	67.65 ± 1.85 β	3.82 ± 0.07 ab, α	16.56 ± 1.74 α	646 ± 25 α
		Sign [a]	**	ns	**	ns	ns	*	ns	ns
		Sign [b]	ns,ns,*,***,**,*	ns,**,**,*,***,**	ns,ns,*,**,**,**	ns,**,***,ns,***,**	*,***,***,***,***,**	*,*,ns,**,***,***	*,**,**,***,***,**	***,***,***,***,***,***
Cabernet sauvignon	6	control	6.86 ± 0.99 b, β	1.66 ± 0.71 ab	5.14 ± 0.36 b, β	6.06 ± 1.13	50.10 ± 1.79 b, β	26.20 ± 1.99 a, α	3.98 ± 0.44 β	294 ± 27 γ
		grape seeds	7.26 ± 0.70 ab	1.38 ± 0.18 b, α	5.70 ± 0.34 ab, β	6.33 ± 0.84 α	54.28 ± 0.60 a, α	20.01 ± 2.00 b, β	3.40 ± 0.67 β	290 ± 20 γ
		grape skins	6.85 ± 0.63 b, $\alpha\beta$	1.31 ± 0.19 b, α	5.56 ± 0.29 ab, β	6.35 ± 0.26 α	55.45 ± 1.33 a, α	20.84 ± 2.22 b	4.48 ± 0.65 β	275 ± 27 γ
		acacia	8.91 ± 0.57 a, $\alpha\beta$	2.40 ± 0.17 a, α	6.24 ± 0.32 a, β	7.23 ± 0.54 α	53.04 ± 0.37 ab, α	18.72 ± 0.39 b, β	3.46 ± 0.90 β	295 ± 8 γ
		quebracho	8.37 ± 0.57 ab, β	1.64 ± 0.16 ab, α	6.14 ± 0.34 a, β	6.54 ± 0.06 α	53.00 ± 1.07 ab, α	21.28 ± 2.75 ab	3.03 ± 0.66 β	294 ± 35 γ
		biosurfactant	7.70 ± 0.39 ab, β	1.87 ± 0.33 ab, α	5.74 ± 0.19 ab, β	6.84 ± 0.43 α	52.80 ± 0.91 ab, $\alpha\beta$	20.81 ± 0.49 b	4.24 ± 0.56 β	313 ± 21 γ
		Sign [a]	*	*	*	ns	**	**	ns	ns
	72	control	9.50 ± 0.21 ab, α	1.32 ± 0.04 b	7.38 ± 0.21 ab, α	5.13 ± 0.57	51.21 ± 0.53 a, β	19.64 ± 0.83 de, β	5.85 ± 0.32 b, α	846 ± 24 ab, α
		grape seeds	8.27 ± 0.65 b	1.02 ± 0.12 b, $\alpha\beta$	6.72 ± 0.28 b, α	4.73 ± 0.58 $\alpha\beta$	49.31 ± 1.11 ab, β	23.92 ± 0.57 a, α	6.04 ± 0.63 b, α	758 ± 11 b, α
		grape skins	8.19 ± 0.61 b, α	0.98 ± 0.17 b, $\alpha\beta$	6.64 ± 0.33 b, α	4.76 ± 0.25 β	49.38 ± 1.18 ab, β	22.97 ± 0.30 ab	7.09 ± 0.23 a, α	762 ± 59 b, α
		acacia	10.41 ± 0.63 a, α	1.82 ± 0.05 a, β	7.36 ± 0.30 ab, α	5.43 ± 0.38 β	47.01 ± 0.51 b, γ	21.59 ± 0.49 bc, α	6.38 ± 0.31 ab, α	851 ± 19 ab, α
		quebracho	10.16 ± 0.69 a, α	1.30 ± 0.12 b, $\alpha\beta$	7.50 ± 0.21 a, α	5.14 ± 0.12 β	48.67 ± 0.81 ab, β	20.88 ± 0.30 cd	6.35 ± 0.35 ab, α	865 ± 33 a, α
		biosurfactant	9.57 ± 0.69 ab, α	1.39 ± 0.28 ab, $\alpha\beta$	7.30 ± 0.28 ab, α	5.10 ± 0.33 $\alpha\beta$	51.02 ± 1.07 a, β	19.12 ± 0.73 e	6.51 ± 0.22 ab, α	807 ± 34 ab, α
		Sign [a]	**	***	*	ns	**	***	*	**

Table 3. *Cont.*

Grape Cultivar	Time (h)	Treatment	Dp-3-G (%)	Cy-3-G (%)	Pt-3-G (%)	Pn-3-G (%)	Mv-3-G (%)	∑ Acetyl (%)	∑ Cinnamoyl (%)	Total (mg/kg grapes)
		control	7.19 ± 0.02 abc, $\alpha\beta$	0.98 ± 0.03 ab	6.84 ± 0.25 α	4.77 ± 0.71	57.48 ± 1.12 a, α	17.46 ± 1.73 d, β	5.30 ± 0.37 b, α	668 ± 11 ab, β
		grape seeds	6.55 ± 0.92 bc	0.82 ± 0.14 b, β	6.43 ± 0.52 $\alpha\beta$	4.48 ± 0.60 β	57.24 ± 1.73 a, α	18.67 ± 0.60 cd, β	5.81 ± 0.22 ab, α	591 ± 37 b, β
		grape skins	6.35 ± 0.76 c, β	0.74 ± 0.15 b, β	6.00 ± 0.45 $\alpha\beta$	4.20 ± 0.25 β	52.34 ± 1.45 bc, $\alpha\beta$	23.91 ± 0.22 a	6.45 ± 0.24 a, α	579.51 ± 55 b, β
	168	acacia	8.51 ± 0.68 ab, β	1.36 ± 0.07 a, γ	6.91 ± 0.40 $\alpha\beta$	4.88 ± 0.40 β	49.73 ± 0.41 c, β	22.47 ± 0.65 ab, α	6.15 ± 0.44 ab, α	657 ± 23 ab, β
		quebracho	8.77 ± 0.78 a, $\alpha\beta$	1.04 ± 0.13 ab, β	7.21 ± 0.29 α	4.68 ± 0.12 γ	50.27 ± 0.89 c, β	21.67 ± 0.26 b	6.36 ± 0.42 a, α	711 ± 25 a, β
		biosurfactant	7.46 ± 0.90 abc, β	0.99 ± 0.24 ab, β	6.64 ± 0.39 α	4.37 ± 0.38 β	53.89 ± 1.24 ab, α	20.46 ± 0.90 bc	6.19 ± 0.30 ab, α	619 ± 32 ab, β
		Sign [a]	*	**	ns	ns	***	***	*	**
		Sign [b]	*,ns,*,*,*,*	ns,*,*,***,**,*	**,*,*,*,**,**	ns,*,***,**,***,***	*,***,**,*,***,***	**,**,ns,***,ns,ns	*,**,***,**,***,***	***,***,*,*,***,***,***

All data are expressed as average value ± standard deviation ($n = 3$). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, for the differences among treatments for each maceration time ([a]) and among different maceration times for each treatment ([b]) according to ANOVA, Welch's ANOVA, or Kruskal–Wallis tests. Different Latin letters within the same column indicate significant differences ([a]) and different Greek letters within the same column indicate significant differences ([b]) according to Tukey HSD, Games–Howell, and Conover's tests ($p < 0.05$) for ANOVA, Welch's ANOVA, and Kruskal–Wallis tests, respectively. Dp-3-G: delphinidin-3-glucoside, Cy-3-G: cyanidin-3-glucoside, Pt-3-G: petunidin-3-glucoside, Pn-3-G: peonidin-3-glucoside, Mv-3-G: malvidin-3-glucoside. Total anthocyanins were expressed as malvidin-3-glucoside chloride.

Regarding acylated anthocyanin derivatives, these forms are generally worse extracted than the non-acylated anthocyanins as a consequence of the higher retention by cell wall polymeric material [50]. A lower percentage concentration of cinnamoylated forms was observed in macerating solutions than that of grapes, but it increased progressively during maceration. Malvidin-3-glucoside percentage decreased when cinnamoylated derivatives increased in agreement with other previously published studies [53]. Nevertheless, no treatment significantly affected the concentration of these forms regardless of the maceration time. Finally, quebracho tannin and CSL biosurfactant seem to have slowed down the diffusion of acetylated anthocyanin derivatives in the early stage of maceration, but the relative concentration of these forms increased significantly as maceration progressed when these two treatments were carried out.

In the case of Cabernet sauvignon, some variations were also found in the anthocyanin profile of the extracts obtained from skin simulated maceration with respect to that of the grapes, depending on the ease of anthocyanin extraction (Tables 1 and 3). As already observed for Aglianico, the most abundant anthocyanin compound was malvidin-3-glucoside with an average relative concentration of 53.11%, 49.43%, and 53.48% at 6, 72, and 168 h of maceration, respectively. In the untreated sample, an increase of +6.28%, +7.39%, and +13.66% at 6, 72, and 168 h of maceration, respectively, with respect to grape berries. Nevertheless, the percentage concentration of malvidin-3-glucoside varied differently during maceration depending on the treatment tested. Untreated samples, as well as the samples treated with grape seed tannin and CSL biosurfactant, showed an increasing trend whereas those added with grape skin, acacia, and quebracho tannins evidenced the opposite trend, even though the differences were not always significant. Delphinidin-3-glucoside and petunidin-3-glucoside showed the highest relative concentrations in the samples treated with acacia and quebracho tannins, this increase in the concentration being greater at the beginning of maceration when compared to control (respectively +2.05% and +1.10% for acacia, +1.51% and +1.00% for quebracho). As already

observed in Aglianico, the concentration of cyanidin-3-glucoside and peonidin-3-glucoside decreased significantly throughout maceration and a progressive increase in the malvidin/peonidin ratio was observed regardless of treatment. Acacia-derived tannin allowed us to preserve better also disubstituted anthocyanin forms, although the increase observed in the relative concentration was significant with respect to control only for cyanidin-3-glucoside at 72 h of maceration. The changes observed in the anthocyanin profile during Cabernet sauvignon skins maceration are in agreement with those described by Río Segade et al. [54] for simulated macerations in wine-like solutions and by Gil-Muñoz et al. [55] for the wine at the end of alcoholic fermentation.

During maceration, acylated anthocyanins showed different trends in Cabernet sauvignon depending on the treatment tested, as also observed for malvidin-3-glucoside. All treatments slowed down significantly the diffusion of acetylated anthocyanin derivatives in the early stage of maceration. Then, acetylated anthocyanins decreased in control and grape seed formulation, whereas the percentage concentration of these compounds increased significantly for acacia tannin, and it remained statistically unchanged for grape skin and quebracho tannins as well as CSL biosurfactant. As occurred in the Aglianico variety, a lower relative concentration of cinnamoylated forms was observed in macerating solutions compared to that of grapes. Cinnamoylated derivatives increased throughout maceration and, after 168 h of maceration, all treatments showed relative concentrations higher than the control, even if only significantly for grape skin and quebracho tannins.

The anthocyanin profile of the two varieties studied is different, hence they responded quite differently to the treatments tested. A recently published study has highlighted that grape cultivar features are strictly connected with the tannin addition efficacy in skin simulated maceration conditions [12]. For Cabernet sauvignon, the only significant effect reported was the higher delphinidin-3-glucoside content at 72 h of maceration for grape seed tannin formulation when compared to the control sample. In contrast, no differences were found in non-acylated anthocyanins for Aglianico with the addition of different exogenous

tannins (ellagitannins, quebracho, grape seeds, and grape skins). In the present study, similar results were obtained and small variations in the effectiveness of oenological tannins may be due to the different grape ripeness grades influencing the release of anthocyanin forms that have to be protected [39].

The preservation of extracted anthocyanin forms from the first stages of maceration is of great relevance since they influence the colour stability over time through their participation in several chemical reactions [56]. In skin simulated maceration conditions, the CSL biosurfactant played a protective role on delphinidin-3-glucoside and petunidin-3-glucoside in Aglianico winegrape variety whereas on acylated derivatives in Cabernet sauvignon. Nevertheless, its effectiveness resulted to be slightly less than that corresponding to quebracho tannin. The CSL biosurfactant has a high antioxidant activity derived from its phenolic composition, including protocatechuic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, epicatechin, and quercetin [22]. However, quebracho tannins are characterized by not only high antioxidant capacity but also fast oxygen consumption, even higher than grape-derived proanthocyanidins [13,57].

3.4. Phenolic Composition at the End of Maceration

Table 4 shows the phenolic composition of the extracts obtained at the end of the maceration process (168 h) for Aglianico and Cabernet sauvignon berry skins using the different treatments above mentioned. For Cabernet sauvignon variety, the results obtained highlight that the addition of CSL biosurfactant significantly increased the percentage concentration of copigmented anthocyanins, with respect to control, in detriment of free forms. In fact, the greatest richness in copigmented anthocyanins corresponded to the CSL biosurfactant. In Aglianico, the copigmentation phenomenon was not reduced significantly when the CSL biosurfactant was used, contrary to what was observed for grape seed tannin. However, no significant difference was found in the relative concentration of polymeric pigments, particularly long polymeric pigments (LPP), for the two varieties studied, even so, it is possible to evidence

that the samples added with acacia tannin, followed by CSL biosurfactant, grape seed, and quebracho tannins, showed a slight increase in polymeric pigments for Aglianico (+1.91%, +1.09%, +0.81%, and +0.72%, respectively, compared to control). These values agree with those previously reported for simulated maceration of Aglianico skins [12]. Furthermore, the highest copigmentation and polymerization percentages are not related to total phenolic compounds, to non-anthocyanin flavonoids, or to total anthocyanins, whose highest concentrations were found in the samples added with quebracho tannin for both the varieties (Table 4)

The red colour of young wines is mainly due to the presence of monomeric anthocyanins, but they are unstable and can be degraded by oxidation. Once extracted from berry skins, they can take part in copigmentation and polymerization reactions forming more stable pigments. In the present study, most anthocyanins (59.6–67.6%) were in the monomeric form, as expected at the first stages of winemaking [34]. An anthocyanin fraction of about 19.9–36.6% consisted of polymeric pigments, with a greater contribution of small polymeric pigments (SPP) than large polymeric pigments (LPP). In fact, polymerization reactions are destined to increase at later stages of winemaking. These polymeric pigments are formed as a result of the reactions between anthocyanins and condensed tannins, starting from the beginning of maceration and increasing during wine ageing [58,59]. The addition of exogenous tannins can promote the formation of polymeric pigments through two mechanisms. The antioxidant activity of these products may preserve grape anthocyanins and tannins that can react together, or oenological tannins can combine directly with released anthocyanins stabilizing colour before endogenous tannins are extracted. In the case of oenological tannins, these mechanisms have been widely studied [13,57]. However, the chemical structure and dosage of the oenological tannins used as well as the ratio of tannins to anthocyanins in the wine influence their effectiveness on colour stabilization. Particularly, an imbalance in the anthocyanin/tannin ratio may favour the tannin polymerization and thus increasing the yellow hue [16]. The CSL biosurfactant is a novel alternative and

therefore specific studies are needed. Nevertheless, it can be hypothesized a copigment function with a protective role on wine anthocyanins against oxidation as commented below.

The remaining colour fraction, ranging from 18.4% to 26.1%, corresponds to copigmented anthocyanins. Copigmentation has a positive effect on the wine colour because it helps to stabilize the structure of anthocyanins. With regard to the CSL biosurfactant, this is the first time that its effectiveness on colour preservation was studied on a wine-like solution. Therefore, it is interesting to analyse the differences with respect to untreated (control) and tannin-treated samples, since different tannin formulations are used in winemaking for their positive effects on wine colour stability [16]. A very interesting aspect of the CSL biosurfactant is its ability to increase significantly the relative concentration of copigmented anthocyanins in Cabernet sauvignon variety with respect to control (+6.53%, Table 4), surpassing that of all exogenous tannins evaluated (grape skin, grape seed, acacia, and quebracho). This fact explains the significantly higher values of colour intensity and lower L* coordinate for CSL biosurfactant, when compared to control and grape-derived tannins (Tables 2 and S1). This improvement in colour properties agrees with a bathochromic shift and hyperchromic effect on absorbance at 520 nm associated with copigmentation, involving a blueness hue [37]. The bathochromic effect occurs as a consequence of the affinity of the copigment for the quinoidal forms of anthocyanins. The hyperchromic effect is due to the formation of the flavylium cation–copigment complex [56].

Table 4. Phenolic composition of skin extracts at the end of maceration with tannins from different origins and corn steep liquor (CSL) biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Grape Cultivar	Treatment	Copigmented Anthocyanins (%)	Free Anthocyanins (%)	Polymeric Pigments (%)	LPP (%)	SPP (%)	IPT (mg/kg grapes)	TA (mg/kg grapes)	FNA (mg/kg grapes)
Aglianico	control	26.13 ± 1.14 a	63.55 ± 0.25 b	19.90 ± 0.63	8.89 ± 0.68	11.01 ± 0.14	2014 ± 148 c	542 ± 57	639 ± 30 c
	grape seeds	21.62 ± 0.72 b	67.57 ± 0.31 a	20.71 ± 0.95	9.95 ± 0.97	10.75 ± 0.02	2285 ± 68 bc	532 ± 19	828 ± 21 b
	grape skins	24.50 ± 0.64 ab	64.37 ± 1.16 ab	20.19 ± 0.35	8.10 ± 1.06	12.09 ± 1.17	2320 ± 89 b	535 ± 5	814 ± 22 b
	acacia	25.24 ± 1.40 ab	64.11 ± 1.14 ab	21.81 ± 0.29	9.44 ± 1.207	12.37 ± 1.21	2283 ± 37 bc	498 ± 13	831 ± 25 b
	quebracho	24.34 ± 3.42 ab	65.32 ± 3.54 ab	20.62 ± 0.34	10.14 ± 0.47	10.48 ± 0.39	2641 ± 18 a	544 ± 6	1045 ± 28 a
	biosurfactant	25.70 ± 1.20 a	64.12 ± 1.66 ab	20.99 ± 1.45	9.44 ± 2.11	11.55 ± 0.72	2141 ± 184 bc	533 ± 46	632 ± 70 c
	Sign	*	***	ns	ns	ns	***	ns	***
Cabernet sauvignon	control	18.42 ± 2.15 b	64.88 ± 0.97 a	36.60 ± 10.66	16.07 ± 11.33	20.53 ± 1.55 a	2192 ± 222 c	510 ± 58 b	482 ± 38 d
	grape seeds	19.98 ± 2.30 ab	64.23 ± 2.75 a	29.20 ± 1.31	13.46 ± 1.49	15.74 ± 0.19 b	2733 ± 139 ab	543 ± 13 ab	713 ± 6 c
	grape skins	22.62 ± 3.82 ab	61.96 ± 3.03 ab	28.83 ± 0.67	12.27 ± 0.92	16.56 ± 0.86 b	2671 ± 51 bc	534 ± 28 ab	751 ± 21 c
	acacia	20.14 ± 1.20 ab	64.93 ± 1.32 a	29.62 ± 0.31	14.53 ± 0.52	15.09 ± 0.38 b	2856 ± 166 ab	582 ± 11 ab	860 ± 19 b
	quebracho	23.30 ± 0.65 ab	63.02 ± 0.66 ab	30.53 ± 1.19	13.79 ± 3.62	16.74 ± 2.54 b	3191 ± 208 a	606 ± 41 a	1145 ± 57 a
	biosurfactant	24.95 ± 1.55 a	59.62 ± 1.92 b	32.41 ± 3.37	16.65 ± 3.14	16.65 ± 0.77 b	2659 ± 238 bc	529 ± 16 ab	406 ± 19 d
	Sign	*	***	ns	ns	**	***	*	***

All data are expressed as average value ± standard deviation (n = 3). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, for the differences among treatments according to ANOVA or Welch's ANOVA tests. Different Latin letters within the same column indicate significant differences according to Tukey HSD and Games–Howell tests ($p < 0.05$) for ANOVA and Welch's ANOVA, respectively. LPP: long polymeric pigments, SPP: small polymeric pigments, IPT: total phenolic index expressed as (-)-epicatechin, TA: total anthocyanins expressed as malvidin-3-glucoside chloride, FNA: non-anthocyanin flavonoids expressed as (+)-catechin.

The fact that the CSL biosurfactant allowed to encourage copigmentation reactions may be due to its surface-active properties and its phenolic composition consisting of quercetin, epicatechin, sinapic, ferulic, *p*-coumaric, caffeic, protocatechuic, and vanillic acids [22], which are important cofactors [37]. Therefore, the CSL biosurfactant contains phenolic acids and it can justify that the respective macerating solutions had a concentration of total phenolic compounds (IPT) comparable to the solutions added with tannins (Table 4), despite the low content of non-anthocyanin flavonoids (FNA). Considering that the samples treated with quebracho tannin were the richest in IPT and FNA, the nature of the cofactor is of great importance to promoting copigmentation. In fact, this phenomenon depends on the structure of copigments. Particularly, the planar polyphenolic nucleus of flavonols favours π–π stacking with the planar anthocyanin chromophore [56]. A variety effect was also observed since the CSL biosurfactant did not enhance copigmentation reactions for Aglianico skins when compared to the untreated sample. Nevertheless, it is important to evidence that the percentage concentration of copigmented anthocyanins was higher for the CSL biosurfactant than that found for tannins (+4.08% compared to grape seed tannins). In fact, regarding the tannins tested in this experiment, the percentage concentrations of copigmented anthocyanin forms were not significantly different among them or with respect to control, excepting for the low values associated to grape seed tannin in Aglianico (Table 4). This could be due to the richness in coumaroylated anthocyanins in Aglianico skins, which could diminish the effect of added copigments [60,61]. These results are in accordance with the highest values of colour intensity and lowest L* coordinate reported for the CSL biosurfactant also in Aglianico (Tables 2 and S1). The combined contribution of copigmentation and polymerization reactions could help to better understand the small improvement in the colour properties for Aglianico on simulated skin maceration in the presence of CSL biosurfactant.

Although it is well known that exogenous tannins influence positively colour copigmentation, their effectiveness as copigments depends on the botanical origin, dose, pH level, and ethanol content as reported for a model wine solution

containing malvidin-3-glucoside [62]. Within the same type of copigments, a higher tannin dosage resulted in a greater effectiveness on copigmentation because the copigment concentration increased. Moreover, an increase in pH and ethanol strength reduced the tannin effect on red colour. Therefore, copigmentation occurs mainly during the first days of fermentation [63]. Nevertheless, the higher solubility of some compounds in the wine at higher ethanol concentration may have a countering effect enabling a significant contribution of copigmentation also after fermentation [37].

To better understand the differences among treatments for each variety, a principal component analysis (PCA) was performed (Figure 3). Regarding Aglianico variety, principal component 1 (PC1) accounted for 35.6% of the explained variance, whereas principal component 2 (PC2) explained the 32.6% with a total explained variance by the first two components of 68.2%. PC1 was correlated, in order, with total anthocyanins, tonality, petunidin-3-glucoside (−0.936, 0.903, and −0.883, respectively, all $p < 0.02$), and PC2 were strongly influenced by free and copigmented anthocyanins with a correlation of −0.909 and 0.881 (both $p < 0.02$). Figure 3 confirmed that quebracho tannin protects better anthocyanins and colour with respect to other tannins and CSL biosurfactant, particularly delphinidin-3-glucoside and petunidin-3-glucoside. Moreover, the CSL biosurfactant has not reported differences when compared to control. For Cabernet sauvignon, the multivariate analysis explained 72.2% of the total variance, accounting for PC1 for 40.8% and PC2 for 31.4%. The first principal component was correlated to cinnamoylated and acetylated anthocyanin derivatives (−0.977, $p < 0.001$ and −0.863, $p < 0.03$, respectively), as well as to polymerized pigments and small polymeric pigments (both 0.840, $p < 0.04$). The second principal component was mainly associated with delphinidin-3-glucoside (−0.960, $p < 0.01$), petunidin-3-glucoside (−0.929, $p < 0.01$), cyanidin-3-glucoside (−0.888, $p < 0.02$), and peonidin-3-glucoside (−0.869, $p < 0.03$). As can be observed in Figure 3, all treatments increased total anthocyanin concentration, colour intensity, acylated anthocyanin forms, and copigmented anthocyanins with respect to control. CSL biosurfactant showed an intermediate

improvement between that of tannins from exotic oak (acacia and quebracho) and that corresponding to grape-derived tannins (skins and seeds) regarding the preservation of individual anthocyanins, but it was more strongly related to copigmented anthocyanins.

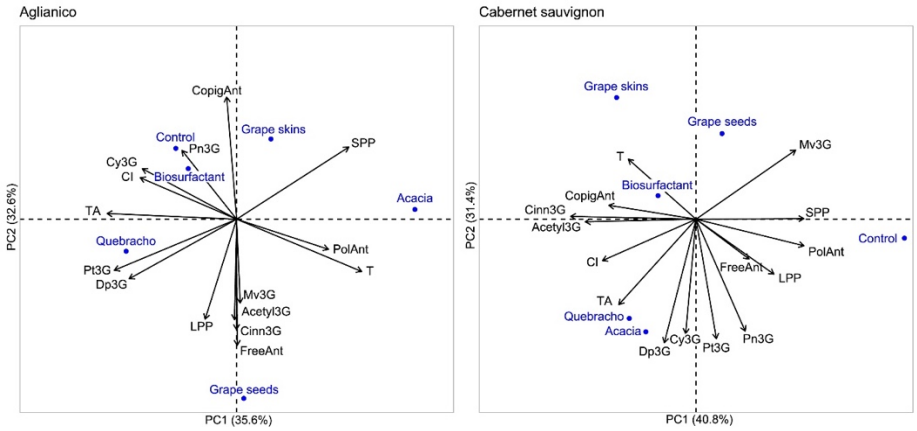

Figure 3. Principal component analysis (PCA) of anthocyanin compounds and colour characteristics of macerating solutions at 168 h of simulated skin maceration for control and for the addition of exogenous tannins and CSL biosurfactant. CI: colour intensity, T: tonality, Dp3G: delphinidin-3-glucoside, Cy3G: cyanidin-3-glucoside, Pt3G: petunidin-3-glucoside, Pn3G: peonidin-3-glucoside, Mv3G: malvidin-3-glucoside, Acetyl3G: acetylated derivatives, Cinn3G: cinnamoylated derivatives, CopigAnt: copigmented anthocyanins, FreeAnt: free anthocyanins, PolAnt: polymerized anthocyanins, LPP: long polymeric pigments, SPP: small polymeric pigments, TA: total anthocyanins.

5. Conclusions

Anthocyanins are phenolic compounds responsible for the colour of red wine, which is the first attribute perceived by consumers and a major factor determining the quality. These red pigments are released in the first steps of maceration from grape skins and they can undergo chemical reactions influencing colour stability. This study has highlighted that the prefermentative addition of a biosurfactant from a corn steep liquor (CSL), which is a residual stream of the corn wet-milling industry, could represent a promising tool in order to improve

colour properties of young red wines. Its effectiveness was variety dependent on skin simulated maceration conditions. After 168 h of maceration, a higher colour intensity was observed in agreement with lower values of lightness (L* colour coordinate). These colour differences can be visualized as shown by the high values of ΔE* parameter, achieving 5.02 and 11.53 units for Aglianico and Cabernet sauvignon, respectively, with respect to untreated samples. The more significant improvement in colour properties for the second winegrape variety seems to be mainly due to the copigmentation effect rather than the protection of specific individual anthocyanin forms, for which quebracho tannin resulted to be more effective regarding delphinidin-3-glucoside and petunidin-3-glucoside. Instead, a combined contribution of copigmentation and polymerization reactions could justify the improved colour of Aglianico skin extracts induced by the CSL biosurfactant. The knowledge of the effectiveness of CSL biosurfactant to preserve the wine colour may open a new field of research on its potential for winemaking. Future research will focus on evaluating the effectiveness of the CSL biosurfactant for colour stability during a real winemaking and wine ageing.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: CIEL*a*b* parameters of skin extracts during maceration with tannins from different origin and CSL biosurfactant for Aglianico and Cabernet sauvignon winegrapes, Table S2: ΔE* colour parameter of skin extracts at 168 h of maceration without and with addition of tannins from different origin and CSL biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Table 1. CIEL*a*b* parameters of skin extracts during maceration with tannins from different origin and CSL biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Colour index	Grape cultivar	Treatment	6 h	24 h	48 h	72 h	96 h	168 h	$Sign^b$
L*	Aglianico	control	59.96 ± 2.84 α	39.28 ± 1.74 a, β	33.39 ± 0.94 a, γ	31.35 ± 1.92 ab, γ	32.92 ± 1.77 a, γ	33.39 ± 1.39 ab, γ	***
		grape seeds	57.01 ± 2.92 α	36.91 ± 1.33 ab, β	33.06 ± 0.72 ab, $\beta\gamma$	31.53 ± 0.87 ab, β	32.41 ± 0.44 a, $\beta\gamma$	35.28 ± 1.18 ab, $\beta\gamma$	***
		grape skins	55.00 ± 3.94 α	36.05 ± 1.21 ab, β	31.92 ± 0.08 ab, $\beta\gamma$	30.33 ± 0.63 ab, γ	31.21 ± 1.24 a, γ	33.41 ± 2.50 ab, $\beta\gamma$	**
		acacia	55.56 ± 2.57 α	37.58 ± 0.33 ab, β	33.12 ± 0.66 ab, $\gamma\delta$	32.04 ± 0.55 a, δ	33.14 ± 0.78 a, $\gamma\delta$	36.05 ± 0.64 a, $\beta\gamma$	***
		quebracho	52.07 ± 2.57 α	34.98 ± 0.84 b, $\beta\gamma$	30.87 ± 0.69 b, δ	29.27 ± 0.33 b, ε	30.38 ± 0.31 a δ	33.31 ± 0.73 b, β	**
		biosurfactant	55.48 ± 2.79 α	37.22 ± 1.60 ab, β	31.61 ± 1.43 ab, $\beta\gamma$	30.20 ± 1.85 ab, γ	30.77 ± 1.73 a, γ	29.80 ± 3.01 ab, γ	***
		$Sign^a$	ns	*	*	*	*	*	
	Cabernet sauvignon	control	65.26 ± 2.61 a, α	41.54 ± 2.02 a, β	35.17 ± 1.90 γ	33.64 ± 2.08 γ	36.04 ± 2.18 a, $\beta\gamma$	37.00 ± 1.13 a, $\beta\gamma$	***
		grape seeds	61.94 ± 1.65 abc, α	37.81 ± 2.39 ab, β	33.07 ± 1.11 β	35.50 ± 3.13 β	34.97 ± 1.58 ab, β	35.96 ± 1.47 ab, β	**
		grape skins	64.35 ± 2.13 ab, α	36.46 ± 1.76 b, β	29.97 ± 1.60 γ	35.21 ± 0.95 β	32.64 ± 1.41 abc, $\beta\gamma$	33.66 ± 2.30 ab, $\beta\gamma$	***
		acacia	60.86 ± 0.85 abc, α	37.80 ± 0.92 ab, β	31.46 ± 1.49 γ	32.85 ± 2.15 γ	32.80 ± 0.59 abc, γ	33.16 ± 0.61 ab, γ	***
		quebracho	58.67 ± 1.66 c, α	34.99 ± 1.29 b, β	31.58 ± 1.77 $\beta\gamma$	32.21 ± 1.71 $\beta\gamma$	30.64 ± 0.83 bc, γ	31.43 ± 0.44 bc, $\beta\gamma$	***
		biosurfactant	59.84 ± 0.70 bc, α	35.26 ± 0.98 b, β	28.84 ± 4.94 β	30.26 ± 4.86 β	28.97 ± 3.25 c, β	27.94 ± 2.94 c, β	***
		$Sign^a$	**	**	ns	ns	**	***	
a*	Aglianico	control	54.14 ± 2.51 γ	63.86 ± 0.95 α	61.62 ± 0.48 $\alpha\beta$	60.19 ± 1.33 β	61.19 ± 0.46 $\alpha\beta$	61.01 ± 0.26 ab, $\alpha\beta$	***
		grape seeds	55.00 ± 1.16 γ	62.00 ± 1.34 $\alpha\beta$	61.53 ± 0.61 $\alpha\beta$	60.59 ± 0.96 β	61.00 ± 0.49 β	63.66 ± 0.76 ab, α	***
		grape skins	56.46 ± 1.45 β	62.12 ± 0.73 α	60.74 ± 0.08 $\alpha\beta$	59.90 ± 0.75 $\alpha\beta$	60.04 ± 1.30 $\alpha\beta$	62.16 ± 2.62 ab, $\alpha\beta$	*
		acacia	55.70 ± 1.34 γ	62.58 ± 0.10 $\alpha\beta$	61.40 ± 0.58 β	61.03 ± 0.47 β	61.42 ± 0.64 β	64.11 ± 0.41 a, α	***
		quebracho	58.40 ± 1.58 γ	62.11 ± 0.64 $\alpha\beta$	60.33 ± 0.64 $\beta\gamma$	59.38 ± 0.33 γ	59.94 ± 0.30 $\beta\gamma$	63.16 ± 0.76 ab, α	***
		biosurfactant	57.31 ± 1.22 β	63.00 ± 1.50 α	60.18 ± 1.37 $\alpha\beta$	59.17 ± 0.10 $\alpha\beta$	59.38 ± 1.59 $\alpha\beta$	57.52 ± 2.77 b, β	*
		$Sign^a$	ns	ns	ns	ns	ns	**	
	Cabernet sauvignon	control	48.58 ± 3.22 γ	63.25 ± 0.87 a, α	60.99 ± 1.14 a, α	58.57 ± 1.10 $\alpha\beta$	58.86 ± 1.39 a, $\alpha\beta$	55.25 ± 2.50 a, β	***
		grape seeds	49.89 ± 1.52 β	59.06 ± 2.47 ab, α	57.58 ± 0.95 ab, α	61.03 ± 2.83 α	57.87 ± 0.76 a, α	54.69 ± 0.08 a, β	**
		grape skins	48.88 ± 2.73 β	57.71 ± 2.12 b, $\alpha\beta$	54.64 ± 0.75 b, β	60.90 ± 0.19 α	55.54 ± 0.49 ab, β	53.24 ± 1.24 a, β	***
		acacia	51.15 ± 0.11 γ	60.99 ± 1.51 ab, α	57.54 ± 1.73 ab, $\alpha\beta$	59.89 ± 2.48 α	57.53 ± 1.17 a, $\alpha\beta$	54.23 ± 1.46 a, $\beta\gamma$	***
		quebracho	51.61 ± 2.17 γ	58.11 ± 0.37 b, α	58.06 ± 1.76 ab, α	59.12 ± 1.36 α	55.59 ± 0.19 ab, $\alpha\beta$	53.30 ± 1.29 a, $\beta\gamma$	***
		biosurfactant	51.22 ± 0.60	58.33 ± 1.98 b	54.87 ± 5.42a b	56.32 ± 5.34	53.04 ± 3.29 b	48.13 ± 2.52 b	ns
		$Sign^a$	ns	*	*	ns	**	**	
b*	Aglianico	control	6.07 ± 0.80 b, γ	21.00 ± 2.14 β	27.39 ± 2.41 ab, α	28.54 ± 1.84 b, α	27.30 ± 2.22 ab, α	26.37 ± 2.51 bc, $\alpha\beta$	***
		grape seeds	12.93 ± 1.27 a, γ	24.94 ± 0.10 β	30.66 ± 0.97 ab, α	31.73 ± 1.16 a, α	30.78 ± 1.26 ab, α	30.59 ± 1.48 ab, α	***
		grape skins	10.99 ± 1.26 a, γ	24.69 ± 0.70 β	30.98 ± 0.40 a, α	32.15 ± 0.63 a, α	30.81 ± 0.63 ab, α	30.53 ± 1.39 ab, α	***
		acacia	10.91 ± 1.05 a, γ	22.55 ± 1.17 β	28.92 ± 0.55 b, α	30.07 ± 0.56 ab, α	29.52 ± 0.43 b, α	29.87 ± 0.62 abc, α	***
		quebracho	11.27 ± 1.03 a, γ	24.99 ± 0.50 β	31.02 ± 0.28 a, α	32.33 ± 0.10 a, α	31.32 ± 0.34 a, α	31.84 ± 0.11 a, α	***
		biosurfactant	7.43 ± 1.28 b, γ	23.59 ± 0.97 β	28.74 ± 1.33 ab, α	29.59 ± 1.49 ab, α	28.35 ± 1.51 ab, α	26.05 ± 2.06 c, $\alpha\beta$	**
		$Sign^a$	***	ns	*	**	*	**	
	Cabernet sauvignon	control	3.46 ± 0.77 d, β	18.11 ± 2.74 b, α	22.88 ± 2.96 α	21.55 ± 2.70 b, α	18.37 ± 2.82 b, α	16.38 ± 2.38 b, α	***
		grape seeds	10.54 ± 0.37 a, γ	21.86 ± 0.98 ab, β	23.80 ± 0.58 α	24.83 ± 0.64 ab, α	21.65 ± 0.41 ab, β	20.38 ± 0.41 ab, β	***
		grape skins	6.82 ± 0.24 c, γ	20.10 ± 2.01 ab, $\alpha\beta$	22.48 ± 1.17 $\alpha\beta$	23.67 ± 1.62 ab, α	20.27 ± 1.55 ab, $\alpha\beta$	19.36 ± 0.27 ab, β	***
		acacia	9.20 ± 0.42 ab, δ	23.40 ± 0.89 a, $\beta\gamma$	25.95 ± 1.12 $\alpha\beta$	27.24 ± 1.00 a, α	23.64 ± 1.06 a, $\beta\gamma$	21.54 ± 1.37 a, γ	***
		quebracho	8.93 ± 0.71 b, δ	21.54 ± 1.41 ab, γ	25.64 ± 0.94 $\alpha\beta$	25.88 ± 1.20 a, α	22.63 ± 1.39 ab, $\beta\gamma$	20.97 ± 1.23 ab, γ	***
		biosurfactant	5.47 ± 0.34 c, ε	20.20 ± 0.88 ab, $\beta\gamma$	24.96 ± 1.59 α	23.64 ± 1.47 ab, $\alpha\beta$	20.15 ± 1.69 ab, γ	16.52 ± 1.07 b, δ	***
		$Sign^a$	***	*	ns	*	*	*	

All data are expressed as average value ± standard deviation (n=3). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, for the differences among treatments for each maceration time ([a]) and among different maceration times for each treatment ([b]) according to ANOVA, Welch's ANOVA, or Kruskal-Wallis tests. Different Latin letters within the same column indicate significant differences ([a]) and different Greek letters within the same row indicate significant differences ([b]) according to Tukey HSD, Games-Howell, and Conover's tests ($p < 0.05$) for ANOVA, Welch's ANOVA, and Kruskal-Wallis tests, respectively. L*: lightness, a*: red/green colour coordinate, b*: yellow/blue colour coordinate.

Table S2. ΔE* colour parameter of skin extracts at 168 h of maceration without and with addition of tannins from different origin and CSL biosurfactant for Aglianico and Cabernet sauvignon winegrapes.

Grape cultivar		control	grape seeds	grape skins	acacia	quebracho	biosurfactant
Aglianico	control	0					
	grape seeds	5.33	0				
	grape skins	4.31	2.39	0			
	acacia	5.37	1.15	3.34	0		
	quebracho	5.88	2.39	1.65	3.51	0	
	biosurfactant	5.02	9.40	7.40	9.85	8.82	0
Cabernet sauvignon	control	0					
	grape seeds	4.18	0				
	grape skins	4.91	2.90	0			
	acacia	6.52	3.07	2.45	0		
	quebracho	7.48	4.77	2.75	2.04	0	
	biosurfactant	11.53	11.05	8.18	9.46	7.66	0

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Chapter V

Impact of oenological processing aids and additives on the genetic traceability of 'Nebbiolo' wine produced with withered grapes

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ABSTRACT

'Nebbiolo' is a well-known grapevine variety used to produce prestigious monovarietal Italian red wines. Genetic traceability is an important tool used to protect the authenticity of high-quality wines. SNP-based assays are an effective method to reach this aim in wines, but several issues have been reported for the authentication of commercial wines. In this study, the impact of the most common commercial additives and processing aids used in winemaking was analysed in 'Nebbiolo' wine using SNP-based traceability. Gelatine and bentonite had the strongest impact on the turbidity, colour and phenolic composition of wines and on residual grapevine DNA. The DNA reduction associated with the use of bentonite and gelatine (>99% compared to the untreated control) caused issues in the SNP-based assay, especially when the DNA concentration was below 0.5 pg/mL of wine. This study contributed to explaining the causes of the reduced varietal identification efficiency in commercial wines.

Keywords: Grapevine, Wines, Oenological additives, Sfursat, Genetic traceability, SNPs

1. Introduction

‘Nebbiolo’ (Vitis vinifera L.) is an important Italian winegrape variety used to produce high-quality wines. It is diffused in north-western Italy, where it is used to produce well-known worldwide DOCG (Denomi- nazione di Origine Controllata e Garantita) wines, such as Barolo, Bar- baresco, Roero, Gattinara, Ghemme, and Sfursat (Raimondi et al., 2020). ‘Nebbiolo’ wines play an important role in the Italian wine market due to their high economic value (Miglietta & Morrone, 2018). The wine market is often plagued by fraud, which can occur in many forms, and adulteration is defined as the fraudulent alteration of wine composition. However, other types of fraud have increasingly spread in recent years. Among them, the misrepresentation on the label regarding the origin and variety of wine is very common (Holmberg, 2010). Thus, there is the need to protect ‘Nebbiolo’ wines from fraud that could damage the image and market of these premium wines. To protect the authenticity and verify the truthfulness of what is claimed on the label, models that allow the recognition of wines are needed.

During the past two decades, several authors have studied the authenticity of wines, to identify traceability methods to associate the chemical composition of wine with its varietal, geographic, and productive origin (Versari et al., 2014; Villano et al., 2017; Solovyev et al., 2021). Nevertheless, chemometric

approaches are often expensive in terms of time and resources, and they present some inaccuracies caused by the great influence of viticultural and winemaking methods on the qualitative and quantitative composition of wine (Versari et al., 2014). Therefore, the results cannot be considered reliable if the models are applied to commercial wines (Zhang et al., 2010). Biological traceability techniques based on a genetic approach appear very interesting. By extracting DNA from wine and using variety-specific molecular markers, it is possible to discriminate musts and wines (Siret et al., 2000; Pereira et al., 2012). However, the results can be very different depending on the wines, the DNA extraction technique, the type of marker, and the amplification technique used. Single sequence repeats (SSRs) represent the most common markers used in grapevine for fingerprinting (This et al., 2004). Several authors used SSRs as markers for genetic traceability and varietal recognition starting from residual DNA in musts and wines (Boccacci et al., 2012; Pereira et al., 2012; Recupero et al., 2013; Siret et al., 2000; Zambianchi et al., 2021). However, due to DNA degradation in the winemaking process, the results of amplification are often not reliable (Catalano et al., 2016). Indeed, several studies have reported issues with using these methods for assessing the traceability of commercial wines (Agrimonti & Marmiroli, 2018; Recupero et al., 2013).

After the first sequencing approach (Jaillon et al., 2007), several projects involving the sequencing or re-sequencing of grapevine cultivars, including

'Nebbiolo', have been performed (Gambino et al., 2017). The comparison between different available genomes allowed the identification of several mutations and polymorphisms between different genotypes, such as single nucleotide polymorphisms (SNPs). SNPs are particularly interesting because they are spread throught the grapevine genome and have the potential to become a valid alternative to SSRs for cultivar identification (Cabezas et al., 2011). Therefore, SNPs have also been used for genetic traceability of varieties in wine (Barrias et al., 2019; Pereira et al., 2017; Fanelli et al., 2021) since they can be detected in low-quality fragmented DNA (Catalano et al., 2016). However, while SSRs are the optimal markers for fingerprinting in grapevine and a limited number of markers is sufficient for varietal identification, many SNP markers are required, which can be analysed by sequencing or hybridisation techniques that are not applicable in wine (Cabezas et al., 2011; Myles et al., 2011). In wine, the most effective approach is the analysis of a limited number of SNPs using qPCR, which allow the identification of a specific cultivar within a group of genotypes (Catalano et al., 2016; Pereira et al., 2017). For example, SNPs for the authentication of 'Nebbiolo' were identified, and a way for assessing the molecular traceability of this cultivar in experimental wines, based on the SNP TaqMan® assay was developed (Boccacci et al., 2020). Two markers, SNP_15082 and SNP_14783, were sufficient to distinguish 'Nebbiolo' from a group of more than 1100 genotypes. Nevertheless, the efficiency of the assay

decreased at the end of malolactic fermentation and in commercial wines due to the reduction of the amplification efficiency and to the increased presence of PCR inhibitors.

After malolactic fermentation, wine can undergo several winemaking practices before bottling, which may modify its composition. In winemaking, the use of additives and oenological adjuvants or processing aids to enhance wine stability is well diffused, and several oenological products have been allowed for this purpose (OIV, 2016a). To produce high-quality wines, stabilisation and clarification are essential. Different products can be employed as fining agents; among them, the most frequently used are bentonite, chitosan, vegetable proteins, animal proteins, and polyvinylpolypyrrolidone (Castro Marin & Chinnici, 2020; Ficagna et al., 2020; Río Segade et al., 2020). The main products used as stabilisers are potassium polyaspartate, yeast mannoproteins, and Arabic gum (Bosso et al., 2020; Rinaldi et al., 2019).

These problems for the amplification efficiency of DNA in commercial wines (Boccacci et al., 2012; Boccacci et al., 2020; Recupero et al., 2013; Zambianchi et al., 2021) are probably associated with aging, clarification, fining agents, and/or DNAse yeast activity (Catalano et al., 2016). However, to date, no work has analysed in detail these procedures and agents, which may potentially drastically reduce the quality and quantity of DNA in the wine after malolactic

fermentation (Faria et al., 2008; Siret et al., 2000; Siret et al., 2002). The effect of the most common additives on wine's chemical composition has been widely studied, but to the best of our knowledge, there are no studies about their effect on wine DNA traceability. Therefore, this study aimed to determine the impact of the most common commercial additives and processing aids on the SNP-based traceability of 'Nebbiolo' wine.

2. Materials and methods

2.1. Plant material

Young leaves of 'Nebbiolo', 'Barbera', and 'Freisa' were collected, and DNA was extracted using a Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's in- structions. Accessions were genotyped with six SSR markers (This et al., 2004) to confirm their cultivar identity, together with ampelographic observations.

2.2. Experimental vinification

Partially dehydrated 'Nebbiolo' grapes from the Valtellina wine re- gion (Sondrio, Italy) were crushed in a TEMA de-stemmer–crusher (Enoveneta, Piazzola Sul Brenta, Italy) in December 2019, and 10 mg/L SO_2 was added to the grape must. After 24 h, the must was inoculated with *Saccharomyces cerevisiae* active dry yeast (ACTIFLORE® BO213, Laffort, Bordeaux, France)

at the dose suggested by the producer (30 g/ hL). Maceration lasted for 14 days; the cap was punched down once the first day, and two punches down were carried out daily until the 6th day. During the second week of fermentation, two pumpings per day were performed in the first two days, while only one per day was carried out in the following days. At the end of maceration, free-run wine was obtained, and then the pomace cap was gently pressed using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy). Malolactic fermentation was induced by the inoculation of Oenococcus oeni (MalotabsTM, Lallemand Inc., Montreal, Canada). After malolactic fermentation, 50 mg/L SO_2 were added, and the wine was subsequently racked to remove the lees. The first control wine (CONTR20) was sampled in 0.5 L bottles and frozen for two weeks at –20 °C before DNA extraction, as described below in Section 2.5. Every 6 months the wine was racked and of 10 mg/ L SO_2 was added, and in March 2021, the wine was used for the oenological treatments.

2.3. Wine treatment with oenological additives and processing aids

In March 2021, 10 winery treatments were tested (Table 1), with three replicates each, on the same 'Nebbiolo' wine. The most common additives and processing aids used in winemaking were selected for this experiment. For each treatment, the preparation was carried out according to the instructions reported on the product's technical sheet (Table 1). The dose used was calculated as 85% of the

maximum dose suggested by the producer. In each sample, a small quantity of water was added to reach the same final volume of the treatment that required more water in the preparation phase (bentonite). The treatment time was kept constant at 7 days for all treatments according to previous experience, and to information available in the literature (Table 1). At the end of the treatment, each trial was racked with a small laboratory peristaltic pump, avoiding the collection of lees deposited on the bottom of the container. The clear wine was collected for chemical analysis, and a 0.5 L bottle was frozen for two weeks to enhance nucleic acid precipitation.

2.4. Chemical-physical analysis of 'Nebbiolo' wines

After treatment, 250 mL of wine was collected to carry out the chemical-physical analysis. Total acidity was determined by titrimetry according to the OIV-MA-AS313-01 method, while pH was evaluated by potentiometry using an InoLab 730 calibrated pHmeter (WTW, Weilheim, Germany) following the OIV-MA-AS313-15 method (OIV, 2016b). Ethanol, glycerol, and organic acids (malic, lactic, tartaric, citric and acetic acid) were determined by HPLC (Agilent Technologies, Santa Clara, USA) with a diode array detector set to 210 nm, following the method proposed by Schneider et al. (1987).

Table 1
'Nebbiolo' wine samples and treatments used for the study. CONTR20: untreated wine sampled in 2020, one year before application of treatments; CONTR: untreated wine sampled in 2021 at the time of application of treatments.

Sample	Treatment	Product	Used Dose	Reference
CONTR20	–	–	–	–
CONTR	–	–	–	–
BEN	Bentonite	Gelbentonite Dal Cin, Concorezzo, Italy	25.5 g/hL	Ficagna et al., 2020
GEL	Gelatine of animal origin	Premium Gel Grado 1 Vason, S. Pietro in Cariano, Italy	25.5 g/hL	Cosme et al., 2007
VEG	Vegetable protein	Vegecoll Laffort, Bordeaux, France	4 g/hL	Río Segade et al., 2020; Ficagna et al., 2020
PVPP	Polyvinylpolypyrrolidone	PVPP Alea Evolution, Molinella, Italy	25.5 g/hL	Cosme et al., 2012; Ficagna et al., 2020
YST	Yeast hulls	Aleavit Help Alea Evolution	32 g/hL	Costa et al., 2019
CHT	Chitosan	Chitogel AEB, Brescia, Italy	25.5 g/hL	Castro Marin & Chinnici, 2020
MAN	Yeast mannoprotein	Oenoless MP Laffort	25.5 g/hL	–
ARG	Arabic gum	Arabique L30 Alea Evolution	85 mL/hL	–
POL	Potassium polyaspartate	Zenith Uno Enartis, Trecate, Italy	85 mL/hL	–
TAN	Grape skin tannin	Protan Raisin AEB	25 g/hL	–

anthocyanins (TA) and total flavonoids (TF) were quantified by diluting the sample with ethanol:water:37% hydrochloric acid (70:30:1, v/v) and subsequently measuring absorbance at 536–540 nm and 280 nm, respectively. TA were quantified as mg/L of malvidin-3-O-glucoside chloride, and TF were expressed as mg/L of (+)-catechin. The total polyphenol index (TPI) was evaluated by measuring absorbance at 280 nm in a sample diluted in water, and it was expressed in mg/L of (−)-epicatechin, as reported by Scalzini et al. (2020). Wine colour parameters were evaluated through the acquisition of the visible spectra (380–780 nm) of the undiluted samples using 2-mm optical path cuvettes. Subsequently, colour intensity (CI) ($A_{420} + A_{520} + A_{620}$) and hue ($A_{420}/A_{520}$) were calculated on an optical path of 10-mm, following the OIV-MA-AS2-07B method (OIV, 2016b). Wine colour was also evaluated by CIELab parameters, according to the OIV-MA-AS2-11 method (OIV, 2016b). L* represents lightness, whereas a* and b* are red/green and yellow/blue colour coordinates, respectively. The total colour difference (ΔE*) between the control and treated samples was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Then, the CIELab coordinates were converted to RGB values.

Turbidity was analysed using a turbidimeter (Model TB1, Velp Scientifica, Usmate, Italy) and expressed in nephelometric turbidity units (NTU) in accordance with the OIV-MA-AS2-08 method (OIV, 2016b). Total and free-SO2 were quan- tified by titration after the extraction using a Solfotech apparatus (Exacta + Optech Labcenter Spa, San Prospero, Italy) according to the OIV-MA-AS323-04A method (OIV, 2016b).

Wine phenolic composition and colour parameters were evaluated following the methods reported by Petrozziello et al. (2018) using a UV- 1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). Total anthocyanins (TA) and total flavonoids (TF) were quantified by diluting the sample with ethanol:water:37% hydrochloric acid (70:30:1, v/v) and subsequently measuring absorbance at 536–540 nm and 280 nm, respectively. TA were quantified as mg/L of malvidin-3-O-glucoside chloride, and TF were expressed as mg/L of (+)-catechin. The total polyphenol index (TPI) was evaluated by measuring absorbance at 280 nm in a sample diluted in water, and it was expressed in mg/L of (−)-epicatechin, as reported by Scalzini et al. (2020). Wine colour parameters were evaluated through the acquisition of the visible spectra (380–780 nm) of the undiluted samples using 2-mm optical path cuvettes. Subsequently, colour intensity (CI) ($A_{420} + A_{520} + A_{620}$) and hue ($A_{420}/A_{520}$) were calculated on an optical path of 10-mm, following the OIV-MA-AS2-07B method (OIV, 2016b). Wine colour was also evaluated by CIELab parameters, according to the

OIV-MA-AS2-11 method (OIV, 2016b). L* represents lightness, whereas a* and b* are red/green and yellow/blue colour coordinates, respectively. The total colour difference (ΔE*) between the control and treated samples was calculated as follows: ΔE* = [(ΔL*)2 + (Δa*)2 + (Δb*)2]1/2. Then, the CIELab co-ordinates were converted to RGB values.

2.5. Grapevine DNA extraction from wines

The total DNA from wine was extracted with two different methods: i) Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Can- ada) (Norgen protocol) and ii) the cetyltrimethylammonium bromide (CTAB)-based method by Siret et al. (2002) with several modifications (SirM protocol). The wine aliquots for DNA extraction were collected from wine conserved at –20 °C and homogenised by inverting the bottle several times. Each replicate was extracted from 50 mL (Norgen) and 100 mL (SirM) wine pellets obtained after centrifugation at 4000*g* at 4 °C for 1 h. In the Norgen protocol, before the extraction, the pellet was frozen in liquid nitrogen and ground using a TissueLyser II (Qiagen, Hilden, Germany). All DNA extractions were performed by following the man- ufacturer's instructions, excluding the RNase step, and the final elution occurred in 45 µL of elution buffer. In the SirM protocol, DNA was extracted according to a modified CTAB-based method by Siret et al. (2002) and following some modifications proposed by Agrimonti and Marmiroli (2018). The

pellet obtained after centrifugation, as reported above, was dissolved in 5 mL TEX buffer (20 mM EDTA, pH 8.0; 1.4 M NaCl; 1 M Tris–HCl, pH 8.0; 3% CTAB: and 1% β-mercaptoethanol) and incubated at 65 °C for 1 h, with mixing every 10–15 min. Then, 1 vol of chloroform:isoamyl alcohol (24:1) was added and homogenised. After centrifugation at 8000*g* for 10 min at 4 °C, the supernatant was mixed with 0.1 vol of 10% CTAB and extracted again with 1 vol of chloroform: isoamyl alcohol. The DNA-containing upper phase was precipitated overnight at –25 °C with 2 volumes of ethanol. Then, DNA was collected by centrifugation at 10,000*g* for 30 min at 4 °C, resuspended in 250 µL TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0), and treated with 20 µL Proteinase K (20 mg/mL) at 48 °C for 30 min. Then, 1 vol of phenol:chloroform:isoamyl alcohol (25:24:1) was added and samples were centrifuged at 11,000*g* for 15 min at 4 °C. DNA was precipitated with 2 volumes of ethanol and 2.5 M ammonium acetate (7.5 M) at –25 °C for at least 2 h. After centrifugation at 22,000*g* for 30 min at 4 °C, the pellets were washed twice with 500 µL 70% ethanol and resuspended in 45 µL TE buffer. Final purification was performed with the NucleoSpin® Plant Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. DNA quantity and quality were estimated by determining the spectrophotometric absorbance of the samples at 230, 260, and 280 nm and the ratios of A260/A280 and A260/A230. A NanoDrop

1000 spectrophotometer (Thermo Fisher Scientific,Wal- tham, MA, USA) was used. DNA was stored at − 20 °C.

2.6. Grapevine DNA quantification by qPCR and determination of PCR inhibitors

To quantify grapevine DNA, all DNA samples were analysed by qPCR amplification of the 9-*cis*-epoxycarotenoiddioxygenase gene (*VvNCED2*, VIT_10s0003g03750), using the primers and TaqMan® FAM-labelled probe reported by Savazzini and Martinelli (2006). The presence of PCR inhibitors in the extracted DNA was evaluated according to Boccacci et al. (2020), by adding TaqMan® Exogenous Internal Positive Control (EIPC) reagents (Thermo Fisher Scientific) into the qPCR mixture. The qPCR reaction was performed in a final volume of 10 µL, consisting of 2.5 µL DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.3 µM of each primer and 0.2 µM of FAM probe, 0.2 µL EIPC DNA, 1 µL EIPC mix (containing premixed forward, reverse primers, and VIC probe specific for EIPC), and 0.1 µL sterile water. Amplification cycles were characterised by an initial denaturation step at 95 °C for 10 min, followed by 55 cycles of 95 °C for 15 s, and 60 ° C for 1 min. A calibration curve of the *VvNCED2* TaqMan® assay was constructed with samples of 'Nebbiolo' DNA extracted from leaves and obtained by serial dilution. Grapevine DNA quantification took place by plotting the Ct values obtained from

the DNA extracted from wines together with the standard curve using the CFX96 Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of PCR inhibition was calculated from a calibration curve with serial dilution of EIPC, according to Boccacci et al. (2020). All samples were analysed in duplicate.

2.7. SNP genotyping protocol and data analysis

DNA extracted from 'Nebbiolo' wines was analysed by SNP_15082 and SNP_14783. As reported by Boccacci et al. (2020), 'Nebbiolo' alleles and non-'Nebbiolo' alleles were marked with different dyes (FAM and VIC) (Table S1). 'Barbera' and 'Freisa' were selected as examples of homozygous and heterozygous non-'Nebbiolo' cultivars (Boccacci et al., 2020), respectively, which were necessary to product allelic discrimation plots. The qPCR reaction for TaqMan® SNP assays was performed in a final volume of 10 µL, consisting of 2.5 µL DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.25 µL 40X TaqMan® SNP Genotyping Assay mix (containing pre-mixed forward and reverse primers, VIC probe, and FAM probe), and 2.25 µL sterile water. The amplification profile was the same as that reported in Section 2.6. Allelic discrimination plots were constructed using the CFX96 Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were analysed in duplicate.

2.8. Statistical analyses

Statistical analyses were performed using R statistic software (R Foundation for Statistical Computing, Vienna, Austria). For each variable, one-way analysis of variance (ANOVA) with the Tukey HSD posthoc test was used to evaluate significant differences among treatments. The normality and homoscedasticity ANOVA assumptions were tested using Shapiro–Wilk's and Levene's tests, respectively. When the ANOVA assumptions were not met, the Kruskal–Wallis non-parametric test with Conover's All-Pairs Rank Comparison Test was performed. Differences were considered statistically significant at p-value < 0.05.

3. Results and discussion

3.1. Impact of treatments on chemical-physical parameters of 'Nebbiolo' wines

The chemical composition and colour characteristics of the 'Nebbiolo' wine used in this experiment are reported in Table S2. The impact of additives and processing aids on 'Nebbiolo' wine turbidity, phenolic composition, and colour parameters is reported in Table 2. Bentonite (BEN), gelatine (GEL), polyvinylpolypyrrolidone (PVPP), and yeast hulls (YST) strongly decreased wine turbidity, while mannoprotein (MAN), chitosan (CHT), and Arabic gum (ARG) slightly increased the NTU level compared to the untreated control

(CONTR). BEN, a commercial product mainly composed of a natural clay known as montmorillonite, is widely used as a fining agent in wine due to its ability to adsorb and precipitate proteins. In our study, BEN had the greatest impact on wine turbidity (–80%). These results agree with those of Ficagna et al. (2020), in which 'Merlot' wine clarification with BEN showed the most intense reduction in turbidity, while PVPP and vegetable proteins (VEG) treatments led to a minor reduction in the NTU level. GEL also had a great impact in terms of turbidity reduction (–43%), reported by Gonza´lez- Neves et al. (2014).

The wine phenolic composition changed after treatment with different processing aids and additives. 'Nebbiolo' wines treated with grape skin tannin (TAN) showed a higher TPI and TF content compared to CONTR, whereas GEL, PVPP, CHT and BEN treated wines showed significantly lower TPI values. The TA content was not significantly affected by the treatments with TAN, ARG, MAN, and CHT, whereas GEL, PVPP, VEG, potassium polyaspartate (POL), YST and BEN caused a decrease in anthocyanin content. 'Nebbiolo' wines treated with GEL showed the lowest TA values, which were about 15% lower compared to CONTR. GEL and PVPP treatments significantly affected the TF content with respect to CONTR, leading to a reduction of 12.3% and 4.3%, respectively, while TAN increased this parameter by 4.4%. Instead, other treatments did not significantly change the TF content. Among the treatments tested in our study, GEL had the strongest impact on wine phenolic composition, showing the lowest

TPI, TF, and TA values (Table 2). Our results agree with the literature; BEN and GEL strongly affected the phenolic composition of wine through a reduction of an- thocyanins and tannins in 'Tannat' red wine (Gonza´lez-Neves et al., 2014). In 'Nebbiolo' wine, a significant reduction of about 9% was observed in TPI values after GEL treatment due to the removal of oligomeric and polymeric flavanols (Río Segade et al., 2020). PVPP treatment also caused a strong decrease (>55%) in flavanol content (Ficagna et al., 2020). In addition to protein removal, BEN can bind other posi- tively charged molecules, like anthocyanins, leading to a loss of colour, while VEG has a minor impact on the anthocyanin content (Ficagna et al., 2020). Other additives, such as CHT, can marginally decrease phenolic composition as a side effect (Castro Marin & Chinnici, 2020).

Wine colour can decrease as a secondary effect of treatment with fining agents (Río Segade et al., 2020). In our experiment, only TAN treatment significantly ($p < 0.05$) increased the colour intensity (CI), while the lowest values were shown in wines treated with GEL, followed by BEN and PVPP. Regarding hue values, VEG-, YST-, and CHT-treated wines did not show any significant differences compared to CONTR. BEN and TAN treatments showed higher hue values, while GEL and PVPP showed the lowest values. However, the changes in hue induced by the treatments were minimal, resulting in values in the range 0.74–0.76.

Table 2
Turbidity, phenolic composition and colour parameters of 'Nebbiolo' wines treated with different additives and processing aids. CONTR: untreated control; BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin. CONTR20: untreated wine sampled in 2020, data not available. Data are mean values ± SDs of three replicates. Values followed by different letters within a column are significantly different ($p < 0.05$, one-way ANOVA with Tukey HSD post-hoc test). L*: lightness; a*: red/green colour coordinate; b*: yellow/blue colour coordinate.

Sample	Turbidity	TPI – Total phenolic index	TA - Total anthocyanins	TF -Total flavonoids	Colour intensity (AU)	Hue	L*	a*	b*
	NTU	mg (-)-epicatechin/L	mg malvidin-3-glucoside chloride/L	mg (+)-catechin/L					
CONTR 20	–	–	–	–	–	–	–	–	–
CONTR	11.59 ± 0.04 cd	3070 ± 17 bcd	146 ± 1 a	1051 ± 8b	8.25 ± 0.01 bc	0.75 ± 0.00c	16.6 ± 0.1 ef	47.49 ± 0.09 ef	2781 ± 0.10 ef
BEN	2.30 ± 0.61 g	2974 ± 22 efg	140 ± 2 bcd	1042 ± 8 bc	7.82 ± 0.01f	0.76 ± 0.05 a	18.1 ± 0.1b	49.01 ± 0.08b	29.93 ± 0.09b
GEL	6.58 ± 1.31 e	2739 ± 37 h	124 ± 0 e	922 ± 6 e	6.97 ± 0.03 g	0.74 ± 0.01 d	20.9 ± 0.1 a	51.43 ± 0.07 a	33.31 ± 0.12 a
VEG	9.93 ± 1.16 d	3010 ± 42 cdef	139 ± 3 d	1019 ± 17 bcd	8.08 ± 0.01 d	0.75 ± 0.00c	17.2 ± 0.1 d	48.05 ± 0.09 d	28.61 ± 0.08 d
PVPP	4.15 ± 0.27f	2917 ± 16 g	137 ± 2 d	1005 ± 11 d	7.89 ± 0.02 e	0.74 ± 0.01 e	17.6 ± 0.1c	48.39 ± 0.07c	29.16 ± 0.08c
YST	7.21 ± 0.49 e	2996 ± 9 def	139 ± 2 bcd	1029 ± 9 bcd	8.06 ± 0.00 d	0.75 ± 0.00c	17.2 ± 0.0 d	48.09 ± 0.02 d	28.69 ± 0.03 d
CHT	14.55 ± 0.24 a	2963 ± 25 fg	145 ± 4 ab	1035 ± 23 bcd	8.06 ± 0.00 d	0.75 ± 0.01c	17.2 ± 0.0 d	48.04 ± 0.04 d	28.62 ± 0.05 d
MAN	14.56 ± 0.61 a	3081 ± 33 bc	143 ± 1 abcd	1018 ± 4 bcd	8.30 ± 0.03 ab	0.75 ± 0.02b	16.5 ± 0.1 fg	47.27 ± 0.14f	27.57 ± 0.17f
ARG	13.61 ± 0.37 ab	3097 ± 22 ab	144 ± 2 abc	1014 ± 19 bcd	8.24 ± 0.01c	0.75 ± 0.03b	16.6 ± 0.0 ef	47.47 ± 0.03 ef	27.82 ± 0.03 ef
POL	12.58 ± 0.04 bc	3048 ± 22 bcde	139 ± 1 cd	1023 ± 9 bcd	8.23 ± 0.03c	0.75 ± 0.04b	16.7 ± 0.1 e	47.50 ± 0.10 e	27.86 ± 0.11 e
TAN	12.35 ± 0.01 bc	3163 ± 25 a	142 ± 0 abcd	1097 ± 6 a	8.35 ± 0.00 a	0.76 ± 0.05 a	16.4 ± 0.0 g	47.28 ± 0.06 ef	27.56 ± 0.06f

The reduction of wine CI and the increase of hue values are likely due to the lower TA values reported after the treatment with fining agents, which led to a reduction of the red colour component (A520). In contrast, the decrease observed in hue values after GEL and PVPP treatments could be due to their higher effectiveness in removing the flavanic component, as mentioned above.

To further explore the colour of treated wines, CIELab characterisation of 'Nebbiolo' wines was performed. L* (lightness), a*, and b* (red/green and yellow/blue colours, respectively) values are reported in Table 2, while their colour outcome (after conversion in 24-bit RGB values for publication purposes) is available in Fig. 1. A slight increase in L*, a*, and b* values was shown after treatment with CHT, YST, PVPP, and VEG, while a more prominent increase in these parameters inter- ested BEN and GEL treatments, which was visually confirmed by rep- resentation in Fig. 1; only TAN showed a significant ($p < 0.05$) decrease in L* values with respect to CONTR. From the obtained CIELab data, the ΔE* parameter was calculated for all treatments compared to CONTR (Fig. 1). GEL gave the highest ΔE* value (8.01), followed by BEN (2.98).

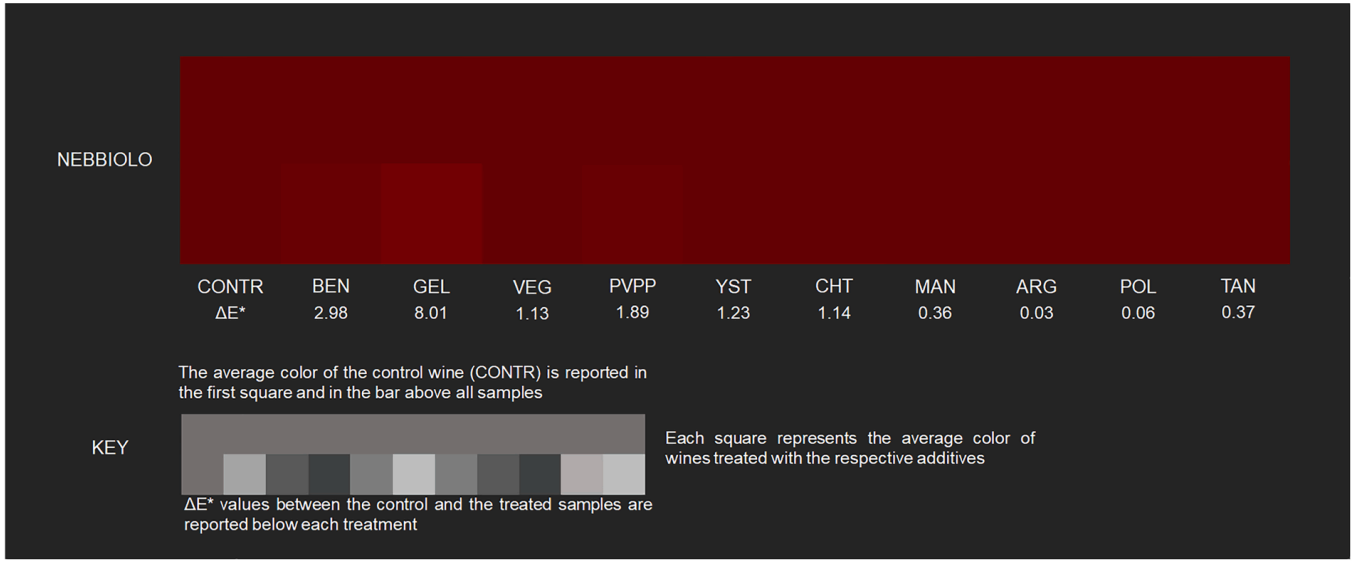

Fig. 1. 'Nebbiolo' wine colour detected after the treatment with different additives and processing aids. BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin. Each colour was acquired by spectrophotometry, expressed in CIELab coordinates, and then converted to RGB values. The untreated control (CONTR) sample was extended on the top side of the bar to facilitate comparisons with treated wines.

Wines treated with BEN and GEL reached a visual perceived colour reduction, whereas all other treatments did not approach a value of three ΔE* units, which is the threshold estimated to allow a visual recognition of wine colour difference by the human eye (P´erez-Magarin˜o & Gonza´lez-Sanjos´e, 2003). Therefore, BEN and GEL had the strongest impact on the turbidity, colour, and phenolic composition of 'Nebbiolo' wines. These results confirmed that the ΔE* were higher than 5, which were obtained when 'Nebbiolo' wines were treated with a GEL fining agent (Río Segade et al., 2020).

3.2. DNA extraction from wine after application of oenological additives

The DNA yield and quality ratio of DNA extracts were initially estimated through a spectrophotometric analysis using NanoDrop. The spectrophotometric quantification of wine extracted using the Norgen protocol is reported in Table S3, while the quantification results of wines extracted with the SirM method are reported in Table 3. In general, low- quality DNA was found in all wine samples using both extraction methods. Concerning the Norgen protocol, no significant differences in DNA yield were discovered between CONTR and the treatments; only CONTR20 ('Nebbiolo' wine sampled in 2020, one year before application of oenological additives) showed a high DNA yield (Table S3). In addition, significant differences were found in wine samples extracted with the SirM method; CONTR20 showed the highest DNA yield, while CONTR had the lowest

concentration. In contrast with the results obtained after the Norgen protocol, all wines treated with different additives and processing aids showed a significantly higher DNA yield compared to CONTR (Table 3). The quality of the extracted DNA was estimated from traditional absorbance ratios (A260/A280 and A260/A230). 'Nebbiolo' wines extracted using both protocols did not show any significant differences in terms of A260/A280 and A260/A230 ratios. However, DNA extracted with the SirM protocol showed higher quality than DNA extracted with the Norgen protocol, likely due to the application of a more intense DNA cleaning operation using phenol and chloroform (Table 3, Table S3). The presence of polysaccharides and phenolic substances (including tannins), which are extremely common in grapes, negatively affects the quality of DNA extracted from wine.

Table 3
DNA purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*. Percentage ratio between DNA quantification by *VvNCED2* and the yield measured by NanoDrop. Loss of DNA after treatment expressed as percentage ratio between the DNA (quantified by *VvNCED2*) of the control and the treated wine. The ratio was calculated as follows: (DNA of the control - DNA of' wine after treatment)/ DNA of the control. Allelic profile of genotyping assay SNP_14783, SNP_15082. For each treatment replicate, one sample was extracted (R1, R2, and R3). For each sample, genotyping was performed twice (1st and 2nd repetition). '-' in the allelic profile denotes an incorrect allelic call; '+' indicates samples that correctly amplified, and 'nd' stands for 'not detected'. Data are means of 3 replicates ± standard deviation. Values followed by different letters within a column are significantly different ($p < 0.05$, Kruskal-Wallis test with Conover's Comparison test). CONTR20: untreated control sampled one year before the application of additives; CONTR: untreated control; BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin.

Sample	NanoDrop Quantification			*VvNCED2* quantification DNA yield [pg/mL of wine]	% Grapevine DNA	DNA treatment / DNA CONTR (%)	SNP_14783			SNP_15082			SNP_14783			SNP_15082		
							1st repetition						2nd repetition					
	DNA yield [ng/ mL of wine]	A_{260}/A_{280}	A_{260}/A_{230}				R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
CONTR20	55.23 ± 31.13 a	2.05 ± 0.04 a	2.29 ± 0.09 a	9.55 ± 0.97 a	0.01 ± 0.00 ef	+41.92 ± 12.58 a	+	+	+	+	+	+	+	+	+	+	+	+
CONTR	2.42 ± 0.50 e	1.40 ± 0.17 a	0.72 ± 0.02 a	6.73 ± 1.13 ab	0.29 ± 0.07 a	–	+	+	+	+	+	+	+	+	+	+	+	+
BEN	4.27 ± 0.11 abcd	1.44 ± 0.03 a	0.66 ± 0.03 ab	0.03 ± 0.04 g	0.00 ± 0.00f	−99.56 ± 0.35 fg	–	–	–	–	+	–	nd	–	–	nd	nd	–
GEL	4.97 ± 0.55 ab	1.52 ± 0.03 a	0.65 ± 0.03 ab	0.02 ± 0.04 g	0.00 ± 0.00f	−99.67 ± 0.36 h	nd	+	–	nd	–	nd	–	nd	+	+	nd	–
VEG	4.69 ± 0.55 ab	1.48 ± 0.03 a	0.67 ± 0.02 ab	1.30 ± 0.78 efg	0.03 ± 0.02 cdef	−80.65 ± 12.24 defg	+	+	+	+	+	+	+	+	+	+	+	+
PVPP	4.41 ± 0.45 abc	1.43 ± 0.06 a	0.61 ± 0.09 ab	1.90 ± 0.47 abcd	0.04 ± 0.01 bcde	−71.67 ± 6.67 cde	+	+	+	+	+	+	+	+	+	+	+	+
YST	3.80 ± 0.52 bcde	1.45 ± 0.09 a	0.65 ± 0.02 ab	4.13 ± 0.68 abc	0.11 ± 0.02 ab	−38.57 ± 8.27 abc	+	+	+	+	+	+	+	+	+	+	+	+
CHT	3.02 ± 0.12 cde	1.88 ± 0.26 a	043 ± 0.08b	0.56 ± 0.32 fg	0.02 ± 0.01 def	−91.65 ± 5.20 efg	+	+	+	+	+	+	+	+	+	+	+	+
MAN	4.57 ± 0.85 ab	1.52 ± 0.06 a	0.50 ± 0.26 ab	1.47 ± 0.26 cdef	0.03 ± 0.01 bcde	−78.09 ± 3.77 cdef	+	+	+	+	+	+	+	+	+	+	+	+
ARG	3.50 ± 0.05 bcde	1.58 ± 0.13 a	0.69 ± 0.03 ab	2.51 ± 1.49 abcd	0.07 ± 0.04 abcd	−62.76 ± 21.13 bcd	+	+	+	+	+	+	+	+	+	+	+	+
POL	4.76 ± 1.19 ab	1.60 ± 0.08 a	0.66 ± 0.07 ab	3.97 ± 1.92 abcd	0.10 ± 0.07 abc	−41.00 ± 29.33 abc	+	+	+	+	+	+	+	+	+	+	+	+
TAN	2.67 ± 0.52 de	1.99 ± 0.44 a	0.60 ± 0.11 ab	1.77 ± 0.39 cdef	0.07 ± 0.01 abc	−73.69 ± 6.17 cde	+	+	+	+	+	+	+	+	+	+	+	+

Several previous works (Savazzini & Martinelli, 2006; Vignani et al., 2019) reported the presence of yeast DNA and phenolic substances in the DNA extracted from the wine, which can decrease the precision of the measurement; thus, spectrophotometric quantification is often not reliable for the quantification of grapevine DNA in wine. Consequently, we adopted a more specific quantification of grapevine DNA based on VvNCED2 amplification using TaqMan® probes, as previously suggested (Boccacci et al., 2020; Savazzini & Martinelli, 2006; Vignani et al., 2019). No amplification of VvNCED2 was observed in DNA samples collected in 2021 and extracted with the Norgen protocol, only in CONTR20, collected in 2020 after malolactic fermentation, VvNCED2 was amplified in qPCR. The grapevine DNA present in the samples was probably too limited and/or too impure to allow amplification during qPCR of VvNCED2, while after malolactic fermentation the protocol was more efficient, as reported previously (Boccacci et al., 2020). Instead, the DNA extracted with the SirM protocol was successfully amplified using VvNCED2 TaqMan® probes (Fig. 2C). Values of grapevine DNA and its percentage ratio, with respect to the total DNA yield measured by NanoDrop, are reported in Table 3. In general, the data obtained with Nanodrop quantification were overestimated. CONTR had the highest concentration of grapevine DNA with 6.73 ± 1.13 pg/mL of wine; this amount corresponds only to the 0.29% of the DNA yield quantified by NanoDrop. In the other treatments, the percentage of grapevine DNA was

lower. Most of the DNA yield quantified by Nanodrop is likely not from grapevine. Spectrophotometric quantification is a non-reliable method to quantify DNA extracts from wine, regardless of the extraction method. The use of dehydrated grapes did not influence the extraction of DNA from wine; moreover, these data confirmed the overestimation previously reported in 'Nebbiolo' wines produced with fresh grapes (Boccacci et al., 2020). The results of previous work showed that grapevine DNA can be up to 25 times less than the DNA estimated with a spectrophotometer in the musts, and 20,000 times less in the wine after 1 year.

Most of the treatments showed a lower grapevine DNA concentration with respect to CONTR. All the clarification treatments played a role in removing DNA from the wine, but the intensity of the reduction differed depending on the treatment. Interestingly, the products with the highest chemical-physical impact (Table 2) caused the highest loss of DNA compared to CONTR (Table 3). Samples treated with BEN or GEL had the strongest reduction, with a DNA loss of 99.56% and 99.67%, respectively. Whereas, POL, ARG, PVPP, and YST did not have an impact on grapevine DNA extraction with respect to CONTR. Moreover, CONTR20 had 41.92% more grapevine DNA compared to CONTR. Nevertheless, the reduction that occurred during one year of storage in stainless steel casks with three rackings after malolactic fermentation was not significant.

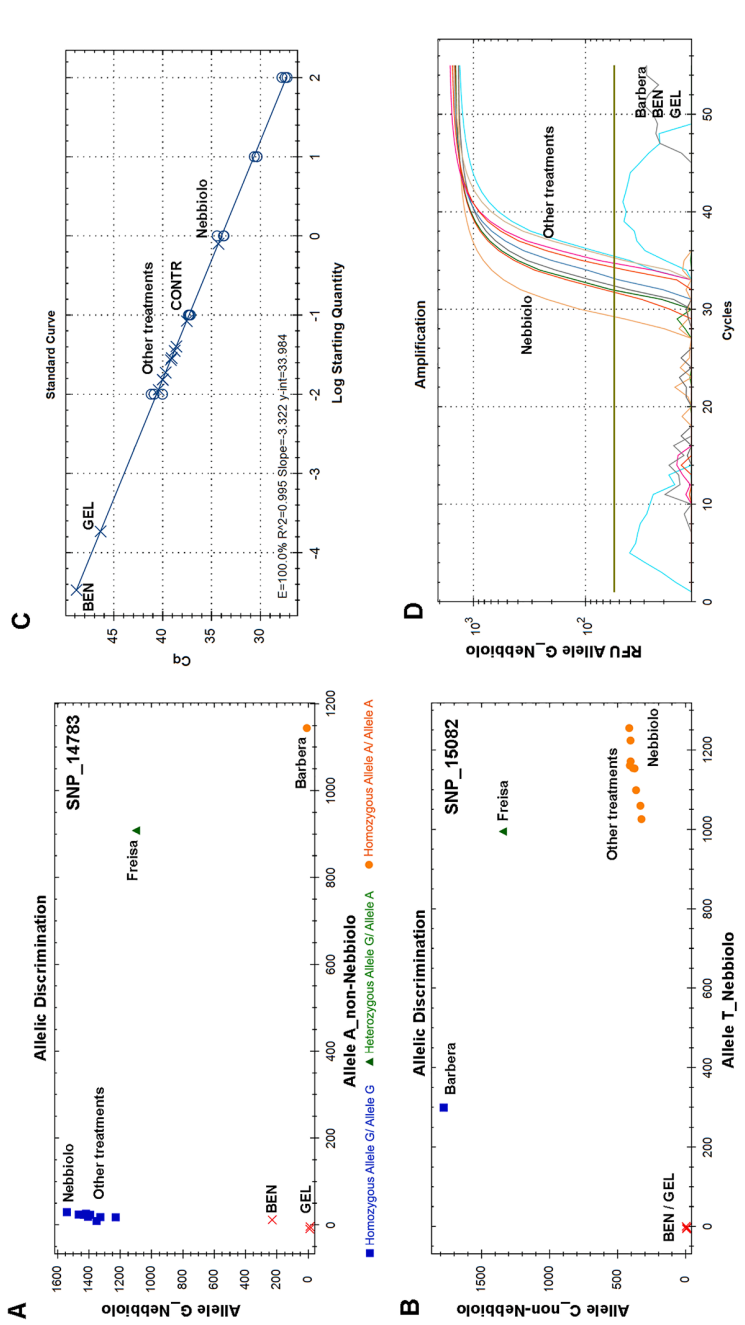

Fig. 2. SNP genotyping in 'Nebbiolo' wines extracted with SirM method and previously treated with different oenological additives and processing aids. (A) Scatterplot of TaqMan® SNP 14783 genotyping assay with 'Nebbiolo' wines. (B) Scatterplot of TaqMan® SNP 15082 genotyping assay with 'Nebbiolo' wines. (C) Standard curve of *VvNCED2* TaqMan® probe used to quantify grapevine DNA in 'Nebbiolo' wines. DNA from 'Nebbiolo' leaves was used as a calibrator for the standard curve. (D) Relative fluorescence unit (RFU) of the TaqMan® SNP_14783 probe tagged with VIC dye (allele G 'Nebbiolo'). The yellow line in the amplification plot indicates the RFU level of 'Barbera' (non-'Nebbiolo' control), above which, it was possible to detect 'Nebbiolo' wines. The control DNA from 'Nebbiolo', 'Barbera' and 'Freisa' were extracted from leaves. CONTR: untreated control; BEN: bentonite; GEL: gelatine.

Moreover, the literature reports greater efficiency in reducing the turbidity of wine after the use of mixes of different fining agents (Ficagna et al., 2020). Interestingly, the loss of DNA caused by aging was lower compared to the loss due to treatment with fining agents (i.e. BEN and GEL). Thus, fining operations play the most important role in decreasing residual DNA in 'Nebbiolo' wine. Therefore, it can be expected that the combined effect of these treatments may strongly reduce DNA quality and quantity in the wine, explaining why several authors did not successfully find traces of DNA in commercial wines (Boccacci et al., 2012; Boccacci et al., 2020; Catalano et al., 2016).

3.3. SNP genotyping in 'Nebbiolo' wines

The combination of allelic calls of two specific 'Nebbiolo' SNPs (SNP_15082 and SNP_14783) is enough to distinguish 'Nebbiolo' from more than 1100 genotypes. In a precedent study, the TaqMan® assay based on these two SNPs allowed the recognition of 'Nebbiolo' musts and wines with high sensitivity (Boccacci et al., 2020). Nevertheless, as reported by several studies (Baleiras-Couto & Eiras-Dias, 2006; Siret et al., 2002), due to the lack of quality and DNA integrity, commercial wines and aged wine also showed a reduced identification efficiency for 'Nebbiolo'.

TaqMan® assays for the detection of SNP_15082 and SNP_14783 were applied to the DNA extracted with the Norgen protocol, only CONTR20 correctly

amplified both alleles, while all other samples collected in 2021 lacked amplification or had incorrect calls using the genotyping assays (Table S3). These results confirm that the problems observed with the amplification of *VvNCED2* are likely due to the extremely low quality of DNA extracted from wine. Commercial kits, which are extensively used in the extraction of plant material, are not reliable tools for DNA extraction from aged wine. The use of commercial kits is fully effective only with musts and young wines (Boccacci et al., 2020); nevertheless, in aged wine and clarified samples, the quality of DNA is too low and can cause incorrect amplification during the Taq- Man® assay. The genotyping of DNA extracted with the SirM protocol was more successful (Table 3, Fig. 2A, B, D). CONTR and CONTR20 correctly amplified both alleles. Several authors (Boccacci et al., 2020; Catalano et al., 2016) reported aging time as one of the causes of the reduction of identification efficiency in commercial wines, as was also confirmed in this work using an ineffective extraction method (Table S3). However, according to our results using SirM protocol, the reduction in the quantity of DNA that occurs over time is not alone responsible for the incorrect amplification of commercial wines. Despite the aging of the wine and the low quality of the DNA, this TaqMan® assay was confirmed to be very robust and effective in identifying 'Nebbiolo' wines in experimental conditions.

Interestingly, there is a clear correlation between the treatments and the success of genotyping; ARG, TAN, CHT, YST, MAN, VEG, POL, and PVPP treatments

did not have any effect on the assay. All repetitions had correct amplification and allelic discrimination was always possible with a precision of 100%. Nevertheless, BEN and GEL resulted in incorrect or absent SNP amplification (Table 3, Fig. 2A, B, D). This is probably due to the low quantity of residual DNA in the wine. Indeed, according to the VvNCED2 quantification results, the TaqMan® assay used in our study loses efficacy if the samples have less than 0.5 pg of DNA per mL of wine. This threshold can be deduced from the CHT treatment, with 0.56 ± 0.32 pg of DNA per mL of wine being the treatment with the least amount of DNA in which the genotyping assays worked correctly (Table 3). Serial dilutions of DNA extracted from CONTR, ARG, TAN, CHT, YST, MAN, VEG, POL, and PVPP confirmed that under 0.5 pg of DNA per mL of wine, both TaqMan® assays lost their effectiveness in all treatments showing incorrect or absent of SNP amplification. In addition to the low DNA concentration, the presence of PCR inhibitors in the extracted DNA can influence the PCR efficiency and the results of the TaqMan® assay. The amplification efficiency, verified by adding an EIPC in all DNA extracts, was 100% in all samples extracted with SirM protocol without significant differences. This result suggests that the amplification issues in BEN and GEL treatments were uniquely caused by the low quantity of DNA in wine after treatment with fining agents and not by the presence of PCR inhibitors in the extracts.

The use of BEN and GEL represent very common practices widely used in the production of most commercial red wines, including 'Nebbiolo' wines. Moreover, it is not unusual to use these products together. Considering our results, one of these fining agents alone can reduce the grapevine DNA by 99%; therefore, their effect, alone or combined, on the residual DNA can explain why the TaqMan® assay and other molecular assays do not properly work on aged commercial wines (Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; Boccacci et al., 2020; Catalano et al., 2016; Recupero et al., 2013). To the best of our knowledge, this is the first time an experiment has investigated the causes of the reduced efficiency of genetic traceability in wine.

4. Conclusions

In this study, we investigated the impact of the most common additives and processing aids used in winemaking on the efficiency of the TaqMan® assay for the varietal authentication of 'Nebbiolo' wines. As already reported by Boccacci et al. (2020), using two SNP markers (SNP_14783 and SNP_15082) it is possible to identify 'Nebbiolo' from a group of 1157 non-'Nebbiolo' genotypes. Nevertheless, the winemaking process can affect the precision of varietal identification. All oenological operations at reaching the clarity and stability of the wine after malolactic fermentation can reduce the quality and the amount of DNA in the wine. In the present study, the results showed an impact of fining

agents on turbidity and phenolic composition in line with other studies reported previously. BEN and GEL had the strongest impact on turbidity, phenolic composition, and colour parameters. The efficiency of the TaqMan® assay for varietal identification was also confirmed in aged wines; indeed, under experimental conditions, recognition was possible in 2-years-old wine with 100% precision. Identification was also possible for most of the wines treated with additives or processing aids. Nevertheless, recognition failed in wines treated with BEN and GEL. 'Nebbiolo' wines that have undergone these treatments showed the lowest concentration of grapevine DNA. Therefore, there is a clear correlation between the efficiency of the assay and the quantity of DNA in the wine. These results allowed us to identify a threshold DNA concentration (0.5 pg/mL of wine) below which the TaqMan® assay loses efficiency. Moreover, one year of aging in stainless steel did not significantly affect either the DNA quantity or the identification efficiency. This study contributed to explaining the reasons for the decreased identification efficiency in commercial wines and confirmed the need for future improvements of DNA extraction techniques from wine. Furthermore, these data suggest how an integrated molecular approach using different markers (SSRs and SNPs) with different characteristics could probably be useful for wine authentication by overcoming the limitation of a single class of molecular markers (Vignani et al., 2019; Fanelli et al., 2021).

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CRediT authorship contribution statement

Giorgio Gambino: Conceptualization, Supervision, Funding acqui- sition, Methodology, Visualization, Writing – review & editing. **Lorenzo Ferrero:** Investigation, Formal analysis, Data curation, Validation, Visualization, Writing – original draft. **Giulia Scalzini:** Investigation, Methodology, Formal analysis, Data Curation, Validation. **Camilla De Paolis:** Investigation, Formal analysis, Data curation, Validation. **Maria Alessandra Paissoni:** Investigation, Formal analysis, Data curation, Validation. **Susana Río Segade:** Investigation, Methodology, Validation, Writing – review & editing. **Simone Giacosa:** Investigation, Methodology, Validation, Writing – review & editing. **Paolo Boccacci:** Investigation, Methodology, Validation, Writing – review & editing. **Luca Rolle:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110874.

Table S1. Primers and probes used for the SNP genotyping

ID marker	SNP position	Allele Nebbiolo	Allele non-Nebbiolo	ID Oligo	Primer and Probe sequences 5'-3'	Length of the fragment (bp)
SNP_14783	chr8_13053532	G	A	For	GAGCACAATCAACAATTATCCATTT	83
				Rev	TGGTTGTGTTAATAGCAGGCAA	
				Probe Allele A	FAM-TAAAAAAGTGTTAAGGTGATAAT-NFQ	
				Probe Allele G	VIC-TAAAAAAGTGTTAAGGTGATGAT-NFQ	
SNP_15082	chr8_19402046	T	C	For	TCTCTTCTGGCATGGAAATCAAT	89
				Rev	TAGATTACGGGCCAAGCTGA	
				Probe Allele T	FAM-TCTCATTTTCCTCATTAT-NFQ	
				Probe Allele C	VIC-TCTCATTTTCCTCATCATG-NFQ	

Table S2. DNA quantity and quality extracted from 'Nebbiolo' wines treated with different additives and processing aids using Plant/Fungi DNA Isolation Kit (Norgen). For each treatment repetition, one sample was extracted (R1, R2, and R3). Purity and yield measured using NanoDrop 1000. Allelic profiles of genotyping assays SNP_15082 and SNP_14783. '-' in the allelic profile denotes an incorrect allelic call; '+' indicates samples that correctly amplified, and 'nd' stands for 'not detected'. Data are means of 3 replicates ± standard deviation. Values followed by different letters within a column are significantly different ($p < 0.05$, Kruskal-Wallis test with Conover's Comparison test).

Sample	Treatment	NanoDrop Quantification			SNP_14783			SNP_15082		
		DNA yield [ng/µl]	A260/A280	A260/A230	Alleles			Alleles		
					R1	R2	R3	R1	R2	R3
CONTR	-	5.2±1.1 a	1.13±0.11 a	0.26±0.04 a	nd	-	nd	-	-	-
BEN	Bentonite	3.4±1.8 a	1.13±0.08 a	0.23±0.14 a	-	-	-	-	-	-
GEL	Gelatine	6.8±3.1 a	1.19±0.07 a	0.31±0.01 a	-	-	-	-	-	-
VEG	Vegetables protein	7.6±1.8 a	1.27±0.05 a	0.31±0.03 a	-	-	-	-	-	-
PVP	Polivinipolidon	5.8±0.9 a	1.05±0.19 a	0.24±0.09 a	-	-	-	-	-	-
YST	Yeasts hulls	7.5±3.1 a	1.18±0.06 a	0.27±0.02 a	nd	nd	nd	nd	nd	nd
CHT	Chitosan	7.4±2.4 a	0.98±0.06 a	0.21±0.04 a	-	nd	nd	nd	nd	-
MAN	Yeast Mannoprotein	11.9±5.1 a	1.17±0.09 a	0.25±0.03 a	-	-	-	-	-	-
ARG	Arabic Gum	16.6±19.9 a	1.11±0.06 a	0.29±0.06 a	nd	nd	nd	nd	nd	nd
POL	Potassium Polyaspartate	6.7±0.8 a	1.08±0.09 a	0.26±0.06 a	+	nd	nd	nd	+	nd
TAN	Grape Tannin	5.5±1.2 a	1.03±0.14 a	0.31±0.12 a	-	nd	-	nd	nd	-
FLT	Filtration	5.3±0.1 a	1.08±0.03 a	0.29±0.07 a	-	nd	nd	-	nd	+

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General Conclusions and Future Perspectives

This PhD thesis was focused on two main aspects:

1. The withering of red winegrapes, through the study of techniques currently used in order to introduce innovations in traditional processes.

2. The exploration of new processing aids and the impact of additives on traceability techniques.

Concerning the first part, the results obtained contributing to the comprehension of the postharvest withering of red winegrapes, which is a very complex process, involving grape modifications from both a chemical and physical points of view.

The bibliographic research conducted with the systematic review method provided useful results for withering and winemaking management. Phenolic acids evolution throughout grape withering is a complex phenomenon affected by several genetic and technological variables. Indeed, the withering conditions applied, and grape cultivar genotype have a key role in the modification of hydroxycinnamic and hydroxybenzoic acids amounts; yet there is still much to understand, particularly towards the combined effect of both factors through genetic responses to environmental stresses and their consequent chemical implications.

The three-years study of the combined effect of ripeness degree and withering process length on the physio-chemical composition of partially withered Nebbiolo grapes showed that these two variables can be modulated according to the desired oenological objective, searching for the valorization of grape potentialities. As for dry red wines, harvest time represents a step of great importance for the potential quality of wines produced with partially dehydrated grapes. Interestingly, contrary to what the producers traditionally applied for the production of Sforzato di Valtellina DOCG wine, the results obtained during the 2019-2020-2021 consecutive vintages suggested that the choice to anticipate the harvest time for red grapes destined to withering process could be preferred in view of long ageing of the respective wines.

Moreover, the study conducted on the Italian winegrape variety Aleatico under two different withering conditions pointed out new insights about the extractability of red withered winegrapes. After withering, the phenolic extractability decreased in skins and increased in seeds, in both cases greatly for sun-exposed grapes than those withered in controlled conditions. Skins extractable phenolic profiles are affected by the combination of their loss/concentration balance, together with the modified possibility to extract them, likely due to cell-wall gel network changes for the rearrangement of skins polysaccharides, strongly influenced by the withering conditions. Pectin modifications affect also the mechanical properties of the grapes, especially for

skins rigidity. For the first time, it was demonstrated that the differences shown by the seeds extractable phenolic profiles in wine-like conditions are due to a modified extractability caused by the different withering conditions and not to chemical modifications of phenolic compounds occurred during the withering. Given the difficulty of studying seeds tissues, specific studies are necessary to understand the causes of the differences observed in extractability.

Regarding the second part, the effects of several processing aids on grape-identity potential wine features were assessed.

A corn-derived biosurfactant extract was evaluated as a novel processing aid and compared with oenological tannins as a solubilizing and stabilizing agent of anthocyanins in red wine. The results showed that biosurfactant addition interestingly improved the colour properties of skin extracts, with a variety-dependent effectiveness, mainly due to copigmentation and polymerization effects. The knowledge of the effectiveness of this biosurfactant to preserve the wine colour may open a new field of research on its potential for winemaking, but more research are needed to evaluate the effectiveness in real winemaking and wine ageing conditions.

Finally, the impact of oenological processing aids and additives on the genetic traceability of ‘Nebbiolo’ wines have been studied, contributing to explaining the causes of the reduced varietal identification efficiency in commercial wines.

Among the treatments assessed, Bentonite and Gelatine had the strongest impact on turbidity, phenolic composition and colour parameters. Moreover, Nebbiolo wines that have undergone these treatments showed the lowest concentration of grapevine DNA. These results identify the weakness of the DNA extraction techniques from wine and confirm the need for future improvements of the SNP-based assay

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Annex I – Research Products

ISI index journals articles

▪ Scalzini, G., López-Prieto, A., Paissoni, M. A., Englezos, V., Giacosa, S., Rolle, L., Gerbi, V., Río Segade, S., Pérez Cid, B., Moldes, A. B., Cruz, J. M. (2020). Can a Corn-Derived Biosurfactant Improve Colour Traits of Wine? First Insight on Its Application during Winegrape Skin Maceration versus Oenological Tannins. *Foods*, *9*(12), 1747. DOI: https://doi.org/10.3390/foods9121747

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Thesis

▪ Conoscenza delle caratteristiche chimico-fisiche dei vini 'Sforzato di Valtellina DOCG' presenti in commercio (2022). Candidato: Alberto Agagliati. Relatore: Prof. Luca Giorgio Carlo Rolle. Correlatrice: Dott.ssa Giulia Scalzini. Correlatrice: Dott.ssa Maria Alessandra Paissoni. (Tesi di Laurea Magistrale CdLM Scienze Viticole ed Enologiche – Università di Torino).

▪ Influenza della data di raccolta e del tempo di appassimento delle uve per la produzione di vini atti a Sfursat di Valtellina: effetti sulla composizione chimica (2022). Candidato: Marco Lagori. Relatore: Dott. Giacosa Simone. Correlatrice: Dott.ssa Giulia Scalzini (Tesi di Laurea Magistrale CdLM Scienze Viticole ed Enologiche – Università di Torino).

▪ Postharvest dehydration of Nebbiolo grapes in Valtellina: effect of grape bunch placement on phenolic parameters (2023). Candidato: Li Yunhan. Relatore: Dott. Giacosa Simone. Correlatrice: Dott.ssa Scalzini Giulia (Tesi di Laurea Master VINIFERA).

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