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CYCLE: XXXVII

NEW APPROACHES FOR THE PRODUCTION AND TRACEABILITY OF HIGH-QUALITY WINES

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A tutti voi, che siete stati parte di questo viaggio.

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General introduction

Agri-food is a nature-based and climate-dependent industry, strongly affected by the trend of weather and environment. Nowadays the agricultural sector is facing on the effects of climate change, that are affecting significantly the viability of the productions, with an impact on the whole supply chain (Costa et al., 2023). Likewise, the wine industry is impacted by climate change, with both economic and technological consequences (Gonen et al., 2024).

Wine production is straightly linked with the Italian traditions and leads the national agribusiness, representing the Made in Italy in the world (Corsi et al., 2019). Wine economic value and quality are often related to the concept of '*terroir'*, which is defined as the result of the interaction of a high number of factors including soil, topography, and climate, as well as the human impact. Together those elements create a unique environment that is used to characterize wine regions (Sun et al., 2022; Ollat et al., 2016). However, these factors are influenced by the effects of climate change and this can lead to alteration the grape chemical composition and consequently affect the choice of winemaking technology and final wine quality (Rogiers et al., 2022).

These factors related to '*terroir*' are able to affect both wine primary and secondary metabolites. However, various winemaking practices contribute to enhancing wine quality, modifying quantity and compositions of these compounds (Selli et al., 2006a). Particularly for white wines, new oenological trends are focused on products able to express their aromatic characters, with rich structure and strong varietal imprint.

Nowadays wine producers have to deal with consumers more and more careful to wine authenticity, typicity, and quality. To ensure authenticity, methods, that have the ability to track the entire path, have to be develop and improve (Geana et al., 2016). Indeed, wine, due to its high economic value and to the elevate number of intermediates that are involved during the chain, is easily subject to fraud, adulteration and manipulation which can damage its market and image (Ranaweera et al., 2021). Moreover, traditional techniques used to detect wine manipulation are affected by external condition, such as climate seasonal patterns, vineyard management and winemaking processes. Factors, as well as berries chemical composition, that, in recent years, have been strongly influenced by climate change (Palade et al., 2018; Villano et al., 2017).

The aim of these three-years work was the enhancing the knowledge for the production of high-quality wines, preserving and increasing the varietal characteristics of different autochthonous grape varieties, applying new winemaking methods. Moreover, it aimed to improve techniques of wine traceability, including also the study of the impact of different oenological treatments on wine DNA.

During the PhD study course, in collaboration with Araldica Castelvero company, the work was focused on different topics. Therefore, two main research lines have been followed, to carry out these objectives.

In the first section (Experimental section – PART A) the primary aim was to understand the development of the oenological sector in the context of climate change. Through a bibliographic research, the goal was to have an overview of the impact of the effects of climate change on the wine composition and on the winemaking processes (Chapter 1). This allowed to deeper the understanding to improve the production of high-quality

wines. Indeed, a new oenological winemaking technique was evaluated, the so called '*cold liquid stabulation*'. This was applied on two autochthonous Italian white grape varieties 'Arneis' and 'Cortese' to detect the impact of these innovative approach on the phenolic, antioxidant and aroma characteristics of produced wines (Chapter 2). In addition, during my PhD course, a three-year study has been conducted with the propose to investigate the combined effect of different ripeness degree and withering rates on the chemical composition, mechanical properties, and phenolic profile of Nebbiolo grapes, used for the production of a high-quality Italian DOP wine: '*Sforzato di Valtellina DOCG*" (Chapter 3).

The second section (Experimental section – PART B) concerned about genetic traceability of monovarietal wines. First a series of new single nucleotide polymorphism (SNPs) markers have been identified for some north-western Italian grape varieties, used for the production of high-quality wines, and validated with two real-time PCR techniques: TaqMan® genotyping assays and high-resolution melting analysis (HRM) (Chapter 4). Then, to contribute to clarify the reasons behind the lower efficiency of varietal identification in commercial wines, the impact on genetic traceability of 'Nebbiolo' wines, of most common commercial additives and processing aids, used in winemaking industry, was evaluated, through a SNP-based method (Chapter 5). Finally, different filtration treatments have been tested on 'Nebbiolo', to detect the DNA traceability (Chapter 6).

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Experimental section – PART A

Sustainability and new technologies in winemaking industry

Chapter 1

The effects of climate change on wine composition and winemaking processes

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Abstract

Climate change strongly affects the wine industry, with impacts on grapevine vegetative behavior, grape primary and secondary metabolites and wine composition. The increase of ethanol is one direct consequence, creating the necessity of new oenological strategies. Nowadays, a challenging objective is the production of wines with reduced or removed alcohol content. Different strategies are developing, divided in pre-fermentative, fermentative and post fermentative. Those are also technologies able to reduce or remove alcohol content through physical methods. This review examines the effects of climate change on wine composition and winemaking processes, considering new technologies used to produce removed or low-alcohol-content wines.

Keywords: Alcohol, Climate change, Grape composition, pH, Volatile organic compounds, Wine composition

Introduction

Climate change (CC) has become one of the most important topics being debated in recent years. It is well known that CC impacts political decisions, government policies, and human activities, including the agricultural sector. Agriculture is a nature-based and climate-dependent sector; hence, it is strongly influenced by CC. The viticulture and wine industry are affected by this situation, with technological and economic consequences (Costa et al., 2023). It is easy to understand that climatic variations impact grapevine vegetative behavior and therefore on grape musts and composition of wines.

Wine is a complex beverage and its composition and final quality depends on various factors. Quality is the result of a balance between wine and its characteristics, and this balance defines the typicity (Drappier et al., 2017). The typicity reflects the terroir, defined as the result of an interaction between climate, soil, and topography, creating together a unique environment that characterize each vineyard's area (Rogiers et al., 2022). Moreover, agronomical and technological choices, such as vineyard management, varieties, clones, and winemaking techniques, influence quality of the final product and its value on the market (van Leeuwen et al., 2019).

The wine composition is the result of numerous molecular compounds present at the time of harvest. The main objective of a winemaker is to modulate these compounds through the choice of optimal grape maturity (van Leeuwen et al., 2022). In the past, maturity was referred only to technological parameters, that is, sugar accumulation and ratio of acids; nowadays the concept of maturity has evolved and other types of maturity parameters have been defined, such as physiological, technological, phenolic, and aromatic. These depend on climatic

conditions, particularly temperature, water, and sun exposure. So, in the context of CC, finding the perfect grape maturity, and able to obtain balanced wines, is a new challenge for winegrowers and winemakers (Allamy et al., 2023). The aim of this review was to analyze the effects of CC on grape composition, and their consequences on winemaking and the final quality of wine.

1. The impact of climate change on grape composition

1.1 Temperature

Grape maturation, and consequently the produced wines, is governed by climatic factors. Among these, temperature is one of the most important factors influencing the physiology of grapevine. In fact, during the growing season, temperature is a key factor for vegetative cycle. Several viticultural climatic indices, based on temperature recorded in the vineyard and developed with the aim to relate the needs of cultivar to climatic conditions, are widely used to assess the effects of CC (Piña-Rey et al., 2020). For example, the Winkler Index (WI), calculated as the sum of daily mean temperatures above 10°C from 1st April to 31st October, provides information on heat accumulation during the growing season. It is well known that temperature above 10°C drives budburst (Amerine and Winkler, 1944), defining commonly a new vegetative cycle. The value of WI is related to the rate of vine growth, influencing the final wine quality. Other bioclimatic indices, such as the Huglin Index (HI), calculated as daily average between mean and maximum temperatures above 10°C from 1st April to 30th September, are connected with the rate of vine-growing. Indeed, a climate with HI above 3000 on a day is considered as ‘very warm’ and can create stress in the physiology of vine. In fact, extreme

temperature of above 35°C induces leaf or bunch damages and reduces photosynthesis and anthocyanin concentration, with repercussion on berry composition and wine quality (De Rességuier et al., 2020; Rogiers et al., 2022). On the contrary, HI below 1200 on a day is considered 'too cold' for vine growth (Massano et al., 2023). In addition, during the growing season, temperature is a key factor. Mean temperature during vegetation period (TmVeg), which is the daily mean temperature between 1st April and 31st October, determines the timing of phenological phases. For example, higher TmVeg leads to an anticipation of phenological cycle and TmVeg above 24°C and below 13°C is classified as unfavorable for grapevine cultivation (Massano et al., 2023).

In the context of global warming, increase in temperatures observed in the last decade is expected to continue. Different studies undertaken globally have underlined the impact of temperature and change in climate on quality of wine. Figure 1 shows differences in global temperature from 1976 to 2023, compared to the 1901–2000 average. This increasing trend has influenced both phenology and metabolism of grapevine, inducing an earlier response from plants, with an acceleration of their phenological phases and maturation (Drappier et al., 2017; Petrie and Sandras, 2008). Some studies have observed that more days with temperature >30°C during flowering and veraison can lead to an early harvest by up to 17 days (Jones et al., 2005a, 2005b). Furthermore, a heat stress can reduce phenological intervals and length of the growing season (Jones et al., 2000).

Temperature has a huge influence on grape ripening and berry composition. Heat stresses are able to impact the concentration of primary metabolites, namely, sugars, acids, and their ratios, as well as secondary compounds, such as amino acids, flavonoids, and aroma compounds, with

an effect on the produced wines (Rogiers et al., 2022). Regarding primary metabolism, it is directly related to photosynthesis. Because of increasing temperatures, many studies underline higher sugar accumulation, with a decrease of organic acids and an increase in pH. Temperature above 30°C generates stress in the plant, leading to reduced berry weight and size, and ceasing of sugar accumulation, but high levels of sugar are not due to photosynthesis but to the concentration by evaporative loss (Mira de Orduña, 2010). Indeed, one of the impacts of high temperature is an important phenomenon, called 'berry shriveling'. It occurs through berry water loss because of an alteration in grape water budget when transpiration and potential water backflow exceeds phloem unloading. Different types of berry shriveling are reported in literature, such as sun burn, resulting in development of poor color in red varieties and raisin formation in severe occasions; late season fruit dehydration, with an increase of total soluble solid concentration; and sugar accumulation disorder, resulting in soft and irregular-shaped berries with low fresh weight, reduced sugar accumulation, and low amount of anthocyanins (Šuklje et al., 2016).

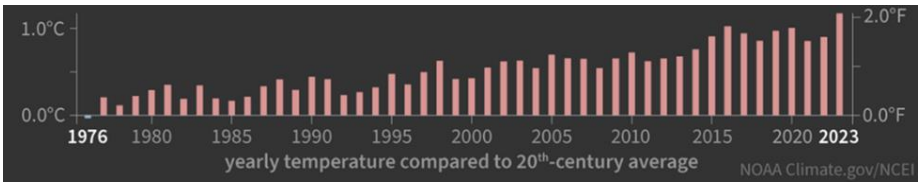

Figure 1. Report of the differences of global temperature from 1976 to 2023 compared with 1901-2000 average (adapted from NOAA National Centers for Environmental Information).

Concerning the titratable acidity, there are different behaviors regarding the two main acids in grapes. Tartaric acid is not affected by temperature and its quantity remains relatively stable after veraison until berry maturation. However, its concentration decreases by dilution with increase

in berry volume. Reduced content of tartaric acid also occurs under particular condition as well as late harvest or grape berry drying (Plantevin et al., 2024). Meanwhile, accumulation and permanence of malic acid is related to maturity and decreases with high temperature because it is easily respired by the berries, making malic acid more unstable (Ganichot, 2002; Neethling et al., 2012). However, heat stress during grape ripening increases phloem transport, resulting in higher accumulation of K+. The overaccumulation of K+ ions leads to an excessive neutralization of organic acids and an increase of pH. The pH increases with increase in the level of ion exchange. Acid degradation reduces titratable acidity and raises the level of exchange. If the tartaric-to-malic acid ratio increases due to malic acid respiration, the pH may stay stable or may rise if there is concurrent mineral uptake. This loss of acidity strongly affects the final wine quality (Boulton 1980; Mira de Orduña, 2010; Monder et al., 2021).

Considering the effect of temperature on secondary metabolites, the flavonoid composition is affected, including tannins, anthocyanins (on red varieties), and flavonols. These components are fundamental to achieve phenolic maturity and to produce quality red wines, influencing color and gustative perception, especially bitterness and astringency (Adams, 2006). Rise in temperatures implies increased sun exposure and consequentially more ultraviolet-A (UV-A) and UV-B radiations, with a subsequent decrease in flavonoid content of grape berries because of a combination of degradation and synthesis inhibition (Martínez-Lüscher et al., 2014).

Regarding flavonols, high temperature could generate a decrease in their metabolism, depending on heat intensity, duration, and phenological stage (Gouot et al., 2019; Rogiers et al., 2022). However, some studies showed that UV-B radiation has particularly strong effect on

the synthesis of flavonols. The total flavonol concentration in berry skins can increase in grapes exposed to UV-B, while individual flavonol concentration is affected by different ways. With an increase of UV-B, the proportions of mono- and disubstituted flavonols increase, while that of trisubstituted flavonols decrease (Martínez-Lüscher et al., 2014; Matus, 2016). Quercetin is a flavonol that shows the strongest response to UV-B radiation. Some studies proposed that some monovarietal wines could develop a quercetin precipitation during wine aging because of the hydrolysis of aglycon. This excess was attributed to a strong copigmentation effect of flavonols, particularly quercetin having with anthocyanins; this helps to maintain quercetin in solution form even at high concentrations, creating a significant commercial problem for global wine market (Gambuti et al., 2020; Waterhouse et al., 2016).

The responses of phenolic compound to UV are different. Flavonols are the most UV-responsive compounds whereas anthocyanins are hardly affected by them. Different responses of the two groups of compounds are due to different regulation systems that control biosynthesis (Del-Castillo-Alonso et al., 2016). Nevertheless, the heat stress generated by high temperatures impacts the biosynthesis of anthocyanin. Anthocyanins have their optimum synthesis at around 30°C, although berry skins under these conditions show a poor coloration. This is due to a combination of factors, such as changes in gene expressions, enzyme activity, and degradations undergone by anthocyanins to protect berries from extreme heat by acting as antioxidants and reducing color in grapes (Gouot et al., 2019). For tannins, the effects are not clear; however, some studies underlined the effects of vintage on their accumulation, observing that high temperatures lead to an increase in the concentration of tannins (Chira et al., 2011; Gouot et al., 2019; Lorrain et al., 2011).

Rise in temperature has a direct effect on grape varietal aroma compounds. Some primary aroma compounds, belonging to the class of isoprenoids, such as monoterpenes, terpenes, and C-13 norisoprenoids, responsible of relevant fruity, floral, and spicy flavors of wines, are affected in different ways by temperature (Ruiz et al., 2019).

Terpenes are present in all grape varieties; they contribute to the aroma with a usual floral, fruity, and muscatel scent. These are present in the exocarp of grapes and occur in many forms, such as free, volatile, or bound glycosidically; however, they have lower concentration in non-Muscat grape varieties (Mele et al., 2021). Monoterpenes are important for aroma and flavor of grapes, as they impart floral and citrus notes to wines (Ebeler, 2001). Terpenes and monoterpenes need sun exposure for their accumulation, but an excessive increase in temperature causes a decrease of their content, limiting the aromatic potential of produced wines (Belancic et al., 1997). Moreover, high temperatures have different effects on some terpene compounds, for example, linalool is affected and its content is reduced, meanwhile the concentration of geraniol does not change in the berries (Duchêne et al., 2016). On the contrary, C-13 norisoprenoids, which are derived from the degradation of carotenoids, increase with exposure to the sun. They are usually found as glucosides and represent a group of flavors. Typical norisoprenoid aromatic compounds include β-damascenone (megastigma-3,5,8-trien-7-one), vitispirane (6,9-epoxy-3,5(13)-megastigmadiene), and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). Their characteristic aroma varies from leafy, minty, and fruity to various floral hints. Syrah's typical varietal aroma of violet is due to these specific compounds. Exposure of grape bunches to sunlight is a key factor that significantly affect the concentration of norisoprenoids in grapes. Indeed, enhanced light and

temperature conditions can break down carotenoid pigments, thereby increasing C-13 norisoprenoids (Asproudi et al., 2016; Li et al., 2024; Reynolds and Balint, 2014).

Methoxypyrazines are a class of chemical compounds responsible for bell pepper, tomato leaf, and vegetal aromas in wines of certain varieties, such as ‘Cabernet Franc’, ‘Cabernet Sauvignon’, and ‘Merlot’. Methoxypyrazines action depends on climatic conditions and decrease with high temperatures (Falcão et al., 2007; Ruiz et al., 2019). The concentration of varietal thiols in wines is related to the concentration of their precursors in grapes and depends on different factors, such as water deficit and grape variety. However, their concentration is not affected by changes in temperature (Roland et al., 2011).

1.2 Water availability

In last few years, winegrowers and winemakers are facing unexpected changes in terms of water availability. Figure 2 shows the mean annual precipitation over the decade of 2011–2020, expressed as a percentage of the mean of the 1951–2000 reference period. It displays that northern part of Europe and Asia experienced significant above-average rainfall from 2011 to 2020, with precipitation levels of 10–20% higher than the 1951–2000 average precipitation. In addition, an increase in the frequency of extreme meteorological and hydrological factors, such as heavy rainfall and flooding, alternated with long periods of drought, impacted the final wine quality (Piña-Rey et al., 2020). In January 2023 itself, 14 different significant climatic anomalies and events were recorded in globally (Figure 3).

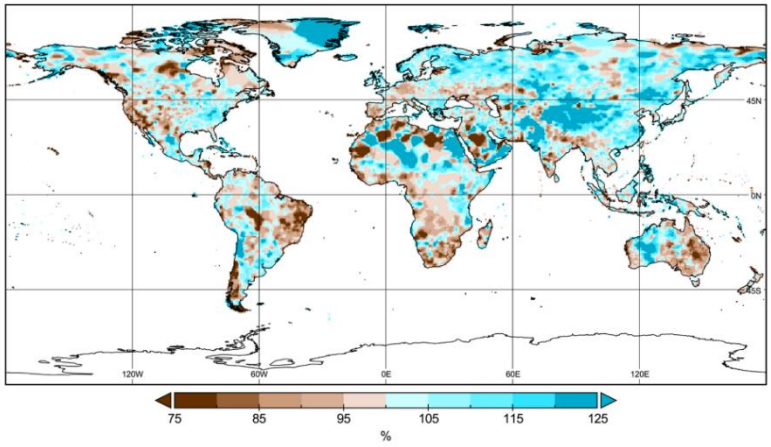

Figure 2. Report of mean precipitation in 2011-2020 decade compared with the 1951-2000 reference period (adapted from World Meteorological Organization WMO).

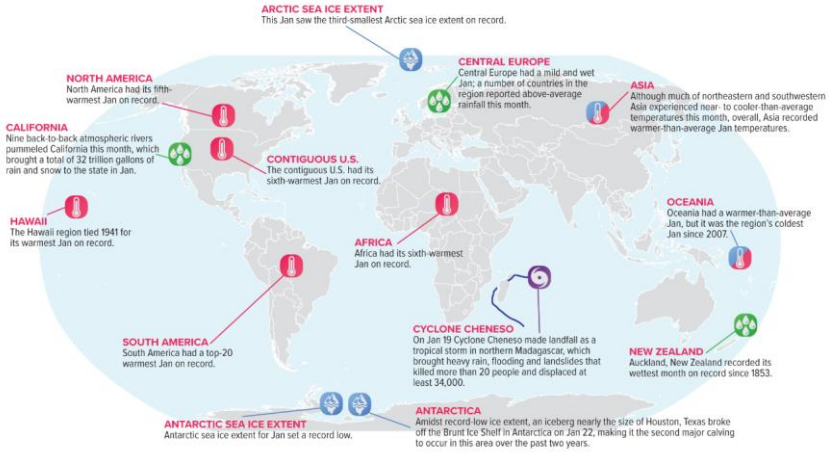

Figure 3. Report of significant climate anomalies and event in January 2023 worldwide (adapted from NOAA's State of the Climate Reports).

As observed for temperature, availability of water also impacted grape composition, influencing the accumulation of both primary and secondary metabolites. Water availability, depending on phenological state, can affect vegetative growth of the plant, on the development, and berry set and its maturation. Flooding, because of extreme and violent rainfall, could

generate hypoxia and/or anoxia, leading to plant oxidative stress that may eventually result in the death of the vine (Rogiers et al., 2022). At the same time, water deficit can impact vine-growing and consequently composition of berries. Different studies have demonstrated that a controlled water deficit helps to improve bunch microclimate, benefitting production of quality wines, although in the prospective of CC, with a drier future, these management techniques need to be revised (Bonada et al., 2015).

Indeed, a controlled water deficit leads to a reduction in berry size, a higher skin-to-pulp ratio, and affects concentration of grape compounds. Moreover, a moderate water deficit during veraison leads to a greater accumulation of sugar, flavanols, flavonoids, and anthocyanidins (Cáceres-Mella et al., 2017; Intrigliolo et al., 2012). The intensity of water stress and the affected vegetative period could have different effects. During pre-veraison stages, it induces metabolic changes in berries and can be maintained up to the harvest. Meanwhile, in post-veraison, the modifications are more variable, with both positive and negative influences. Generally, grapevine response to drought reduces berry weight because of a dehydration effect, and concentrating of sugar and anthocyanin content. At the same time, water stress influences some secondary metabolic pathways, affecting flavor and characteristics of final products (Bonada et al., 2015; Mirás-Avalos et al., 2017). In fact, water availability influences varietal aroma concentration and their precursors. In the case of norisoprenoids, water stress shows an increasing trend (Koundouras et al., 2006). On the other hand, changes in terpenoids, such as in ‘Chardonnay’, seem to be a part of metabolic response, particularly the accumulation of monoterpernes, that is, linalool, nerol, and α-terpineol (Savoi et al., 2016). Concentration of methoxypyrazines is more affected by temperature and exposure to the sun than water availability; however,

their accumulation is higher in highly irrigated vines (Belancic and Agosin, 2007). Concerning thiols, even a light water stress leads to an increase in content, but long periods of drought tend to decrease their concentration (Peyrot des Gachons et al., 2005).

2. Winemaking consequences

2.1 Harvest time

For wine producers, CC has created new challenges because of the modified grape chemical characters. This new scenario has led to the necessity of oenological strategies to obtain quality wines. The first problem that winemakers deal with is the time of harvest. As already described, the main effects of CC on grape composition from the technological point of view are increase in sugar content, decrease of titratable acidity, and consequently a higher pH. Moreover, higher temperature and water stress are able to affect the size of berries, concentrating not only sugar content but also flavonoids.

In addition, CC influences the aromatic composition of grapes; therefore, harvesting of grapes at correct time, with an adequate maturity is the key to produce quality wines (van Leeuwen et al., 2022). For these reasons and according to oenological aim, nowadays the harvest date is anticipated. Particularly in hot vintages, for white wines, it is preferred to choose an early harvest to maintain a lower sugar content and higher acid concentration. In the production of red wine, a good phenolic maturity is preferred. In fact, managing grapes with low amount of anthocyanins or immature tannins is challenging. Often, phenolic and technological maturities do not happen at the same time, so to achieve good phenolic maturity one must tolerate an excessive accumulation of sugars and a

drastic drop in acidity. However, high temperatures affect phenolic maturity, thus reducing accumulation of anthocyanins (Drappier et al., 2017). Hence, winemakers prefer red wines and an earlier harvest date.

Generally, CC affects aroma and their precursor levels, impacting the harvest date. In white wine production, maintenance of higher levels of floral nuances in grapes because of some terpenes, such as linalool, is preferred to harvest when technological maturity is reached, which occurs early if temperature is high. In fact, the concentration of these compounds is moderate prior to verasion, increases during ripening, but decreases with overripening (Costantini et al., 2017). Other classes of aroma compounds, such as methoxypyrazines, are strictly related to grapes maturity and harvest. Allamy et al. (2023) showed that in the case of cv Cabernet Sauvignon wines, delayed harvest date increased cooked fruit notes and induced a decrease of fresh vegetable indications. Moreover, other studies underlined that early harvesting of '*Cabernet Sauvignon'* was marked by fresh fruit and green aromas, while late harvesting resulted in wines with black fruit notes and cooked fruit sensations (van Leeuwen et al., 2022), thus confirming that the aromatic maturity is strictly related to harvest time.

2.2 The effect of high sugar concentration and higher pH

In wine industry, one of the direct consequences of CC is the increased alcohol content of wines. It is estimated that in the past decade, 50% increase in alcohol levels in globally produced wines is related to CC (Jones, 2007); this factor represents a problem not only for technical aspects but also for market trends. Indeed, if a moderate consumption of wine can have beneficial effects on health, higher levels of alcohol

consumption can cause various diseases and injuries; hence, consumers must reduce alcohol beverages (Bucher et al., 2018). From an oenological point of view, ethanol interacts with different wine compounds, thus modifying sensory profile, reducing fruity notes, and amplifying unpleasant notes such as bitterness and astringency (Goldner et al., 2009). During wine production, increased concentration of ethanol may slow down or stop alcoholic fermentation because of its toxic effect on yeasts, and could be a limiting factor for malolactic fermentation (Drappier et al., 2017). Moreover, high sugar accumulation in grape musts leads to yeast cells exposed to high osmotic stress, potentially causing a stuck fermentation (Ishmayana et al., 2011), and thus leading to the production of increased amounts of fermentation secondary products, such as glycerol and acetic acid (Mira de Orduña, 2010).

Climate change during grape ripening has a direct effect on the wine's acidity and thus on the quality of the final product. Increase in pH and the lower content of titratable acidity induces lower biological stability to wine, resulting in more susceptibility to alterations. Particularly during the first stage of alcoholic fermentation, when the amount of ethanol is low, there is a risk of uncontrolled growth of spoilage yeast, such as *Brettanomyces bruxellensis*, which is responsible for off-flavors belonging to the category of volatile phenols (Mira de Orduña, 2010). In addition, increase in pH affects the chemical behavior of different metabolites, including anthocyanins, which are essential for the stability and aging of red wines. At $pH < 3$, the predominant anthocyanin form in solution is flavylium cation, which exhibits red color. However, if $pH \geq 3.7$, the more prevalent form becomes colorless carbinol pseudo bases, reducing the contribution of anthocyanin to red wine color (Brouillard and Dubois, 1977). Moreover, when in their flavylium form, anthocyanins can either

associate with each other or interact with other organic compounds, primarily flavonoids and phenolic acids, to form co-pigments. These copigments typically contribute to blue-purple tones in red wines. Consequently, at higher pH levels, there is a lower concentration of anthocyanins in their flavylium cation form available for copigmentation (Forino et al., 2020).

Furthermore, increase in pH impacts the activity of sulfur dioxide (SO_2). It is well known that SO_2 is a strong antioxidant and important antimicrobial agent used as a preservative in wines. A large proportion of SO_2 is bound to carbonyl compounds. The so-called free SO_2 in wine is predominantly in the form of bisulphite ions (HSO_3^-) and only a small proportion is present as a molecular SO_2. Therefore, the chemical equilibrium of the two species depends on the wine pH, and with increasing pH, the molecular SO_2 fraction decreases, thus reducing the antiseptic activity (Divol et al., 2012; Giacosa et al., 2019). SO_2 also acts as an antioxidant by reacting with hydrogen peroxide, derived by oxidation of polyphenols in wine and by reducing the quinones back to their phenol form. Moreover, SO_2 in sulfurous acid form combines with acetaldehyde to form aldehyde sulfurous acid, competing with hydrogen peroxide to prevent the formation of aldehyde (Boulton et al., 1996; Yildirim and Darici, 2020). Finally, the pH is able to influence the hydrolysis rate of acetate esters and the equilibrium kinetics of ethyl esters of fatty acids. Indeed, these compounds influence the fruity character of young wines. However, during storage, the esters tend to hydrolyze, causing a reduction in some fresh aroma of wine. This behavior is accelerated by low pH and higher temperature. Accordingly, rise in pH due to the effects of CC leads to a greater ester stability and preservation of fruity aroma in wines; nevertheless, this effect must be assessed in the context of overall balance

of wines, also considering the risks associated with microbiological and oxidative stability at higher pH levels (Makhotkina and Kilmartin, 2012; Pérez-Coello et al., 2003; Ramey and Ough, 1980).

2.3 Techniques to reduce or remove alcohol content in wine

Nowadays, to confront the main effects of CC in winemaking, one of the most challenging objectives is the production of wine with reduced or removed alcohol content. Precisely, different strategies have developed that are categorized depending on the vinification time of application. These strategies are divided as pre-fermentative, fermentative, and post-fermentative techniques.

Pre-fermentative techniques

The reasons that have led to an increase in the concentration of sugars in musts are to be discovered for improving vineyard management practices, as for many years it has been attempted to increase the concentration of grapes in primary and secondary metabolites (Smart et al., 1990); however, CC has contributed to exacerbating the effects. The first fundamental choice that an oenologist faces is related to harvest time. In the case of grapes for producing white wines, opting for an early harvest can lead to satisfactory results; however, it is necessary to implement early ripening controls and adopt adequate organizational strategies (Varela et al., 2015).

The advancing of harvest in the case of red grapes for the production of red wines is not always practicable because the content of polyphenols and aromas may not have reached the maximum potential. In particular, in grapes characterized by high levels of tannins contained in

the skins or seeds, the advancing of harvest appears to be impractical because of sensory imbalances that could be generated in wines (van Leeuwen et al., 2022).

Dilution is the easiest way to reduce alcohol content. Water addition in grapes reduces sugar content, but in general, has a negative impact on other parameters, such as reduced acidity, color, and phenolic compounds (Martínez-Moreno et al., 2023). Some studies showed that decreasing the final ethanol content through water addition could increase the fruity notes of wines, producing a fresher product. However, in most wine-producing countries, the practice of grape must or wine dilution is either forbidden or strictly limited and regulated by competent authorities (Harbertson et al. 2009; Varela et al., 2015). International Organization of Vine and Wine (OIV) admit water addition in winemaking only for aromatized wines and wine-based beverages (Resolution OIV-OENO 439-2012, 2012). The only case where water could be reintroduced is the practice of reducing sugar content in musts through membrane coupling (Resolution OIV-OENO 450B-2012, 2012). The water and organic acids filtered by nanofiltration process are reintroduced into the treated must. However, the OIV has no specific guidelines for adding water for technical purposes, such as incorporating permitted additives or processing aids; for this, every country has the responsibility to regulate legislative aspects.

Another strategy that does not foresee special equipment or additional costs is the blending of wines. For this, wines obtained from early-harvest–low-sugar grapes are blended with wines from higher-sugar grapes, obtaining a final product with reduced ethanol content. Blending wines from grapes of different maturity stages is a good method to obtain a quality product with lower alcohol content and improved color, mouthfeel, and flavor perception (Martínez-Moreno et al., 2023).

Moreover, this procedure that requires important volumes of low-alcohol wines reduces pH without impairing other characteristics of the final product (Kontoudakis et al., 2011). Unfortunately, blending of wines is not always permitted. As in the case of dilution, this technique also depends on every state's rules and regulated by state's competent authorities.

Removal of sugar with nanofiltration is another technique to reduce ethanol content in wines. It consists of passing a fraction of grape must into a membrane under a pressure gradient to separate permeate (with a low amount of sugar) and retentate (with a higher content of sugar). At the end of filtration, the two parts are mixed in specific portions to obtain a must with desired characteristics (Varela et al., 2015). Studies on the application of nanofiltration for both red and white musts showed that the final wines obtained after fermentation by a mix of original must and a portion of the must had a lower content of ethanol. However, a significant reduction of flavor and color was detected (García-Martín et al., 2010).

Similar to nanofiltration, the reverse osmosis technique is applied as well to lower sugar contents before alcoholic fermentation. Reverse osmosis is a separation technique based on the application of high pressures (60–80 bar) for purification of water systems. Instead, if a pressure more than osmotic pressure is applied to the system, then water, ethanol, and other small molecules are forced through a semi-permeable membrane, leaving behind the rest of compounds and allowing isolation and removal (Afonso et al., 2024; Sam et al., 2021b; Török, 2023). Mira et al. (2017) used reverse osmosis on different varieties of grape juices to obtain permeate (with low sugars) and retentate (with high sugars), which were then mixed in different proportions to achieve the final wine with alcohol reduction of up to 5% v/v. However, these wines had a decreased color intensity, anthocyanin content, and phenols. Finally, in a pre-

fermentative stage, the enzyme glucose oxidase obtained from the fungus *Aspergillus niger* is used to reduce the content of glucose in grape juices. The enzyme first converts glucose into D-glucono-lactone, producing hydrogen peroxide, and then it catalyzes the conversion of D-glucono-lactone to gluconic acid (Sam et al., 2021b; Varela et al., 2015). Functioning of the enzyme leads to a lower amount of ethanol, although the production of gluconic acid decreases pH and increases total acidity. The sensory perception also is modified, with a lower intensity of fruity flavors (Röcker et al., 2016).

The research community continuously develops new approaches and technologies to produce high-quality wines with a lower alcohol content. Martínez-Pérez et al. (2020) studied the use of high-power ultrasounds to produce quality red wines, starting from slightly less ripe grapes, hence recouping the limited extractability with an enhanced extraction technique. High-power ultrasounds typically operate at frequencies of 20–40 kHz. Acoustic cavitation phenomena are induced, forming bubbles that implode quickly. Plant or microorganism cells in the media are affected by this phenomena, as their cell walls are severely damaged leading to cell death and release of its contents in the media. In enology, this technique was applied on crushed grapes, with reduced sugar content, to facilitate the production of highly colored wines with lower amount of alcohol. The obtained wines, compared to control, had similar color characteristics, and the aroma compounds were judged positively during the sensory analysis.

Fermentative techniques

The fermentation process is considered to reduce ethanol content during wine production. *Saccharomyces cerevisiae* is considered as the most

efficient yeast species to convert glucose into ethanol during winemaking, also considering its alcohol and stress tolerance. In recent years, a new approach comprising research and isolation of new *S. cerevisiae* strains presenting lower ethanol yield, or mixed fermentation with non-*Saccharomyces* yeasts, is able to produce less alcohol and convert carbon metabolism to other pathways, thus developing metabolites without compromising sensory quality of wines (Rolle et al., 2018; Varela et al., 2015).

The developing of low-alcohol *S. cerevisiae* strains is supported by metabolic engineering. Varela et al. (2015) modified two strains, and were able to decrease ethanol content from 15.6% to 13.2% v/v in the first strain, and from 15.6% to 12.0% v/v in the second one. However, both strains enhanced the production of glycerol, acetaldehyde, and acetoin, affecting negatively the resulting wines. Difficulties in using genetically modified microorganisms due to consumer opposition are well known, but authors have also underlined a negative impact of modified strains on wine's flavors (Heux et al., 2006; Sam et al., 2021b; Tilloy et al., 2015).

Regarding the use of non-*Saccharomyces* yeasts in association with *S. cerevisiae* strains, the species most studied are *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, and *Starmerella bacillaris*. *Metschnikowia pulcherrima* is an indigenous yeast with a low fermentative power, and is able to increase the release of varietal aroma compounds because of high enzymatic capacity. Moreover, under aerobic conditions, its respiratory metabolism helps to reduce ethanol content (Morata et al., 2019). A study conducted by Contreras et al. (2014) showed the utilization of *M. pulcherrima* with *S. cerevisiae.* This combination led to a reduction of alcohol content from 0.9% to 1.6% v/v, compared to a control inoculated by *S. cerevisiae* strain only. Similar results were obtained by

Varela et al. (2017). The use of *M. pulcherrima* with *S. cerevisiae* produced wines with a lower amount of ethanol (–1.0% v/v) and higher concentration of ethyl acetate, total esters, and total higher alcohols, affecting positively the sensory profile of wine. Regarding *Torulaspora delbrueckii*, it has a capacity to produce low content of acetic acid, release polysaccharides and mannoproteins, increase mouthfeel perception, and is able to increase the quantity of esters, thiols, and terpenes (Azzolini et al., 2012; Benito, 2018), leading to positive sensory traits. Additionally, the use of *T. delbrueckii*, in combined fermentations with *S. cerevisiae*, showed lower accumulation of alcohol (from -0.45% to -0.52% v/v, compared to control) without compromising the sensory quality (Azzolini et al., 2012; Belda et al., 2017).

Finally, numerous studies were conducted on *Starmerella bacillaris*, for its fructophilic character or the ability to grow at high concentrations of sugar and low temperature, and to produce a high content of glycerol and a low amount of acetic acid and acetaldehyde. In addition, *S. bacillaris* is resistant to ethanol toxicity, surviving until the end of alcoholic fermentation (Englezos et al., 2015; Rantsiou et al., 2012). Mixed fermentations of *S. bacillaris* and *S. cerevisiae* influence the process, producing wines with increased volatile compounds and glycerol, as reported previously, but with a lower level of ethanol (Binati et al., 2020; Englezos et al., 2019). Further, some *S. bacillaris* strains increase total acidity (Englezos et al., 2019), thus influencing organoleptic perceptions.

Post-fermentative techniques

Alcohol content in wines can be reduced or removed at the end of alcoholic fermentation through physical methods, such as membrane processes,

extraction processes, and thermal distillation.

Besides the application of pre-fermentative techniques, some membrane-based techniques, such as nanofiltration and reverse osmosis, are applied directly on wine. Several studies have underlined the effectiveness of nanofiltration and reverse osmosis for both alcohol reduction and dealcoholization (Afonso et al., 2024; Sam et al., 2021b) (Figure 4).

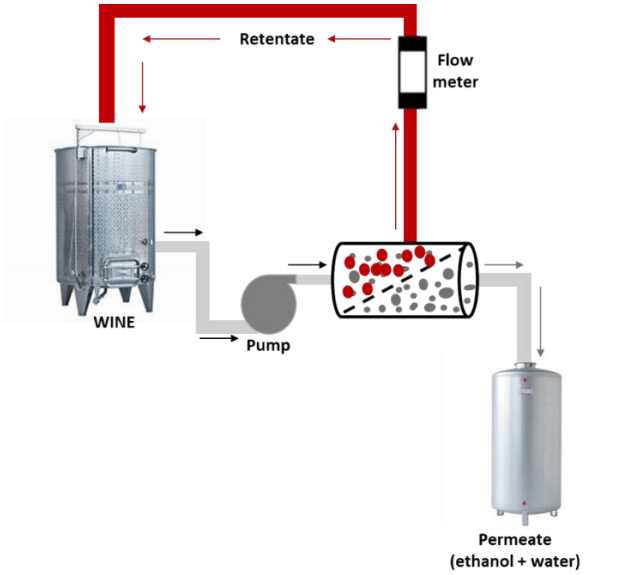

Figure 4. Scheme of reverse osmosis to remove alcohol from wine.

Gonçalves et al. (2013) showed that, similar to the application on grape juice, nanofiltration decreases polyphenols and reduces total and volatile acidity because of a higher passage of acetate ions. Reverse osmosis applied on wines reduces the content of anthocyanins, caused by membrane adsorption, and produces an alteration to wine's body and texture, particularly in red wines, because of the concentration of tannins (Török, 2023).

Osmotic distillation (or evaporative perstraction) is a separation process applies to reduce alcohol in wines. This technology is based on membranes that separate two aqueous phases: wine, containing volatile compounds, and water, used as a stripping liquid. These phases circulate in the opposite direction of a hydrophobic hollow fiber membrane module, guided by the vapor pressure of volatile solute in wine and stripping liquid. Ethanol first evaporates due to increased temperature; then, ethanol vapors diffuse through membrane pores, and finally exits from membrane pores and condenses in water media (Afonso et al., 2024; Sam et al., 2021a) (Figure 5).

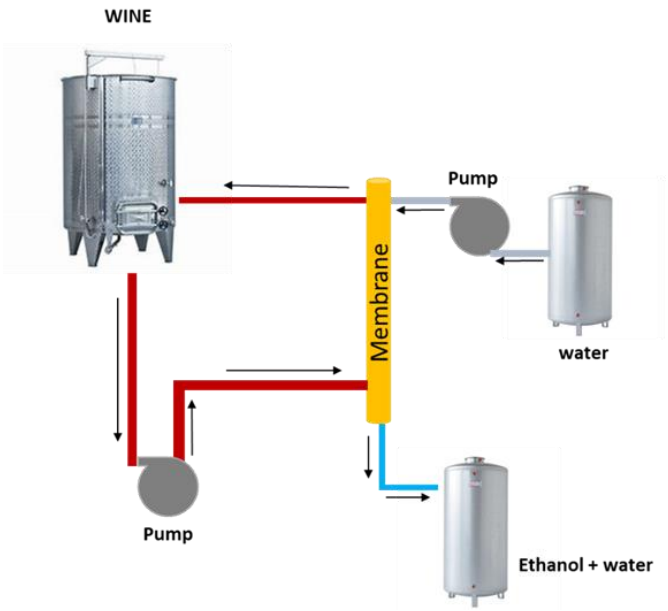

Figure 5. Scheme of osmotic distillation.

Osmotic distillation reduces alcohol content and has a low subtractive impact on wine's final composition, preserving aroma compounds and color as well as phenolic compounds without sharp modification in the quality of wine (Corona et al., 2019; Liguori et al., 2012).

Another membrane separation technique used to reduce alcohol content in wine is pervaporation, also called vapor permeation. Based on the principle of partial evaporation, it separates components from liquid mixtures using dense and non-porous membranes (Afonso et al., 2024). The separation relies on differences in the transport rate of individual components. Substances crossing the membrane change from liquid phase to vapor phase, desorbing from the other side pressured through vacuum stress (Sun et al., 2020; Takács et al., 2007). Studies on pervaporation achieved good results for producing quality wines. This process is able to separate phenolics, residual sugars, and aroma components from ethanol, obtaining alcohol-free or low-concentration wines (Afonso et al., 2024; Sun et al., 2020). In addition, this process has low energy consumption and operates at low temperatures, with more efficiency than other dealcoholization or traditional distillation methods (Sam et al., 2021b).

Decrease in the ethanol concentration of wine is also accomplished by extraction methods by using gasses. Compression of a gas under specific conditions and above its critical point transforms it in a supercritical fluid, which is able to extract organic compounds, such as ethanol. In the winemaking industry, CO_2 is used due to its characteristics, such as no toxicity and low critical temperature (31°C). In its liquid state CO_2 in wine has an affinity with ethanol's carbon chain that facilitates its dissolution, however if CO_2 has a transition back into a gaseous state it carries dissolved ethanol, reducing the wine alcohol content (Afonso et al., 2024; Schmidtke et al., 2021). This technique has the disadvantage of decimating aroma together with ethanol. However, studies conducted by Ruiz-Rodríguez et al. (2010, 2012) demonstrated that the application of supercritical CO_2 extraction is an attractive process because it does not remove or denature water, salts, proteins, and carbohydrates. Furthermore,

this process does not modify the antioxidant power and aromatic profile of wines with reduced alcohol content. Some trials showed that supercritical CO_2 extraction is employed to recover aroma compounds, and ethanol from raffinate is separated in a subsequent distillation column. Finally, alcohol-free wine is produced by mixing extracted aroma compounds into the product of distillation. Differently, ethanol and aroma can be removed in the first step of distillation, and sequentially aroma compounds are extracted from distillate by supercritical CO_2 and recycled to the bottom through distillation to have a no-alcohol product (Ruiz-Rodriguez et al., 2012).

Vacuum distillation and spinning cone column are two thermal distillation methods applied in the wine industry to partially or completely remove alcohol from wines. Vacuum distillation separates ethanol from wine through evaporation. The process is performed at low temperatures, generally between 15°C and 20°C, under vacuum conditions. The operating conditions allow separating alcohol as vapors and then to condense it into a liquid form, producing a distillate with extracted ethanol (Gómez-Plaza et al., 1999; Motta et al., 2017). Vacuum distillation can maintain high concentration of flavonoids, organic acid, and anthocyanins, and can increase total acidity. On the contrary, this technology affects the sensory profile of wines, particularly floral and fruity sensations. The final product results in the depletion of volatile compounds (Gómez-Plaza et al., 1999; Sam et al., 2021a).

Spinning cone column is one of the most common methods to remove alcohol, and is mainly used in the beverage and winemaking industry. It is based on a vertical rotative column, formed by stacked cones, which operate under vacuum and at low temperature to change volatile compounds into gaseous phase. The extraction takes place in two steps: in

the first step, conducted at 26–28°C under reduced pressure (about 0.04 bar), aromatic compounds are extracted. In the second step, ethanol is extracted at high pressure and temperature (38°C). At the end of the process, a recovering system is used for the volatile compounds removed in the first step to reconstitute the final aroma of wine (Belisario-Sánchez et al., 2009; Zamora, 2016) (Figure 6).

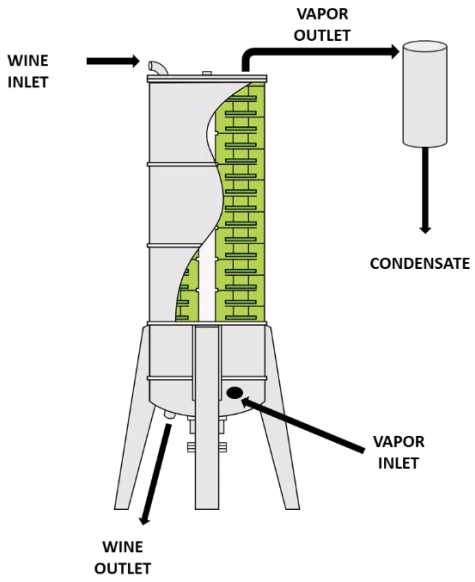

Figure 6. Scheme of a spinning cone columns system

Studies conducted on the use of spinning cone column underlined its low aggressivity to remove or reduce alcohol content in wines. In fact, phenolic compounds, anthocyanins, and flavonols have a low increasing trend due to concentration. In addition, beneficial compounds, such as resveratrol, with antioxidant activity increased after the application of this technique (Belisario-Sánchez et al., 2009). Nevertheless, an important usage of this technique is that it can be paired with adsorbent materials for removing

ash and smoke taint from wines produced from grapes exposed to bushfire smoke; increasing occurrence of wildfires represent another effect of CC. In some regions, the phenomena of wildfires has become more relevant in the last few years, an issue reflected in wine production (Mirabelli-Montan et al., 2021; Puglisi et al., 2022).

3. Conclusions

The current situation that the wine community confronts due to CC has forced producers to implement strategies to reduce alcohol content in wines. Choice of the approach to achieve results is made primarily considering the aims, effectiveness, and sustainability of the process. Therefore, it is essential to adopt innovative and environment-friendly techniques, such as low-alcohol yeasts, optimized management of grape ripening, and usage of more efficient winemaking methods. In addition, the research and development of grape varieties more resistant to high temperatures and drought is crucial. Collaboration between wine producers and wine researchers is essential to find and develop effective and sustainable solutions. This is the only way to guarantee the quality of wine without compromising the ecosystem and well-being of future generations while ensuring that winemaking traditions can adapt and thrive in a changing environmental context.

Author Contibutions

Both authors contributed equally to this paper.

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Conflicts of Interest

The authors declared no conflicts of interest.

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Chapter 2

Cold liquid stabulation: Impact on the phenolic, antioxidant and aroma characteristics of wines from aroma-neutral white grape varieties

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Abstract

Cold liquid stabulation aims to extract valuable compounds from grape lees before juice clarification. In this study, 7, 14, and 21 days of lees contact were tested on aroma-neutral ‘Arneis’ and ‘Cortese’ grape juices vs control. Basic parameters, colour, polyphenols, antioxidant capacity, and volatile organic compounds were assessed throughout winemaking. Wine sensory analysis was performed. The produced wines did not differ in terms of colour and show limited differences in polyphenols, not influencing astringency and bitterness. Variety and treatment length influenced free and glycosylated volatile organic compounds. Free terpenes increased in the 21-day treated ‘Arneis’ wine (+67 %). Lower free esters in ‘Arneis’ with 14 days of stabulation were found (− 10 %). On the contrary, higher values of individual esters were found in 14 and 21-day treated ‘Cortese’ wines, but these showed lower free C6 (−12%) and sulphur compounds (− 23 % and − 24 %, respectively), and higher overall wine quality with respect to non-stabulated wine.

Keywords: Pre-fermentative techniques, *maceration sur bourbes*, volatile organic compounds, Arneis, Cortese, sensory analysis

1. Introduction

Wine primary and secondary metabolites are affected by climate, region, grape variety, ripeness, viticultural and oenological practices (Rienth et al., 2021). Different winemaking techniques help to improve the wine quality modifying the quantity and composition of these compounds (Selli, Canbas, Cabaroglu, Erten and Günata, 2006a).

In white wine production, pre-fermentative maceration on skins, under controlled conditions, low temperature as cold maceration, and usually with the help of exogenous enzymes, improves the extraction of volatile organic compounds (VOCs), as well as some hydrosoluble phenolics (Aleixandre-Tudo et al., 2015; Bestulić et al., 2022; Malićanin et al., 2022; Wang et al., 2016). This aspect results in increased floral and fruity attributes, and in more balanced, round, and full-bodied wines. Alternatively, wine aging in presence of fine lees, produced after alcoholic fermentation, has a significant effect on wine mouthfeel, due to lees being rich in tartaric salts, amino acids, fatty acids, vitamins, and compounds released through yeast autolysis such as mannoproteins, β-glucans, and lipids (Fornairon-Bonnefond et al., 2002).

However, the potentialities of the grape solids corresponding to the grape flesh (*bourbes*) are still scarcely investigated. Usually, this fraction is removed with the juice clarification treatments (e.g. cold settling), applied after pressing and before alcoholic fermentation, and discarded as a winemaking by-product. Only a minor part is kept into juices to increase their lipidic content for yeast growth during fermentation (Casalta, Cervi, Salmon, & Sablayrollers, 2013; Guittin et al., 2021). This solid residue is composed mainly of polysaccharides (70%), lipids (8%), and in minor part

of minerals, pectin, and nitrogen compounds (2.5%), and lastly of phenolic compounds (Alexandre, Nguyen van Long, Feuillat, & Charpentier, 1994). Nevertheless, this solid part can influence the wine production process, through a technique called *maceration sur bourbes* or cold liquid stabulation (CLS). In brief, CLS consists, after grape pressing, in maintaining the grape juice on its lees, kept in suspended condition, at a low temperature (0-8 °C) for a variable period (2-26 days). The expected effect of this technique is: *i)* a higher extraction of substances from flesh particles during must-lees contact, mainly VOC precursors such as terpenes, norisoprenoids, and thiols (Philipp, Jagschitz, Langman, Riegelnegg, & Suchel, 2022; Philipp, Eder, Sari, Korntheuer, & Eder, 2024), and *ii)* an increased content of nutrients for the fermentation development.

The presence of lipids and nitrogen compounds can impact the fermentation progress, being also a source of fermentative VOCs (Casalta *et al*., 2013; Guittin et al., 2021), whereas polysaccharides may influence the mouthfeel, as well as phenolic compounds could in turn affect astringency, bitterness, and colour of the produced wines (Hornedo-Ortega, Reyes González-Centeno, Chira, Jourdes, & Teissedre, 2020). Nevertheless, grape solids may enhance herbaceous notes by increasing the presence of C6 alcohols and aldehydes extracted from skins, or increasing enzymes activities such as polyphenoloxidase or esterase, as well as unwanted microbiological contamination and the risk of pesticide residues into wines due to prolonged skin contact (Casalta et al., 2013).

Few studies on the use of CLS and its impact on finished wines in terms of basic, phenolic, and aroma characteristics are available, showing that in some aromatic varieties, *i.e*. ‘Traminer’ or ‘Sauvignon blanc’, a CLS of 7 days can increase significantly the free monoterpene

concentration when compared to a non-stabulated control, or can have a greater impact on the production of thiols, such as 3-mercaptohexanol (3MH). (Philipp et al., 2022; 2024). Cravero, Bonello, Chiusano, Tamborra, & Savino (2012) showed that CLS application on the Italian autochthonous cultivar 'Bombino bianco' resulted in wine with higher golden yellow hue with respect to the control. Among the available studies, to our knowledge no information on the effect of a CLS length greater than 7 days has been published.

The aim of this study was to evaluate the changes in the volatile composition of stabulated grape juices and wines obtained with different CLS lengths, namely 7, 14 and 21 days, and compared to a non-stabulated control, as well as the influence of these changes on sensory profile of resulting wines. At the same time, the assessment of phenolic, colour and the antioxidant characteristics was done. CLS was applied in the winemaking of two *Vitis vinifera* L. varieties, 'Cortese' and 'Arneis'. These varieties are used to produce relevant volumes of white wine in the northern Italy landscape and are involved in several monovarietal Protected Designation of Origin (PDO) wines, mostly from the Piemonte region (Carlin et al., 2022). These two grape varieties are classified neutral in terms of VOCs (Piano et al., 2014; Piergiovanni et al., 2023), therefore an increase in their aroma precursors from the grape and an improvement in fermentative aroma could strongly influence the final wine sensory characteristics.

2. Materials and Methods

2.1 Winemaking

Approximately 300 kg of grapes *Vitis vinifera* L. cv. 'Arneis' (total soluble solids 23.4±0.3 °Brix; total acidity as g/L of tartaric acid 5.9±0.1, pH 3.20±0.02) and 300 kg of grape 'Cortese' (total soluble solids 22.0±0.3 °Brix; total acidity as g/L of tartaric acid 5.1±0.1, pH 3.30±0.01) were hand-harvested on the 25th of August and 22nd of September 2022, respectively. Once arrived in the experimental cellar of University of Torino '*Bonafous*' in Chieri (Italy), the intact grapes were stored into a thermo-controlled room at 0 °C for 12 h. The grapes were then destemmed and crushed in a TEMA destemmer–crusher (Enoveneta, Piazzola Sul Brenta, Italy) and pressed using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy), with a pressure program consisting in three cycles with growing pressure (0.6, 0.8, and 1 bar, respectively, for a total of 15 minutes of pressing for each variety). On the obtained juice, 50 mg/L of SO_2 (potassium metabisulphite, Alea Evolution S.R.L., Molinella, Italy) and 2 g/hL of pectolytic enzyme (Lallzyme cuvée blanc, Lallemand Inc., Montreal, Canada) were added. The enzymatic preparation contains polygalacturonase (≥ 13,000 U/g) and β-glucosidase (≥ 12 U/g) activities, as reported by the manufacturer. The resulting juice was then divided in twelve 15-liter glass canisters for each grape variety. Four process lengths were tested in triplicate: 0 (control, no CLS), 7, 14, and 21 days. The control samples, after pressing, undergo a cold static clarification process for 24 h at 0 °C in a temperature-controlled room. Afterwards, they were racked and inoculated for the alcoholic fermentation. The treated samples, instead, during the whole period of CLS were kept in a controlled room at 4 °C with the lees manually suspended twice a day by using a food-grade plastic stirrer. At the end of CLS, a 24-h cold static clarification, in a

controlled room at 0 °C, was carried out before racking. The same turbidity value was reached, expressed as nephelometric turbidity units (NTU), for all tests (target of 220 NTU) by adding their respective lees. For the alcoholic fermentation, *Saccharomyces cerevisiae* active dry yeast (Fermol Chardonnay, AEB Group, Brescia, Italy) at 20 g/hL dose were added, following the rehydration procedure from the manufacturer instructions, standardized for all the treatments. Two additions of diammonium phosphate (Agrovin, Ciudad Real, Spain) were done: 50 g/hL were added at the beginning of alcoholic fermentation, and 25 g/hL at one-third of the fermentation. The fermentation was kept at a controlled temperature (18±1 °C), with a daily monitoring of the sugar consumption. At the end of the fermentation, each sample was racked to remove lees and, 50 mg/L of SO_2 were added. One month later, the samples were cold stabilised for two weeks in a controlled room at 0 °C and then bottled.

During the experimental procedure, samples were obtained after pressing (juice), after cold liquid stabulation (PS), after alcoholic fermentation (PAF), and one month after bottling (PWI). For each stage, the samples obtained following 7, 14, and 21 days of CLS are indicated as AR07, AR14, AR21 for 'Arneis' and as CO07, CO14, and CO21 for 'Cortese', respectively.

2.2 Chemicals and standards

All chemicals of analytical reagent grade, gallic acid, Folin-Ciocalteu reagent, (−)-epicatechin 92.0%, gallic acid monohydrate 99.0%, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97.0% (Trolox), disodium phosphate, and HPLC-gradient grade solvents were supplied by Sigma-

Aldrich (St Louis, MO, USA). For HPLC analysis, glucose, fructose, and malic, tartaric, citric, succinic, and acetic acids, ethanol and glycerol (purity > 98.0%) and lactic acid (purity 90.0%) were purchased from VWR International (Milan, Italy). Deionized water was produced by a Milli-Q system (Merck Millipore, Darmstadt, Germany). For the analysis of VOCs, ethanol 99.8%, 1-heptanol 98.0%, 2-octanol 98%, HPLC-grade methanol (MeOH) 99.9%, anhydrous sodium sulphate 99.0%, and anhydrous sodium phosphate dibasic 99.0% were purchased from Sigma-Aldrich. Dichloromethane and citric acid 99.5% were obtained from Carlo Erba (Rodano, MI, Italy).

2.3 Physical-chemical analysis of 'Arneis' and 'Cortese' grapes, musts and wines

Samples were collected at different times: grape samples, juice after pressing, after cold liquid stabulation (PS), after alcoholic fermentation (PAF), and one month after bottling (PWI).

2.3.1 Basic parameters

Total soluble solids content (°Brix) was analysed through a refractometer Atago palette 0-32 °Brix with automatic temperature compensation (Atago Corporation, Tokyo, Japan). The pH was measured by potentiometry using an Inolab 730 calibrated pHmeter (WTW, Weilheim, Germany) according to the OIV-MA-AS313-15 method (OIV, 2016). The total acidity was determined by titrimetry following the OIV-MA-AS313-01 method (OIV, 2016). The organic acids, i.e. malic, tartaric, lactic, citric, succinic, and acetic acid, ethanol and glycerol were quantified by HPLC (Agilent 1260,

Agilent Technologies, Santa Clara, USA) with a UV detector set to 210 nm and a refractive index detector (Giordano, Rolle, Zeppa, & Gerbi, 2009). Turbidity was determined using a turbidimeter (TB1, Velp Scientifica, Usmate, Italy) following the OIV-MA-AS2-08 method and expressed as NTU.

2.3.2 Spectrophometric measurements

The phenolic composition and colour parameters were evaluated using a UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). Total phenolic index (TPI) was determined by measuring absorbance at 280 nm of the sample diluted (1:10) in deionized water, and expressed in mg/L of (−)-epicatechin by an external calibration curve (Scalzini et al., 2020).

The CIELab parameters were evaluated according to the OIV-MA-AS2-11 method, namely lightness (L^*), red/green (a^*), and yellow/blue (b^*) colour coordinates (OIV, 2016). The sample spectrum in the region 370-700 nm was recorded using 10 mm pathway plastic cuvette, and the CIELab values were calculated as reported in OIV-MA-AS2-11 (OIV, 2016). These values were converted to RGB values for visualization purposes. The total colour difference (ΔE^*) between one CLS-treated sample and the respective control was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (OIV, 2016). Total sulphur dioxide (total SO_2) was determined using an Hyperlab Smart automatic analyser (Steroglass, San Marino in Campo, Italy) through a colorimetric method based on the reaction between total sulphur dioxide and a disulfide chromogenic compound with an absorption maximum at 416 nm (Total SO_2 Kit, SQPE060413, Steroglass). With the same instrument, through enzymatic analysis, acetaldehyde was determined (Acetaldehyde Kit, SQPE059576,

Steroglass). The antioxidant potential was investigated on the samples (either juice or wine) diluted 1:10 with deionized water, following the Brand-Williams, Cuvelier, & Berset (1995) method, modified by Romanet et al. (2019). Briefly, 100 µL of diluted sample were added with 2 mL of DPPH solution (25 mg/L) prepared daily. The DPPH radical was dissolved in a solution composed of methanol and buffer (60:40 v/v). The buffer solution contained 0.1 M citric acid and 0.2 M disodium phosphate, and the pH value was adjusted to 3.6. The absorbance of the samples and the reaction blanks (prepared by replacing the sample with water) was measured at 515 nm absorbance after 240 minutes. The results (sample absorbance – blank absorbance) were converted as mmol Trolox equivalents/L using a Trolox-based calibration curve. On wine (PWI samples), the total phenolic content was determined additionally by the Folin-Ciocalteu method (FC) after purification on 1-g Sep-Pak C18 solid phase extraction cartridge (Waters Corporation, Milford, MA, USA). After 70 minutes of reaction, the resulting absorbance was measured at 750 nm and the results were expressed as mg/L of gallic acid through an external calibration curve (OIV-MA-AS2-10 method; OIV, 2016; Scalzini et al., 2020). Total polysaccharides were evaluated in PWI samples, after reaction through a spectrophotometric analysis, measuring the absorbance at 490 nm, according to Marassi et al. (2021) method. The final results were expressed as mg/L of glucose using an external calibration curve.

2.4 Free and glycosylated volatile organic compounds axtraction and determination in 'Arneis' and 'Cortese' musts and wines.

Free and glycosylated volatile organic compounds (VOCs) were investigated for both varieties on the freshly-pressed juice and at two different times during the winemaking process, i.e. after the stabulation (PS) and at the end of alcoholic fermentation (PAF). Moreover, free VOCs were determined one month after bottling (PWI).

2.4.1 Extraction of free and glycosylated volatile organic compounds (VOCs)

The VOCs extraction from juices and wines was performed as described by Giacosa et al. (2019). Briefly, a 50 mL-aliquot of sample was diluted with 100 mL of deionized water and 0.5 mL of 1-heptanol (60 mg/L in 10% v/v absolute ethanol) were added as internal standard. Then, samples were loaded onto a 5-g Sep-Pak C18 cartridge (Waters Corporation, Milford, MA, USA), previously activated with methanol and washed with deionized water. Free volatile organic compounds were eluted with 30 mL of dichloromethane. The free fraction was dried over anhydrous sodium sulphate and then concentrated to 50 µL under a stream of nitrogen for the direct injection.

Glycosylated compounds were subsequently eluted with 25 mL of methanol and the eluate was evaporated to dryness using a vacuum rotavapor (Buchi R-210, Flawil, Switzerland) set to 30-35 °C. The glycosylated fraction was dissolved in 10 mL of 0.2 M citrate-phosphate buffer at pH 5 and enzymatic hydrolysis was performed with 50 mg of glycosidase enzyme (Rapidase Revelation Aroma, Corimpex, Romans

d'Isonzo, Italy) and incubation at 40 °C for 21 hours. After the hydrolysis, two internal standards, 0.5 mL of 1-heptanol and 0.5 mL of 2-octanol (60 mg/L in 10% v/v absolute ethanol for each one), were added. Finally, the glycosylated precursors were recovered with dichloromethane, following the SPE method previously described, and the glycosylated fraction was then dried over anhydrous sodium sulphate and concentrated to 50 µL under a stream of nitrogen for injection.

2.4.2 Determination of free and glycosylated volatile organic compounds

GC/MS analysis was performed through a GC Agilent 7890A (Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler Gerstel MultiPurpose Sampler MPS 2 (Gerstel GmbH & Co., Mülheim an der Ruhr, Germany) and a DB-WAX capillary column (30 m × 0.25 mm × 0.5 µm, Agilent Technologies, Santa Clara, CA, USA). Injections of 1 µL were performed in split mode (split ratio 0.9:1) setting the injector temperature to 250 °C. The carrier gas (He) flow rate was 1 mL/min. The VOCs' detection was carried out by the MS Agilent 5975C (Agilent Technologies, Santa Clara, CA, USA) system, using a positive ionization energy of 70 eV and the acquisition range of 30-350 m/z. The elaboration of GS/MS data was performed by the Software Agilent G1701EA MSD Productivity ChemStation. Where applicable, the identification of volatile organic compounds was confirmed by comparison with the mass spectra of their respective standards, retention indices (Table S1) calculated for each volatile compound using a C7-C30 n-alkanes certified reference material (Sigma-Aldrich, Milan, Italy) or MS data reported in literature and NIST database (www.webbook.nist.gov/chemistry). Semi-

quantitative data were obtained by measuring the relative peak area of each identified compound in relation to that of the added internal standard (µg/L of 1-heptanol).

2.5 Sensory analysis of 'Arneis' and 'Cortese' wines

The sensory analysis has been organised in two phases: the preliminary training step and the formal tasting sessions of the wine samples that were conducted in two different vinification points, i.e. at the end of alcoholic fermentation (PAF) and one month after bottling (PWI).

2.5.1 Training sessions

The training sessions consisted of seven half-hour sessions over four weeks. Twelve judges (7 women, 5 men) were selected among university personnel already involved in previous white wine evaluations following the same procedure and able to sensory recognize the selected attributes in water. The panel was further trained on tastes, mouthfeel perceptions, and aromas in white wines, as well as at the use of the scale for this study. Ethical permission, to conduct a human sensory study, was granted by University of Torino Ethics Committee (protocol number 0194129). The participants acknowledge an informed consent statement to participate in the study prior to the sensory sessions. They were informed that they would participate in the sensory survey about wine production, all data will be de-identified and only reported in the aggregate, they were able to withdraw from the survey at any time without giving a reason and that the products tested were safe for consumption.

The sensory descriptors were selected on the basis of the most cited attributes found in literature among neutral varietal white wines (Campo,

Do, Ferreira, & Valentin, 2008; Fracassetti, Camoni, Montresor, Bodon, & Limbo, 2020) and on 'Arneis' and 'Cortese' wines (Piano et al., 2014). In training, the identification technique (for aroma, taste, and tactile perceptions), ordination task, and unstructured line scale tools (for tastes and tactile perceptions) were adopted on selected aroma descriptors belonging to fruity (pineapple, lime, lemon, grapefruit, green apple, peach, pear, banana), floral (rose, jasmine), and complex (almond and honey) perception classes. The selected reference standards (Table S2) were dissolved in commercial white wine (Caviro, Faenza, Italy). For taste and mouthfeel (bitterness, astringency, acidity, and body), the 1st session was dedicated for the identification of the stimuli in white wines, the 2nd and 3rd to ordination task, the 4th and 5th for scale alignment training. Furthermore, in all sessions (1-7), the aroma training was performed, firstly asking assessors to associate the aromatic stimuli to a descriptor from a given list (1-5), and then without the list (6-7). At the end of each session, the judges were asked to discuss with the panel leader the results and to assess the standard sample again if needed. Before formal session, two sensory sessions were performed with the same tasting sheets and with three neutral varietal wines to assess the panel performance.

2.5.2 Formal sessions

In the official wine sessions, general descriptive analysis (DA) with 10-cm unstructured line scale and Check-All-That-Apply (CATA) methods were adopted for in-mouth descriptors (bitterness, astringency, acidity, and body) and aroma descriptors, respectively (Lawless & Heymann, 2010; Valentin, Chollet, Lelièvre, & Abdi, 2012). A 10-cm unstructured scale was used also for rating the sample overall wine quality. Twenty mL of

wine were poured in three-digit randomly coded standard ISO 3591 glasses (International Organization for Standardization, 1977) covered with a petri dish and served at room temperature (18 °C). Mineral water and unsalted crackers were provided as cleanser between samples.

A monadic samples evaluation at PAF (equal volume mixed of the three replications, 12 judges, obtaining 12 answers, for each variety) was proposed to the panel according to different fermentation ends that were related to the CLS time. The formal sample's evaluation of the final wines was performed one months after bottling for each variety separately accordingly to the harvest date (formal session date: 14th December 2022 and 25th January 2023, for 'Arneis' and 'Cortese', respectively). All the samples produced for each variety were tested in one session (4 treatments in duplicate for each variety, panel of 10 and 7 judges for 'Arneis' and 'Cortese', respectively, obtaining a total of 20 and 14 answers).

2.6 Statistical analysis

Statistical analyses were performed using R statistic software (R Foundation for Statistical Computing, Vienna, Austria). For physical-chemical and VOCs analysis, for each variable, one-way analysis of variance (ANOVA), with Tukey HSD post hoc test, was used to evaluate the significant differences (p <0.05) among treatments. In case of heteroscedasticity, the ANOVA with Welch's correction was used, followed by Games Howell test as post-hoc.

For the sensory analysis and multivariate elaboration, the data analysis was performed using FactoMineR (Lê, Josse, & Husson, 2008a) and SensoMineR (Lê & Husson, 2008b) packages. In the bottled wine tasting, the panel performance was evaluated for CATA tasks through the

reproducibility index (*Ri*) proposed by Campo et al. (2008). The panel performance was evaluated for each sample set ('Arneis' and 'Cortese', *Ri*=0.37 and *Ri*=0.48, respectively), respecting the repeatability requirement (*Ri* > 0.20). For the aroma frequencies from the CATA questionnaire, Correspondence Analysis (CA) was performed, and significant attributes were assessed with Cochran's Q test (Varela & Ares, 2012). For DA's panel evaluation, SensoMineR package was used to assess the agreement and repeatability of the panel, with a three-way ANOVA ("replicates" * "sample" * "judge" as fixed factors and their interaction). The performance was considered adequate when there were no significant differences ($p > 0.05$) in "judges" * "replicates" and "judges" * "sample" interactions, and panel consensus was monitored by principal component analysis (PCA) for each attribute. Samples significant differences were then evaluated by three-way ANOVA with sample and replicates as fixed effect and judges as random effect. For descriptors with significant differences ($p < 0.05$) Tukey HSD was applied.

PCA with free VOCs detected in bottled wines and sensory aroma descriptors (with citation frequency greater than 20%) was performed. VOCs data was standardized as *z-scores* within each variety to minimize the varietal effects. Aroma descriptors were projected as supplementary quantitative variables after standardization in the same way.

3. Results and discussion

3.1 Impact of CLS treatment on physical-chemical parameters

Physical-chemical parameters of 'Arneis' and 'Cortese', after CLS, alcoholic fermentation, and one month of bottling are shown in the Tables 1 and 2, respectively. For 'Arneis', the results showed that after the CLS

all the samples undergo a reduction of the total acidity down to –8% at AR21 with respect to the control. The same trend was followed by pH values at AR21, although the variation was minimal (0.02 pH units). As well, in ‘Cortese’ CLS samples both total acidity and pH values decreased (–6% and –1% at CO21, respectively, compared to control). The low temperature in both varieties led to a significant decrease of tartaric acid contents, that was evident already after 7 days of CLS, due to tartaric salts precipitation, with the consequence of a reduced acidity. It has been reported that pre-fermentative techniques involving the skin contact can influence the pH of the must, increasing the release of cations from the skin cell wall, and therefore increasing the pH values (Aleixandre-Tudo, et al., 2015). Nevertheless, this depends on the varieties and conditions applied (Aleixandre-Tudo et al., 2015; Wang et al., 2016; Alti-Palacios, Martínez, Teixeira, Câmara, & Perestrelo, 2023). In the case of CLS, this phenomenon was not observed. In both varieties, ethanol, glycerol, and other organic acids have been investigated but no changes at the end of CLS were found in their contents with respect to control under our experimental conditions, with the exception of a slight decrease of malic acid in ‘Arneis’ throughout CLS. Overall, these results demonstrate that no microbiological activity affected the juices and no relevant spontaneous fermentations occurred.

After 7 days of CLS, the total polyphenolic index (TPI) decreased in both ‘Cortese’ and ‘Arneis’. In ‘Arneis’, the lowest TPI value was reached at AR14 (–17%), but at AR21 the index grew up. Indeed, in ‘Cortese’ the value of TPI decreased with increasing CLS length (down to –6% at CO14 with respect to control). Accordingly, the antioxidant capacity (DPPH) decreased with the CLS, but no significant differences were found among different CLS length for ‘Arneis’ samples. Although

polyphenols are well-correlated with the antioxidant capacity of must and wines, other sulphur-containing compounds, such as glutathione and cysteine, can influence the antioxidant capacity (Romanet et al., 2019). In ‘Cortese’ samples, the antioxidant capacity strongly decreased after 21 days of CLS, which was in line with the TPI value, possibly due to grape flesh cell wall material adsorption of polyphenolic compounds, in particular tannins with a higher molecular mass (Bindon, Smith, & Kennedy, 2010). Previously, ‘Cortese’ skins were found to be richer in condensed tannins and they were of higher molar mass with respect to ‘Arneis’ ones (Guaita, Motta, Messina, Casini, & Bosso, 2023). In contrast, ‘Arneis’ grapes have been reported to contain up to two-folds the content of flavonols and hydroxycinnamic acids compared to ‘Cortese’ ones (Ferrandino, Carra, Rolle, Schneider, & Schubert, 2012). These polyphenols are oxidized faster than condensed tannins, probably leading to a decrease in DPPH already at AR07 in ‘Arneis’, whereas ‘Cortese’ could have been affected by both oxidation and adsorption of condensed tannins in a time-dependant manner. During prolonged maceration, secondary oxidation reactions, involving o-quinones (yellow-brown colour), can also occur giving colourless or less yellowish pigments, or producing polymer pigments that precipitate (Gómez-Míguez et al., 2007; Carbone & Fiordiponti, 2016). This could be connected also to the fact that the juice resulted clearer (higher L^*) and with a lower yellow hue (b^*) as long as the CLS length increased in ‘Cortese’ except for CO21, whereas in ‘Arneis’ it was already significantly clearer at AR07 (+13%) and less yellow (–23%) and then remained steady.

At PAF, for ‘Arneis’ wines total acidity values were still lower in AR07 and AR14 (but not significantly for AR21) than control due to the yeast contribution in the production of other organic acids. In the case of

AR21 samples, higher contents of succinic acid were found with respect to other CLS samples while those of tartaric acid decreased according to CLS length. For ‘Cortese’ wines, total acidity and tartaric acid content were lower in stabulated samples, compared to the control, independently on CLS length. At PWI, in both varieties total acidity values were significantly higher in AR21 for ‘Arneis’ than control, AR07, and AR14 samples (p <0.01) and for ‘Cortese’ only than control (p <0.05). This may be linked to a higher stability of tartaric acid salts already achieved with the CLS. At PAF, in CLS samples, an increase of acetic acid content was found with respect to control (achieving 0.23 g/L in ‘Cortese’ and 0.11 g/L in ‘Arneis’, both at 21 days CLS samples) and it remained unchanged also in bottled wines (PWI) for both the varieties.

‘Arneis’ wines at PAF, TPI tends to be higher as the CLS length increases, although it does significantly only for AR21 when compared to control. Furthermore, no significant differences were found in the antioxidant capacity. In bottled wines, AR14 and AR21 samples had higher values of TPI and antioxidant capacity, up to +7% and +6%, respectively, with respect to control. In contrast with ‘Arneis’, for ‘Cortese’ wines at PAF, TPI decreased (p <0.001), especially in the CO21 sample (–12% with respect to control). The same applies for the antioxidant capacity (–18% at CO21). For TPI in bottled wines, the values in wines produced after 14 and 21-day CLS were significantly lower with respect to the other samples, although no significant differences were observed in the antioxidant capacity. In line, also Folin-Ciocalteu results for ‘Cortese’ CO14 wines were significantly lower than control (p<0.01). Those differences are probably due to the grape phenolic composition (Motta, Guaita, Petrozziello, Panero, & Bosso, 2014; Guaita et al., 2023): the stabilization of ‘Arneis’ wines, being the grapes richer in

hydroxycinnamoyl tartrates (HCTAs), flavonols, and monomeric flavanols than Cortese ones (Ferrandino et al., 2012), was faster through CLS by the oxidation and removal of these phenolic compounds with racking. In fact, the TPI values are relatively less variable from juice to PWI for 'Cortese' (decrease of 11-18%) throughout winemaking with respect to 'Arneis', the latter showing a decrease of 35-40% from juice to PWI.

The trends of L^*, a^*, and b^* with the increase of the stabulation length at PAF changed with respect to PS. Thereby, CLS 'Arneis' samples at PAF had lower L^* values, while significant differences were not found in 'Cortese'. The a^* and b^* values, contrary to what happens after the CLS, increased with 7 days of treatment in 'Arneis' with respect to control. The same applies for the bottled wines but the differences were significant at longer CLS. In particular, 'Arneis' had a higher b^* value, and therefore more intense yellow hue in AR21 wines after bottling. In 'Cortese' stabulated samples at PWI, L^* value was lower ($p<0.05$) only in CO07, where also a higher b^* was reported with respect to the other samples. The opposite behavior of b^* in the two varieties agrees with the differences found in TPI values. ΔE^* highlights that just at the end of CLS all 'Arneis' samples showed a visually perceived different colour with respect to the control ($\Delta E^*>6$). However, the colours of AR14 and AR21 at PAF ($\Delta E^*=3.2$ and 2.9, respectively) and AR21 at PWI ($\Delta E^*=2.3$) were also markedly different compared to the control, while in contrast AR07 had a minimum difference ($\Delta E^* < 2$). A ΔE^* of 2.3 was reported as a minimum value to clearly discriminate the colour of white wines in a glass (Saenz-Gamasa et al., 2009), and therefore from instrumental data no potential sensory impact of CLS was found at PWI, except for 'Arneis' wines with 21 days of CLS. Similarly, the colour differences among 'Cortese'

treatments were perceivable in juice after CLS, but they become negligible after alcoholic fermentation and bottling ($0.2<\Delta E^*<0.8$). During skin-contact prefermentative treatments, the extraction of water-soluble phenolic compounds occurs, which can be also more easily oxidized giving yellow pigments (Gómez-Míguez et al., 2007; Carbone et al., 2016). Nevertheless, the CLS treatment applied in this study allowed to obtain wines whose colour cannot be easily differentiated from the control probably due to fast oxidative processes, adsorption on fine lees, and also polymerization and precipitation.

Table 1. Chemical-physical parameters of Arneis Juice after cold liquid stabulation (PS), after alcoholic fermentation (PAF), and one month after bottling (PWI).

		Arneis															
		PS					PAF					PWI					
Parameter	Juice	AR-Control	AR07	AR14	AR21	*Sign*	AR-Control	AR07	AR14	AR21	*Sign.*	AR-Control	AR07	AR14	AR21	*Sign*
Sugars (g/L)	246±0	248±2 a	246±1 ab	246±1 a	243±1 b	**	8±3 a	<1 b	<1 b	<1 b	**	<1	<1	<1	<1	ns
Total acidity (g/L of tartaric acid)	3.8±0.0	3.6±0.1 a	3.4±0.0 b	3.4±0.0 b	3.3±0.0 b	**	6.3±0.1 a	6.0±0.0 b	5.9±0.0 b	6.2±0.2 ab	**	5.2±0.0 b	5.1±0.1 b	5.3±0.1 b	5.6±0.2 a	**
pH	3.20±0.05	3.18±0.01ab	3.19±0.00 a	3.18±0.00 b	3.16±0.00 c	***	3.20±0.00 a	3.18±0.01 ab	3.16±0.01 b	3.22±0.02 a	**	3.01±0.01bc	3.02±0.01 b	2.99±0.00 c	3.05±0.01 a	***
Tartaric acid (g/L)	4.74±0.05	4.40±0.03 a	4.08±0.02 b	3.98±0.04 c	3.98±0.02 c	***	4.35±0.02 a	4.11±0.03 b	4.10±0.02bc	3.99±0.06 c	***	2.60±0.04	2.56±0.01	2.63±0.05	2.65±0.16	ns
Malic acid (g/L)	0.59±0.00	0.63±0.03 a	0.59±0.01 ab	0.56±0.01 b	0.60±0.01 ab	**	0.77±0.02 b	0.79±0.03 b	0.74±0.00 b	0.94±0.02 a	***	0.73±0.02 b	0.82±0.04 b	0.76±0.02 b	0.93±0.05 a	**
Lactic acid (g/L)	nd	nd	nd	nd	nd		0.22±0.01 b	0.23±0.01 ab	0.25±0.01 a	0.22±0.01 b	*	0.19±0.01	0.19±0.01	0.20±0.01	0.18±0.01	ns
Citric acid (g/L)	nd	nd	nd	nd	nd		0.11±0.01 a	0.10±0.01 ab	0.06±0.00 c	0.09±0.00 b	***	0.13±0.00	0.13± 0.01	0.09±0.04	0.12±0.00	ns
Succinic acid (g/L)	nd	nd	nd	nd	nd		1.23±0.02 ab	1.15±0.03 c	1.19±0.03bc	1.26±0.01 a	**	1.15±0.02ab	1.09±0.03 c	1.13±0.03 bc	1.20±0.01 a	**
Acetic acid (g/L)	nd	nd	nd	nd	nd		0.07±0.00 b	0.08±0.00 b	0.10±0.01 a	0.11±0.00 a	***	0.07±0.00 b	0.08±0.00 b	0.10±0.01 a	0.11±0.00 a	***
Glycerol (g/L)	nd	nd	nd	nd	nd		8.11±0.07 a	7.79±0.01 c	8.01±0.02ab	7.94±0.05 b	***	8.11±0.08 a	7. 84±0.04 b	8.09±0.00 a	8.06±0.04 a	**
Ethanol (%Vol)	nd	nd	nd	nd	nd		15.03±0.02bc	15.13±0.03 a	15.00±0.04c	15.09±0.03ab	**	15.05±0.06	15.08±0.08	14.97±0.05	15.04±0.07	ns
Acetaldehyde (mg/L)	0±0	0±0 b	1±0 a	1±0 a	1±0 a	***	16±0	16±1	16±0	18±2	ns	19±1	18±1	18±0	20±2	ns
Total sulfur dioxide (mg/L)	38±1	36±1	36±1	35±1	34±1	ns	31±1 a	31±0 a	24±0 c	27±1 b	***	71±1	71±1	68±3	69±1	ns
TPI (A.u.)	10.1±0.0	9.8±0.1 a	8.3±0.1 bc	8.2±0.0 c	8.4±0.1 b	***	6.4±0.0 b	6.5±0.2 b	6.7±0.1 ab	7.0±0.1 a	**	6.2±0.0 c	6.1±0.0 c	6.4±0.0 b	6.6±0.1 a	***
EC (mg/L)	789±3	764±5 a	647±4 bc	636±0 c	653±7 b	***	496±3 b	506±14 b	519±10 ab	543±9 a	**	482±3 c	477±2 c	499±1 b	518±6 a	***
DPPH (mmol Trolox/L)	2.00±0.08	1.96±0.02 a	1.83±0.03 b	1.80±0.03 b	1.79±0.01 b	***	1.05±0.01	1.02±0.06	1.13±0.05	1.07±0.04	ns	1.30±0.01bc	1.28±0.01 c	1.34±0.01 ab	1.38±0.03 a	**
FC (mg/L of gallic acid)		-	-	-	-		-	-	-	-		143	174	173	167	ns
L^*	83.03±0.29	76.46±0.27c	86.02±0.38 a	85.29±0.26ab	84.06±0.89 b	***	98.16±0.12 a	97.47±0.30 b	96.82±0.02c	97.30±0.15bc	***	98.98±0.01a	98.83±0.08a	98.25±0.07 b	98.09±0.46 b	**
a^*	2.41±0.11	3.91±0.11 a	1.44±0.07 b	1.58±0.29 b	1.87±0.28 b	***	-1.43±0.01 c	-1.19±0.07 b	-0.75±0.11 a	-0.91±0.05 a	***	-0.85±0.03 b	-0.80±0.03 b	-0.65±0.04 a	-0.77±0.03 b	**
b^*	29.39±0.24	33.79±0.13a	26.01±0.23 b	24.88±0.01 c	25.21±0.60 bc	***	8.28±0.22 b	9.86±1.28 ab	11.06±0.84a	11.05±0.85 a	*	5.51±0.10 b	5.56±0.10 b	6.60±0.09 ab	7.63±1.06 a	**
Color and ΔE*			12.6	12.8	11.6			1.7	3.2	2.9			0.2	1.3	2.3	
Total polysaccharides (mg/L glucose)			-	-	-		-	-	-	-		319±21	302±28	305±23	364±50	ns

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences between different samples, for each variety and sampling point, according to Tukey's test. nd indicate not detected. TPI (A.u.): total phenolic index (in absorbance units), EC: (-)-epicatechin, DPPH: Antioxidant capacity, FC: Folin–Ciocalteu index, *L**: lightness; *a**: red/green color coordinate; *b**: yellow/blue color coordinate, Δ*E**: total color difference *vs* control. Colour was acquired by spectrophotometry, expressed in CIEL*a*b* coordinates and then converted into RGB values for visualization. "AR- Control", "AR07", "AR14" and "AR21" indicate the non stabulated and the three treatment periods, respectively 7, 14 and 21 days for 'Arneis' at the different vinification stages.

Table 2. Chemical-pysical parameters of Cortese juice after cold liquid stabulation (PS), after alcoholic fermentation (PAF), and one month after bottling (PWI).

	Cortese																	
		PS					PAF					PWI						
Parameter	Juice	CO-Control	CO07	CO14	CO21	*Sign.*	CO-Control	CO07	CO14	CO21	*Sign.*	CO-Control	CO07	CO14	CO21	*Sign.*
Sugars (g/L)	229±1	229±1	230±0	230±0	230±0	ns	<1	<1	<1	<1	ns	<1	<1	<1	<1	ns
Total acidity (g/L of tartaric acid)	3.9±0.0	3.6±0.0	3.1±0.0	3.2±0.7	3.4±0.0	ns	6.9±0.1 a	6.4±0.1 b	6.3±0.0 b	6.4±0.0 b	***	5.7±0.1 b	5.7±0.0 ab	5.8±0.1 ab	5.9±0.0 a	*
pH	3.22±0.00	3.19±0.00 a	3.17±0.00 b	3.16±0.00 c	3.16±0.00 c	ns	3.25±0.00 a	3.19±0.01 b	3.12±0.00 c	3.10±0.01 c	***	3.02±0.01 a	2.99±0.01 b	2.99±0.00 b	2.98±0.02 b	*
Tartaric acid (g/L)	4.72±0.01	4.55±0.02 a	3.72±0.02 c	3.83±0.04 b	3.58±0.00 d	***	4.49±0.02 a	4.00±0.01 b	3.85±0.06 c	3.82±0.01 c	***	2.56±0.03 b	2.64±0.01 ab	2.67±0.02 ab	2.72±0.08 a	**
Malic acid (g/L)	0.71±0.03	0.71±0.03	0.68±0.02	0.67±0.01	0.67±0.04	ns	0.68±0.01 a	0.66±0.00 b	0.57±0.01 c	0.54±0.01 d	***	0.68±0.01 a	0.58±0.02 b	0.53±0.00 b	0.54±0.04 b	***
Lactic acid (g/L)	nd	nd	nd	nd	nd		0.24±0.01 b	0.26±0.00 a	0.25±0.01 a	0.24±0.00 ab	**	0.24±0.01 b	0.26±0.00 a	0.26±0.00 a	0.25±0.01 a	**
Citric acid (g/L)	nd	nd	nd	nd	nd		0.18±0.00 a	0.18±0.00 ab	0.18±0.00 a	0.17±0.00 b	**	0.23±0.00 b	0.26±0.01 a	0.23±0.00 b	0.22±0.01 b	**
Succinic acid (g/L)	nd	nd	nd	nd	nd		1.29±0.01 a	1.18±0.02 b	1.16±0.01 bc	1.14±0.01 c	***	1.25±0.01 a	1.15±0.03 b	1.13±0.01 b	1.12±0.04 b	***
Acetic acid (g/L)	nd	nd	nd	nd	nd		0.15±0.01 c	0.21±0.01 a	0.17±0.00 b	0.23±0.01 a	***	0.15±0.01 c	0.22±0.01 a	0.18±0.01 b	0.23±0.02 a	***
Glycerol (g/L)	nd	nd	nd	nd	nd		8.89±0.11 a	8.90±0.05 a	8.69±0.06 b	8.80±0.03 ab	*	8.95±0.10 a	8.97±0.07 a	8.73±0.05 b	8.93±0.10 ab	*
Ethanol (%Vol)	nd	nd	nd	nd	nd		13.95±0.10	13.98±0.04	14.04±0.03	14.03±0.05	ns	14.03±0.05	14.06±0.02	14.05±0.02	14.06±0.04	ns
Acetaldehyde (mg/L)	1±0	1±0 b	1±0 b	2±0 a	2±0 a	***	18±1 a	18±1 a	15±1 b	17±1 ab	**	22±2	21±1	20±2	20±2	ns
Total sulfur dioxide (mg/L)	36±1	31±1 a	31±2 ab	32±1 a	28±1 b	*	33±1 a	28±1 b	29±1 b	22±1 c	***	66±1 a	64±1 ab	64±1 ab	60±1 b	*
TPI (A.u. x dil)	7.1±0.0	6.5±0.1 a	6.3±0.0 b	6.1±0.0 c	6.2±0.0 bc	***	6.6±0.1 a	6.4±0.1 a	6.0±0.0 b	5.8±0.0 c	***	6.3±0.1 a	6.2±0.1 a	5.9±0.0 b	5.8±0.0 b	***
EC (mg/L)	556±1	508±6 a	493±3 b	478±4 c	482±2 bc	***	512±5 a	502±5 a	470±3 b	451±3 c	***	492±6 a	486±4 a	462±2 b	452±1 b	***
DPPH (mmol Trolox/L)	1.56±0.01	1.35±0.01 a	1.21±0.02 b	1.35±0.01 a	1.12±0.01 c	***	0.95±0.03 a	0.91±0.02 a	0.90±0.01 a	0.78±0.01 b	***	1.99±0.02	1.95±0.01	1.96±0.02	1.91±0.05	ns
FC (mg/L of gallic acid)	-	-	-	-	-		-	-	-	-		157 ab	147 bc	135 c	166 a	**
L^*	90.28±0.55	93.34±0.56 c	95.68±0.24 b	97.63±0.08 a	97.48±0.21 a	***	98.26±0.04	98.16±0.11	98.13±0.38	98.26±0.06	ns	98.84±0.03 a	98.52±0.14 b	98.68±0.10 ab	98.57±0.14 ab	*
a^*	0.04±0.12	-0.47±0.07 a	-0.65±0.01 b	-0.83±0.01 c	-0.62±0.02 b	***	-1.11±0.05 b	-0.87±0.03 a	-0.89±0.11 a	-0.87±0.03 a	**	-0.53±0.05 c	-0.31±0.00 a	-0.42±0.02 b	-0.44±0.03 b	***
b^*	16.85±0.41	13.39±0.38 a	9.89±0.23 b	8.07±0.07 c	9.37±0.27 b	***	6.32±0.09 b	6.70±0.04 a	6.00±0.21 b	5.51±0.05 c	***	4.92±0.09 b	5.13±0.03 a	4.83±0.05 b	4.81±0.05 b	***
Color and ΔE^*			4.2	6.8	5.8			0.5	0.4	0.8			0.4	0.2	0.3	
Total polysaccharides (mg/L glucose)		-	-	-	-		-	-	-	-		315±48	299±75	370±83	338±19	ns

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences between different samples, for each variety and sampling point, according to Tukey's test. nd indicate not detected. TPI (A.u.): total phenolic index (in absorbance units), EC: (-)-epicatechin, DPPH: Antioxidant capacity, FC: Folin–Ciocalteu index, *L**: lightness; *a**: red/green color coordinate; *b**: yellow/blue color coordinate, Δ*E**: total color difference *vs* control. Colour was acquired by spectrophotometry, expressed in CIEL*a*b* coordinates and then converted into RGB values for visualization. "AR- Control", "AR07", "AR14" and "AR21" indicate the non stabulated and the three treatment periods, respectively 7, 14 and 21 days for 'Arneis' at the different vinification stages.

3.2 Impact of CLS treatment on free and glycosylated volatile organic compounds

Glycosylated VOCs were determined in the just pressed juice used for this study. From the aromatic point of view, both 'Arneis' and 'Cortese' are considered neutral grape varieties, although the analysis of grape juices' VOCs showed differences in their glycosylated volatile compositions (Table S3). The concentration of terpenes in 'Cortese' must was almost double than the one in 'Arneis' (467 *vs* 273 µg/L), and it was remarkably higher in volatile phenols (1145 *vs* 703 µg/L). Instead, the juice obtained from 'Arneis' grapes had more than twice glycosylated norisoprenoids (530 *vs* 196 µg/L). Nevertheless, the different flavour of their wines is due to both the varietal characteristics and the formation of fermentative volatile organic compounds. Free VOCs (Figures 1 and 2, for 'Arneis' and 'Cortese', respectively) and their glycosylated precursors (Figure 3) have been investigated in different steps of the winemaking process with the aim of evaluating their evolution and possible correlation with the sensory analysis.

3.2.1 Free and glycosylated volatile organic compounds at the end of CLS

After the CLS treatment, 44 free VOCs (Table S4) and 53 glycosylated VOCs (Table S5) were identified in the juice. In 'Arneis' juice, significant differences were found in the total free ester content, although these compounds were present as expected before alcoholic fermentation in limited quantity in both varieties (0-3.26 µg/L for 'Arneis' and 8.29-19.53 µg/L for 'Cortese'). The same applies for total free higher alcohol content (59.81-82.15 µg/L in 'Arneis' and 228.48-268.26 µg/L in 'Cortese'), with 2-phenylethanol representing the most abundant compound, particularly for 'Cortese'. The free fraction content of 2-phenylethanol was not significantly different among the treated samples for each variety while that of the glycosylated form decreased significantly for 'Arneis' juice with respect to control. Other free and glycosylated higher alcohols such as 2-ethyl-1-hexanol, 1-octanol, and 1-octen-3-ol decreased with CLS in 'Arneis', and the first two also in 'Cortese' only as glycosylated precursors at CO14 and CO21. Therefore, a significant decrease in the total content of glycosylated higher alcohols was observed for CLS treated 'Arneis' juices.

Generally, free volatile acids were significantly lower in all treated samples for 'Arneis' ($p<0.01$, –36-44% with respect to control) whereas they were not affected in 'Cortese'. Phenomena of adsorption have been found between fatty acids and macromolecules deriving from skin contact in Chardonnay variety (Ferreira, Fernández, Gracia, & Cacho, 1995). Except for free dodecanoic acid whose content increased significantly ($p<0.001$) in CLS-treated 'Arneis' juices, the other individual compounds followed a decreasing trend. A lower concentration after CLS treatment was found also on glycosylated volatile acids for 'Arneis' ($p<0.01$, –18-

27% with respect to control), whereas in ‘Cortese’ there was an increase up to two-times in CO14 and CO21 (p<0.001). It was reported previously an increase in bound fatty acids as consequence of skin maceration (15 °C, 24 h) in cv. ‘Narince’ (Selli et al., 2006a).

For free sulphur compounds, a decrease in the total content with CLS was found in both Arneis (down to –84%, p<0.01), with benzothiaziole decreasing significantly in ‘Arneis’, while also methionol showed a significant decrease in CO14 and CO21 for ‘Cortese’ juice compared to control.

‘Arneis’ and ‘Cortese’ free C6-compounds were not significantly affected by the CLS with the exception of a decrease in 2-hexenal for ‘Cortese’ (p<0.01, –24-55% with respect to control). It has been reported that skin contact increases the release of C6-compounds (Selli et al., 2006a). Nevertheless, the lower temperature applied with respect to skin maceration may have limited the activity of lipoxygenase (LOX) involved in lipid oxidation that causes the formation of C6-compounds (Costantini, Bellincontro, De Santis, Botondi, & Mencarelli, 2006). Furthermore, losses of C6-compounds and of their precursors can occur by adsorption on macromolecules and skin components (Ferreira et al., 1995). A decrease (–9-15%) was found for ‘Arneis’ with the lowest content detected at AR14 in the glycosylated fraction, in particular related to 1-hexanol. In ‘Cortese’, C6 compounds did not show significant differences in the glycosylated fraction.

Concerning varietal compounds (Figures 1 and 2), the only free VOCs’ class significantly affected by CLS in both the varieties under evaluation were volatile phenols. Increasing CLS length, their concentrations in ‘Arneis’ increased (p<0.001) with the highest value achieved in AR21 sample (up to 5-fold the control content), whereas the

differences in ‘Cortese’ between control and CLS treated samples were independent of the treatment length ($p<0.01$, +58-80%). On both the varieties, free 4-vinylphenol, 4-vinylguayacol, and syringol increased significantly with all CLS lengths tested. In ‘Arneis’, the content of glycosylated volatile phenols decreased in different extent with the CLS treatment, oppositely to the free ones. The most relevant compound was 4-vinylguaicol, in terms of quantity and decrease, followed by 4-vinylphenol, even though the differences with respect to control were only significant for the former ($p<0.05$, –11-42%). Glycosylated 3,4,5-trimethoxyphenol and *p*-cresol also decreased significantly with the CLS treatment. The reduction is not in line with the free VOC release, therefore some differences related to adsorption and extraction may have occurred. The opposite behavior was found in ‘Cortese’, with the highest content found of glycosylated 4-vinylphenol, 4-vinylguayacol, phenol, syringol, and 3,4,5-trimethoxyphenol at CO14 and CO21 ($p<0.05$, up to 37% higher than control and CO07 samples for total glycosylated volatile phenols). This highlights a varietal effect of the CLS technique, strongly related to the varietal VOCs profile and content. In other studies, prolonged contact with grape solid parts (skin contact) also caused increased quantities of free 4-vinylphenol and decreased ones of both 4-vinylphenol and 4-vinylguaicol in cv. ‘Narince’ (Selli et al., 2006a).

As regards free terpenes, in ‘Cortese’ juices the highest content was found in CO14 and CO21 samples mainly due to the increase of linalool, whereas free terpenes were not detected in ‘Arneis’. Terpenes are influenced by several factors that occur during the winemaking process, including the extraction from grape skin as well as the hydrolysis of the bound precursors that can be favoured by some pre-fermentative techniques, such as the presence of enzymes. Pre-fermentative treatments

influence the total concentration of terpenes, but different trends can be observed depending on the grape variety and also vintage (Alti-Palacios et al., 2023). Anyway, the increase observed in ‘Cortese’ stabulated juices agrees with the results previously reported for free monoterpenes in ‘Traminer’ grape must after 7 days of stabulation at 2 °C (Philipp et al., 2022). More compounds were found as bound fraction (8 terpenes), with higher total concentration in ‘Cortese’ than ‘Arneis’. In the latter, the CLS treatment led to a significant decrease of total bound terpenes ($p<0.001$, –9-21%) as occurred for geranic acid, whereas in ‘Cortese’ the concentration increased at CO14 with respect to control ($p<0.001$, +16%) in agreement with most of individual glycosylated terpenes, in particular 8-hydroxylinalool. Several studies reported a decrease of different extent on free and bound terpenes during skin contact (Selli, Canbas, Cabaroglu, Erten, & Günata, 2006b; Aleixandre-Tudo et al., 2015; Alti-Palacios et al., 2023).

In contrast, ‘Arneis’ showed higher contents of bound norisoprenoids with respect to ‘Cortese’, which decreased significantly down to –23% in the first variety by the treatment ($p<0.001$, –18-23%). Among them, 3-oxo-α-ionol decreased in all the CLS samples with respect to control. Instead, total content of glycosylated norisoprenoids increased in all CLS samples of ‘Cortese’ ($p<0.001$, up to + 44% with respect to the control). Free norisoprenoids were not detected in ‘Arneis’, and their low concentrations were not affected by CLS in ‘Cortese’.

Free benzenoids were not affected by the CLS treatment as well, apart from increased contents of homovanillinic acid in ‘Arneis’ juice and acetovanillone in ‘Cortese’ with increasing the CLS length. The bound fraction changed significantly (for both varieties $p<0.01$) with CLS in a variety-dependant way: in ‘Arneis’, total content of bound compounds

decreased down to –22% in all CLS samples, decreasing significantly 11 of the 12 detected glycosylated compounds with respect to the control; in ‘Cortese’, total glycosylated benzenoid content in CO14 and CO21 samples was higher than control (+29% and +20%, respectively). The major benzenoids in the two fractions were benzyl alcohol in ‘Arneis’ and both homovanillyl alcohol and benzyl alcohol in ‘Cortese’ juices.

In general, pre-fermentative macerated musts show higher contents of mostly free varietal compounds and glycosylated aroma precursors. However, a longer contact time does not always lead to a greater presence of these compounds due to a balance between extraction, adsorption, and interaction with other compounds or medium components (Alti-Palacios et al., 2023).

Overall, no significant differences were observed in total VOCs (representing the sum of the concentrations corresponding to the different chemical classes) in the free fraction for both the varieties with the CLS treatments. In contrast, the total glycosylated compounds were affected by CLS even if in different extent depending on the variety: in ‘Cortese’ samples, at the end of CLS, total content of glycosylated VOCs was significantly higher already in CO14 (+26%) and CO21 (+20%), whereas in ‘Arneis’ the CLS caused a decrease in the total content of glycosylated VOCs even at AR07 (from –14% to –21% for the different treatments with respect to control).

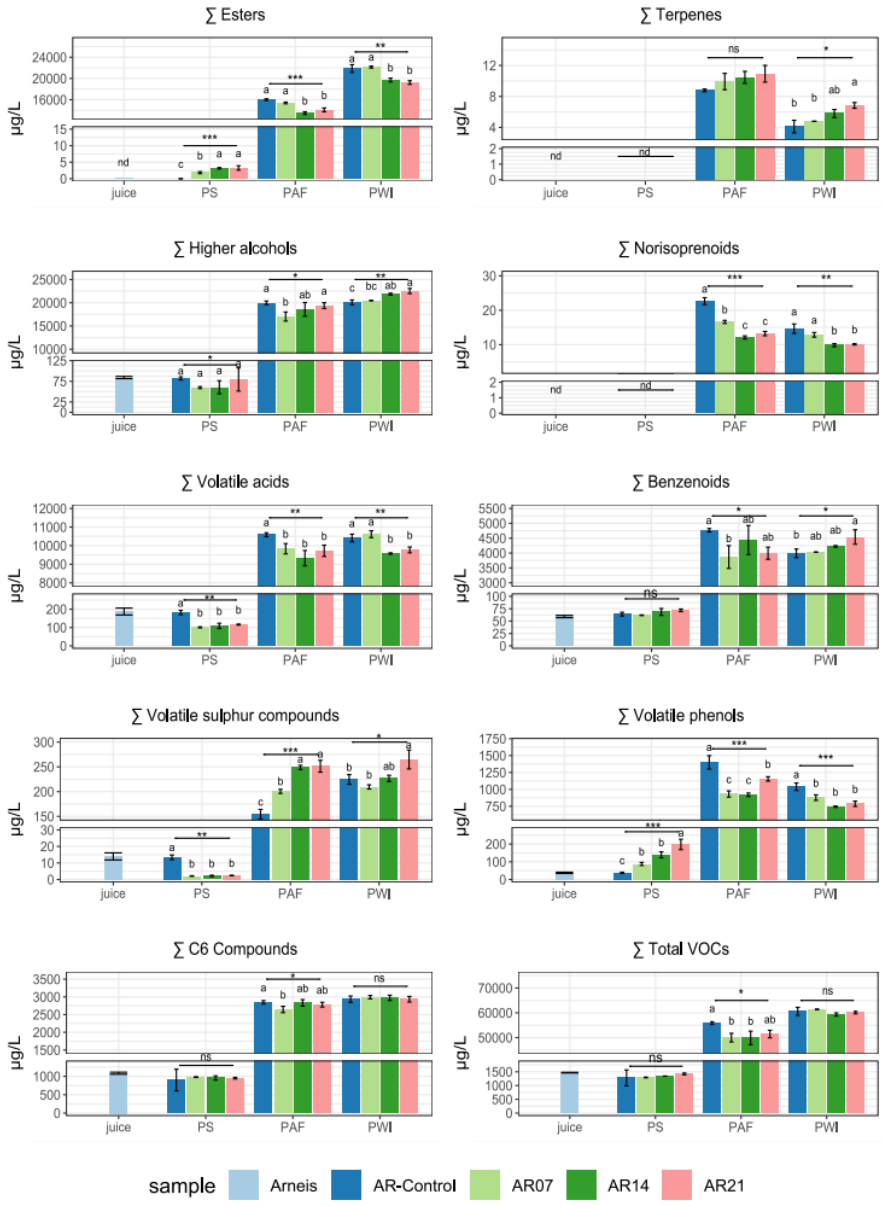

Figure 1. Free VOCs of 'Arneis' juice, after CLS (PS), after the alcoholic fermentation (PAF), and one month after bottling (PWI). Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, according to ANOVA test. Different lowercase letters within the same sampling point refer to the existence of a significant difference among different samples according to Tukey's HSD test. "AR-Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for 'Arneis' at the different winemaking stages.

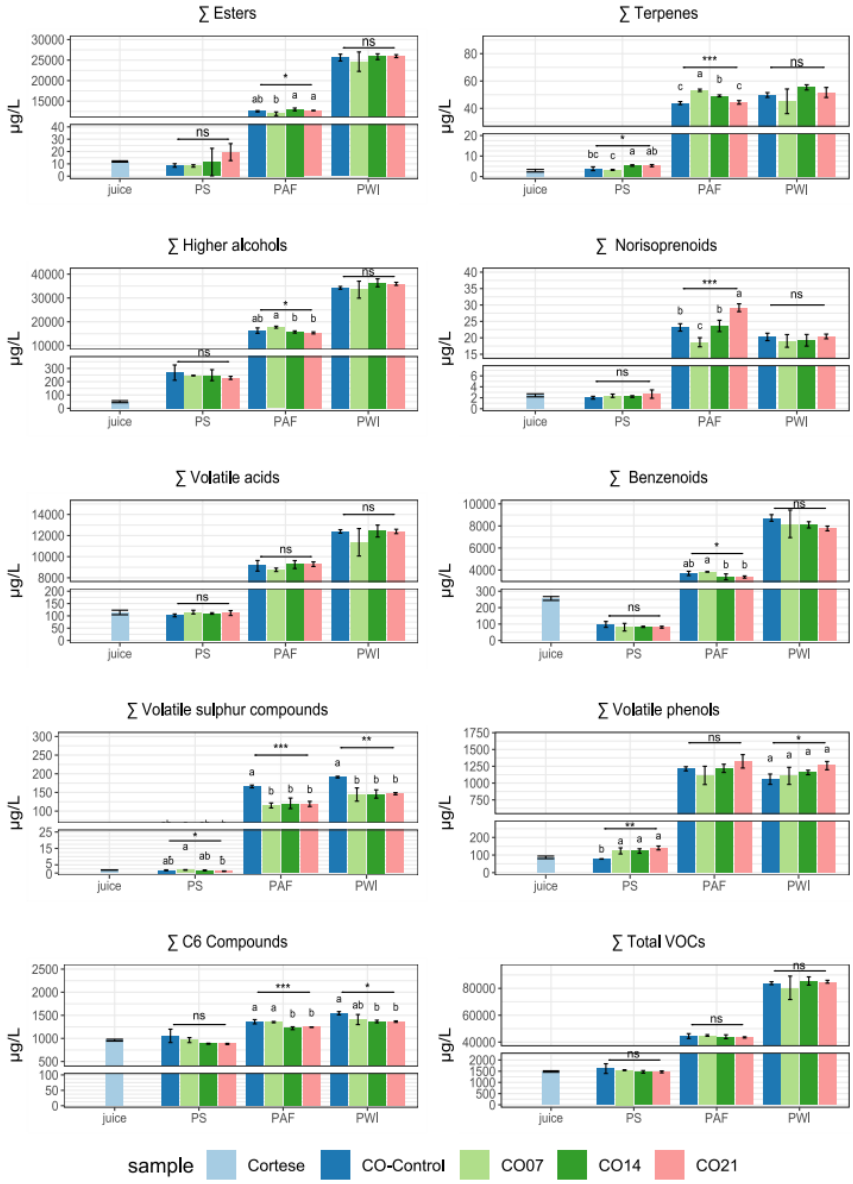

Figure 2. Free VOCs of 'Cortese' juice, after CLS (PS), after the alcoholic fermentation (PAF), and one month after bottling (PWI). "P07", "P14" and "P21" indicate the three treatment periods, respectively 7, 14 and 21 days. Sign: *, **, ***, and ns indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively, according to ANOVA test. Different lowercase letters within the same sampling point refer to the existence of a significant difference among different samples according to Tukey's HSD test. "CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for 'Cortese' at the different winemaking stages.

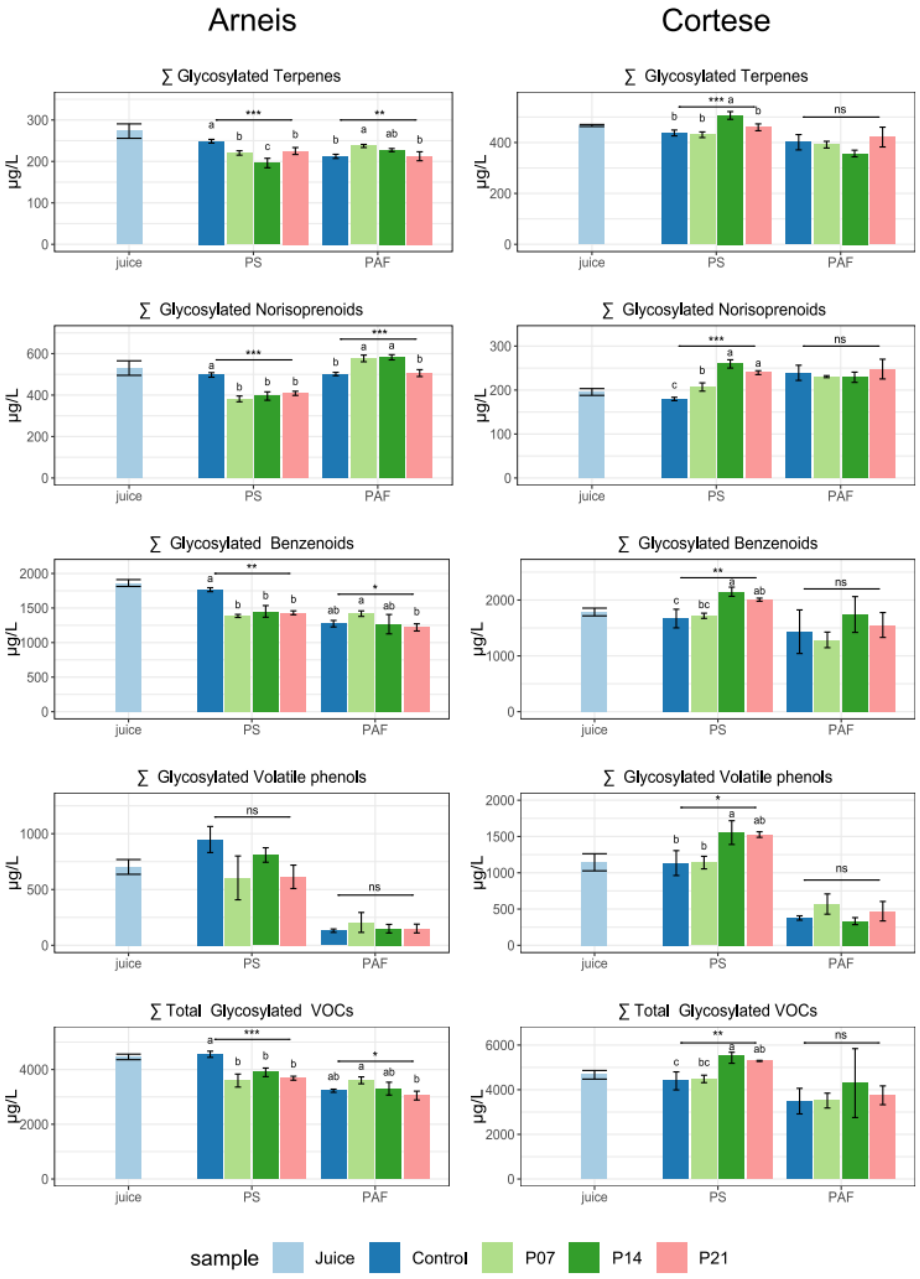

Figure 3. Glycosylated VOCs of 'Arneis' and 'Cortese' juices, after CLS (PS), and after the alcoholic fermentation (PAF). Sign: *, **, ***, and ns indicate significance at $p <$ 0.05, 0.01, 0.001, and not significant, respectively, according to ANOVA test. Different lowercase letters, for each variety, within the same sampling point refer to the existence of a significant difference among different samples according to Tukey's HSD test. "Control", "P07", "P14" and "P21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for 'Cortese' and 'Arneis' at different winemaking stages

3.2.2 Free and glycosylated volatile organic compounds in wines

At the end of alcoholic fermentation (PAF), free fermentative volatile organic compounds (Table S6), e.g. esters, higher alcohols, volatile acids, sulphur compounds, were affected in different extent by the CLS. Esters and higher alcohols are the major chemical classes in young wines produced from neutral varieties. Esters impact on the final fruity aroma of white wines. Instead, higher alcohols can contribute to a positive note when their concentration is less than 300 µg/L but they are related with pungent notes above this threshold (Alti-Palacios et al., 2023). A varietal effect was evident concerning fermentative volatile organic compounds, which were differently influenced by CLS depending on the chemical class and the variety. According to Figure 2, 'Cortese' PAF and PWI wines did not show a clear trend for free esters, higher alcohols, and volatile acids with some few exceptions, such as 3-methyl-1-pentanol, 2-phenylethanol, and hexanoic acid, whose contents decreased in CLS treated samples at PAF (Table S5). In 'Arneis' PAF wines, both esters and higher alcohols decreased significantly with respect to control ($p<0.001$ and $p<0.05$, respectively), reaching the lowest value in AR14 and AR07 samples, respectively (–15-16%). Particularly, AR14 and AR21 samples, and in minor extent AR07, had a significant reduction of the concentration of isoamyl acetate, responsible of *banana* flavour, being the most abundant ester detected. The same trend was maintained in 'Arneis' for free esters also after bottling (PWI, Figure 1), with AR14 and AR21 samples having significantly lower contents of esters ($p<0.01$), but being those of significantly higher contents of higher alcohols ($p<0.01$). Among higher alcohols, 2-phenylethanol has a pleasant aroma descriptor giving *rose* notes and it can contribute to all 'Cortese' wines whereas only to 'Arneis' wine produced from juice stabulated for 21 days whose content is very

close to odour threshold (Table 3). The increase in higher alcohols is often reported when pre-fermentative maceration techniques are applied (Aleixandre-Tudo et al., 2015, Wang et al., 2016; Alti-Palacios et al., 2023), whereas esters are more influenced by variety and treatments (Philipp et al., 2024).

At PAF, the total content of free volatile acids in ‘Arneis’ CLS samples was lower with respect to control and this behaviour was confirmed after bottling for AR14 and AR21 samples ($p<0.01$, Figure 1).

All these fermentative compounds are usually related to the fermentation kinetics and to the yeast strain (Furdíková, Makyšová, & Špánik, 2017). A low quantity of yeast assimilable nitrogen can have significant implications in decreasing the production of higher alcohols via Ehrlich pathway and consequently of esters (Casalta et al., 2013; Aleixandre-Tudo et al., 2015; Guittin et al., 2021). For volatile acids, the lower concentration may be related to a loss of precursors due to adsorption on macromolecules and skin components extracted during CLS (Ferreira et al., 1995). The increase of free sulphur compounds, in all ‘Arneis’ stabulated samples, was significant after alcoholic fermentation ($p<0.001$). The contents of methionol and 3-ethylmercapto-1-propanol increased in wines with increasing the CLS length, whereas those of benzothiazole increased significantly in AR07 and AR14 samples when compared to control (Table S6). This increasing trend was maintained after bottling in ‘Arneis’ only in AR21 sample (Table 3). Despite this increase due to the CLS, fortunately no wine reached values that exceeded the olfactory threshold. The opposite trend was found for ‘Cortese’ wines at both PAF and PWI (all CLS samples showing significantly lower contents of free sulphur compounds, $p<0.001$ and $p<0.01$ for PAF and PWI, respectively, with respect to control, Figure 2).

A significant decrease in total free C6-compounds was observed also in 'Cortese' PAF and PWI wines produced with CLS lengths of 14 and 21 days when compared to control ($p<0.001$ and $p<0.05$ for PAF and PWI, respectively, around –10%, Figure 2) whereas the lowest content in 'Arneis' wines corresponded to AR07 sample after alcoholic fermentation, but no significant differences after bottling (Figure 1). These trends corresponded mainly to the variations in 1-hexanol contents whereas 3-hexen-1-ol was barely affected in both varieties, although were reported concentrations above odour threshold in 'Arneis' that give *green and herbaceous* aromatic notes (Tables 3 and S6). This decrease in the concentration of C6-compounds is valuable with respect to skin contact strategies. Usually, the wines resulting from pre-fermentative skin contact show higher hexanol contents because of the formation of C6-aldehydes and C6-alcohols by the enzymatic and chemical oxidation of fatty acids extracted from the grape skins (Aleixandre-Tudo et al., 2015).

Concerning free and glycosylated varietal VOCs (Tables S6 and S7, respectively), as already previously reported for aromatic 'Traminer' (Philipp et al., 2024), 'Arneis' wines showed a significant increase in some free terpenes in AR21 samples after bottling, particularly geraniol, even if the concentration was below detection threshold (Table 3). After alcoholic fermentation, the content of free geraniol also increased with increasing the CLS length, but the differences were not significant with respect to control (Table S6). Instead, free linalool content increased in CO07 samples for 'Cortese' PAF wines, achieving values over its odour threshold that remained after bottling (Table 3), although significant differences were not found among control and CLS treated samples in 'Cortese' PWI wines (Table 3). Contrarily to the effect observed on CLS musts (Table S5), the highest total concentration of glycosylated terpenes

in the PAF wines was found in AR07 and AR14 samples for ‘Arneis’ while in CO21 samples for ‘Cortese’, although in the latter case the differences were not significant (Table S7). In ‘Arneis’ PAF wines, most of individual glycosylated terpenes followed the same trend of total ones while geraniol showed the opposite trend with the highest content being present in control. Regarding ‘Cortese’ PAF wines, the same trend was observed for the individual and total glycosylated terpenes, particularly for linalool and 8-hydroxylinalool.

Aleixandre-Tudo et al., (2015) found that pre-fermentative skin contact of crushed ‘Chenin blanc’ grapes at 4 °C for 12 hours led to a decrease in terpene concentrations. This agrees with increased terpene concentrations in ‘Muscat of Bornova’ wines, reported after a short period of skin contact at 10 °C while longer periods of up to 12 h caused their decrease (Selli et al., 2006b). In the present study, the advantage of contact with the juice lees instead of grape skins is the possibility of increasing stabulation length without reducing but increasing the concentration of free terpenes in the final wines, as found particularly for ‘Arneis’.

Free norisoprenoids were scarcely present (3-oxo-α-ionol was the only detected compound) and its concentration decreased significantly up to –47% ($p<0.001$) by CLS in ‘Arneis’ PAF wines, following this same behavior after bottling, particularly for AR14 and AR21 samples (Figure 1). The AR07 and AR14 samples had a higher concentration of glycosylated compounds, showing an inverse trend to that previously observed in the treated grape juices (Figure 3). Instead, after an initial decrease for CO07 sample, an increased content of free 3-oxo-α-ionol was achieved for CO21 sample in ‘Cortese’ PAF wines ($p<0.001$, +26%, Table S6). This was in line with a higher content of precursors just after CLS (+30%) with respect to control, Table S5), which were not significantly

different in ‘Cortese’ PAF wines among CLS treated samples and control (Table S7). Nevertheless, this difference was not observed in ‘Cortese’ PWI wines (Table 3).

As regards total free benzenoids, the only difference observed among control and CLS treated samples was for AR07 sample in ‘Arneis’ PAF wines, showing a significantly lower content of them ($p<0.05$, –19%, Figures 1-2). However, a higher quantity was found at AR21 samples for PWI wines. Significant differences with respect to control were not found in ‘Cortese’ PAF and PWI wines. For each variety, some individual free compounds increased significantly with stabulation, such as benzyl alcohol and methyl salicylate for ‘Arneis’, as well as vanillin for ‘Cortese’ at PAF (Table S6), but their concentrations are below their olfactory threshold in final wines (Table 3). Only some small differences in glycosylated benzenoids were observed in ‘Arneis’ and ‘Cortese’ wines after alcoholic fermentation when compared to the musts. Particularly for ‘Arneis’ PAF wines, the highest concentration of glycosylated precursors was found in AR07 samples while non-stabulated musts were richer in glycosylated benzenoids (Tables S5 and S7).

In ‘Arneis’ PAF and PWI wines a significant decrease was found for total content of free volatile phenols for all CLS samples ($p<0.001$, –15-34% with respect to control, Figure 1). 4-vinylguaicol and 4-vinylphenol were the compounds most affected by the CLS technique, reporting a decrease of –25% and –29%, respectively, in bottled ‘Arneis’ wines (Table 3). This may be relevant since the concentration of 4-vinylguaicol is at threshold level in this variety. Contrarily, in ‘Cortese’ PAF and PWI wines, no significant differences were found in both free and bound volatile phenols (Figures 2 and 3). ‘Arneis’ PAF wines also showed no significant differences in total glycosylated volatile phenols or

in main individual compounds (4-vinylguaicol and 4-vinylphenol). Selli et al., (2006b) reported an increase in the volatile phenol concentration in the wines when increasing the length of pre-fermentative skin contact till 12 h. In the present study, the use of a lower temperature and juice lees contact allowed to extend the contact time without increasing the presence of these compounds.

Table 3. Free volatile compounds (µg/L of 1-heptanol) of control and CLS-treated Arneis and Cortese wines analyzed one month after bottling (PWI).

Chemical class/ compound	Ref.	Descriptors	OT[#] (µg/L)	Arneis					Cortese				
				AR-Control	AR07	AR14	AR21	Sign	CO-Control	CO07	CO14	CO21	Sign
Acetate esters													
Isoamyl acetate	[1]	Banana, fruity	30	3859.76 ±8.49 a	4019.11 ±67.32 a	3127.68 ±11.28 b	3008.71 ±92.24 b	***	3485.29 ±201.08 a	2863.69 ±355.02 b	3961.5 ±78.55 a	3948.61 ±118.32 a	*
(E/Z)-3-Hexen-1-ol acetate	[2]	Fresh green, cut grass	-	60.15±1.19 a	63.40±2.05 a	52.48±0.74 b	48.72±1.61 b	***	nd	nd	nd	nd	
1,3-Propanediol diacetate	[2]	Fruity	-	43.39±3.46	46.67±0.98	38.73±2.39	40.37±1.57	ns	nd	nd	nd	nd	
2-Phenylethyl acetate	[1]	Sweet, honey, floral, rose	250	1311.57 ±53.06 a	1232.09 ±5.60 ab	1170.25 ±4.12 b	1133.75 ±44.31 b	**	1234.43 ±78.14 a	999.72 ±112.68 b	1249.79 ±47.21a	1182.11 ±41.00 ab	*
Hexyl acetate	[1]	Lolly, apple,cherry, pear, sweet floral	115	441.35 ±13.57a	471.55±1.07 a	403.18 ±11.52 b	388.17 ±11.35 b	***	204.55 ±14.81 ab	172.81±16.32 b	217.94 ±16.70a	222.10±5.86 a	*
Ethyl esters													
Ethyl hexanoate	[3]	Green apple, fruity, tropical, floral, strawberry	14	1071.21 ±23.42 b	1157.78 ±30.36 a	920.64±2.41 c	941.09 ±20.29 c	***	973.46 ±61.92	913.80±95.50	1038.65 ±28.11	1014.86 ±14.09	ns
Ethyl octanoate	[3]	Fruity, sweet, waxy	5	1928.94 ±92.25 a	2097.97 ±29.68 a	1546.07 ±36.64 b	1561.66 ±52.28 b	***	1990.41 ±114.32 ab	1791.37 ±168.65 b	2083.85 ±79.77 ab	2101.33 ±50.32 a	*
Ethyl decanoate	[2]	Fruity, grape,pear, apple	200	952.14 ±66.42 a	975.85 ±45.35 a	780.95 ±3.61b	717.33 ±22.93 b	**	870.17 ±69.09 a	865.15±72.35 a	959.60 ±35.10 a	986.66 ±22.39 a	*
Ethyl 4-hydroxybutanoate	[4]	Pineapple, rose, tropical fruit	-	1272.87 ±103.51 bc	1607.88 ±10.42 a	989.09 ±101.46 c	1352.67 ±131.05 ab	**	581.69 ±28.80 b	469.92±67.96 c	681.38 ±33.41 ab	771.75 ±20.85 a	***
Ethyl 3-hydroxybutanoate	[2]	Fruity, grape, green	20	206.36 ±32.23 a	225.92 ±17.88 a	137.50±4.51 b	147.44±8.35 b	**	125.68±6.10 b	108.17±9.86 c	137.29±2.09 b	154.52±2.89 a	***
Ethyl 2-hydroxy-4-methylpentanoate	[2]	Fresh blackberry	-	118.02±4.02 b	127.02±8.50 ab	142.55±6.21 a	128.99±6.61 ab	*	110.64±2.22	108.47±10.34	113.74±3.57	102.43±0.29	ns
Diethyl malate	[1]	Over-ripe, peach, prune	760[§]	526.59 ±15.99 a	437.68±3.04 b	355.20 ±10.50 c	339.72 ±11.56 c	***	498.95±9.93 a	459.73±32.44 a	380.72±1.16 b	345.59±9.46 b	**
Ethyl lactate	[5]	Milk, soap, butter, fruits	150[§]	2489.74 ±74.67 a	2461.04 ±36.47 a	2542.85 ±151.51a	2261.72 ±87.18 a	*	3157.91 ±100.76	3413.64 ±331.49	3387.73 ±251.20	3419.30 ±194.72	ns
Monoethyl succinate	[2]	Caramel, coffee	1,000[§]	6328.95 ±361.54	6164.69 ±22.78	6479.59 ±321.85	6232.93 ±223.60	ns	10912.49 ±511.85	11148.63 ±1232.01	10447.37 ±326.14	10648.96 ±397.37	ns
Diethyl succinate	[6]	Oily, fruity, floral, caramel	200[§]	1020.67 ±7.72 a	845.40±8.31 b	754.23±3.28 c	661.85±6.38 d	***	1181.43 ±20.52 a	1053.87 ±60.07 b	942.48±6.20 c	868.03 ±14.37 c	***
Ethyl phenyllactate	[2]	Spicy peppery, black pepper	-	237.62±3.15 b	208.97±2.19 c	268.62±8.98 a	268.27±6.04 a	***	282.22±9.10 a	243.63±22.50 b	229.92±0.77 b	196.16±5.34 c	***
∑ ***Esters***				21869.32 ±729.03 a	22143.02 ±181.31 a	19709.60 ±343.78 b	19233.38 ±345.01 b	**	25609.34 ±830.19	24612.60 ±2365.15	25831.95 ±704.23	25962.42 ±352.63	ns
Higher alcohols													
Isoamyl alcohol	[1]	Harsh, stale, fusel odour	30[§]	11593.41 ±165.53 b	11969.19 ±40.48 ab	12642.92 ±222.22 a	12505.99 ±297.06 a	**	17794.48 ±456.08 a	17872.30 ±1806.45 a	19872.57 ±791.46 a	19688.55 ±518.22 a	*
3-Methyl-1-pentanol	[6]	Vinous, herbaceous, cocoa	50[§]	207.96±3.81 b	240.04 ±13.84 a	172.61±4.62 c	171.51 ±12.76 c	***	215.23 ±11.19 a	172.92±6.11 b	187.70 ±10.51 b	138.44±7.11 c	***
2,3-Butanediol	[4]	Fruity, fresh	150[§]	46.66±14.55	46.62±13.96	41.48±3.35	35.26±6.30	ns	34.78±6.37	89.24±88.36	54.16±22.26	327.77 ±497.98	ns
2-Phenylethanol	[6]	Floral, rose	10[§]	8229.16 ±999.33 b	8214.94 ±44.89 b	8991.14 ±4.49 ab	9818.64 ±347.99 a	**	16210.79 ±321.33	15348.53 ±1681.72	16235.76 ±904.81	15768.86 ±415.92	ns
∑ ***Higher alcohols***				20077.19 ±520.32 c	20470.79 ±23.39 bc	21848.15 ±209.76 ab	22531.40 ±550.61 a	**	34255.28 ± 566.04	33482.98 ±3562.12	36350.18 ±1671.45	35923.61 ±3628.32	ns
Volatile acids													

(continued on next page)

Table 3. (Continued)

Chemical class/ compound	Ref.	Descriptors	OT# (µg/L)	Arneis					Cortese				
				AR-Control	AR07	AR14	AR21	Sign	CO-Control	CO07	CO14	CO21	Sign
Isobutyric acid	[2]	Cheese, pungent	2300	380.87 ±15.68 b	392.86 ±25.77 b	500.15 ±12.95 a	483.37 ±27.46 a	**	482.77 ±46.29	453.86±48.59	486.28 ±74.97	472.80 ±42.58	ns
Butanoic acid	[5]	Pungent	170	392.32 ±13.11 b	461.12±9.16 a	340.21 ±18.71 c	341.92±7.57 c	***	405.16±6.14	389.96±50.07	396.92 ±46.39	414.11 ±12.09	ns
Isovaleric acid	[5]	Taleggio Cheese, rancid, sweaty, stinky	700	953.31 ±34.72 ab	1014.93 ±9.36 a	952.24 ±29.02 ab	932.41±7.83 b	*	1138.30 ±25.66	1052.17 ±117.50	1103.11 ±26.50	1015.00 ±29.17	ns
Hexanoic acid	[6]	Sour, vinegar, cheese, sweaty, chemical	420	3583.02 ±145.35 a	3757.09 ±10.47 a	3250.61 ±67.36 b	3479.42 ±42.02 ab	**	4511.82 ±146.23	3939.79 ±434.00	4347.31 ±179.53	4313.05 ±79.70	ns
Octanoic acid	[3]	Goat rancid cheese, fatty, oily, acetic	500	3610.39 ±68.87 a	3544.46 ±107.84 ab	3231.47 ±118.11 b	3230.14 ±117.95 b	**	4192.15 ±51.01	3912.82 ±449.23	4375.62 ±247.74	4381.17 ±102.82	ns
Nonanoic acid	[1]	Must, fat	-	7.73±0.61	11.75±2.41	6.54±0.14	7.34±0.86	ns	7.89±1.74 ab	5.53±0.65 b	7.82±1.09 ab	8.67±0.92 a	*
Decanoic acid	[5]	Vinegar, animal, fatty, rancid, citrus, phenolic	1000	1338.47 ±47.97 a	1268.88 ±77.52 ab	1184.84 ±3.39 ab	1146.67 ±15.76 b	**	1546.77 ±35.84	1539±199.69	1652.29 ±56.78	1700.54 ±49.87	ns
9-Decenoic acid	[2]	Waxy, creamy, cheesy	-	84.90±21.58	97.93±16.55	58.37±11.23	87.28±9.94	ns	38.15±1.87 a	12.07±1.09 b	6.79±2.31 c	11.88±1.83 b	***
Dodecanoic acid	[6]	Chemical, fatty, rancid	1000	62.85±4.03	68.11±6.35	53.17±6.90	62.28±9.10	ns	69.10±3.16 a	58.93±9.84 a	59.34±3.11 a	71.86±2.06 a	*
∑ Volatile acids				10413.86 ±201.54 a	10617.14 ±182.58 a	9577.60 ±37.33 b	9770.83 ±155.72 b	**	12392.10 ±163.49	11364.16 ±1301.21	12428.68 ±565.60	12389.08 ±202.67	ns
Volatile sulphur compounds													
3-Ethylmercapto-1-propanol	[7]	Sweat odour, roasted, potato, broth	60	19.02±0.36 c	19.74±0.59 bc	20.68±0.41 b	22.01±0.31 a	***	24.79±0.81 a	19.29±2.13 b	20.07±0.50 b	17.71±0.19 b	***
Methionol	[8]	Vegetables, Boiled potato, Cabbage	500	201.29±9.23 b	185.73±3.40 b	201.29±4.67 b	237.03 ±18.34 a	*	160.36±2.04 a	119.28±15.04 b	118.15 ±10.61 b	123.45±3.89 b	**
Benzothiazole	[7]	Burnt Rubber	350	4.25±0.23 b	3.97±0.28 b	4.71±1.20 ab	5.83±0.81 a	*	5.97±2.29	5.82±0.58	7.65±2.59	5.57±0.97	ns
∑ Volatile sulphur compounds				224.56±9.76 b	209.44±4.26 b	226.68±6.28 ab	264.88 ±19.01 a	*	191.12±2.08 a	144.39±17.74 b	145.87 ±10.98 b	146.73±2.81 b	**
C6 compound													
1-Hexanol	[3]	Green, resin, flower	2500	1711.79 ±53.31	1679.92 ±23.73	1732.37 ±44.89	1701.72 ±38.19	ns	1191.35 ±29.97 a	1079.87 ±79.14 ab	1025.70 ±20.01 b	1023.53 ±21.22 b	**
(E/Z)-3-Hexen-1-ol	[3]	Green	(400/ 70)	1222.28 ±36.18	1311.33 ±24.25	1240.97 ±29.18	1232.17 ±40.51	ns	353.42±9.88	328.62±29.81	340.59±7.61	339.54±5.92	ns
∑ C6 compounds				2934.07 ±89.47	2991.25 ±47.98	2973.34 ±74.07	2933.89 ±78.42	ns	1544.77 ±35.52 a	1408.49 ±108.86 ab	1366.29 ±27.62 b	1363.08 ±15.58 b	*
Terpenes													
Linalool	[6]	Rose, Citrus	15	nd	nd	nd	nd	**	49.79±1.73	45.19±9.00	55.37±1.90	51.63±3.65	ns
Geraniol	[6]	Rose, geranium	30	4.10±0.81 b	4.79±0.00 b	5.80±0.52 ab	6.84±0.37 a	**	nd	nd	nd	nd	
∑ Terpenes				4.10±0.81 b	4.79±0.00 b	5.80±0.52 ab	6.84±0.37 a	*	49.79±1.73	45.19±9.00	55.37±1.90	51.63±3.65	ns
Norisoprenoids													
3-Oxo-α-ionol	[2]	Spicy	-	14.64±1.36 a	12.86±0.67 a	9.83±0.45 b	10.10±0.17 b	**	20.28±1.13	19.06±1.93	19.25±1.77	20.42±0.71	ns
Benzenoids													
Vanillin	[6]	Vanilla, sweet pastry	60	3.64±0.30	3.53±0.56	2.73±0.17	2.44±0.12	ns	7.41±0.44	7.74±1.22	8.13±2.59	6.62±0.49	ns
Benzyl Alcohol	[6]	Caramel, fruity, nutty, cherry, rose	200§	27.48±2.94	29.54±0.75	26.57±0.79	26.84±1.19	ns	34.30±1.92	31.48±3.68	29.68±0.50	28.97±0.34	ns
Homovanillinic acid				24.12±2.75 a	20.64±1.42 ab	16.93±1.18 b	17.58±0.89 b	*	31.58±2.10 a	28.29±3.57 ab	22.87±2.63 b	23.08±2.01 b	**
Tyrosol	[5]	Bees wax, honey-like	-	3863.48 ±141.85 b	3908.40 ±5.88 b	4120.21 ±28.08 ab	4435.94 ±243.69 a	*	8382.74 ±312.91	7876.82 ±1216.72	7801.10 ±280.96	7467.79 ±209.25	ns
3,4,5-Trimethoxy-benzenemethanol				7.94±0.25 a	8.34±0.30 a	4.87±0.07 b	5.37±0.16 b	***	14.48±1.00	14.6±1.42	13.89±1.29	15.42±0.17	ns

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Table 3. (Continued)

Chemical class/ compound	Ref.	Descriptors	OT# (µg/L)	Arneis					Cortese				
				AR-Control	AR07	AR14	AR21	Sign	CO-Control	CO07	CO14	CO21	Sign
Homovanillyl alcohol				15.41±0.94 a	15.58±0.34 a	11.71±0.17 b	12.28±0.72 b	**	59.51±2.19	55.89±7.28	53.28±3.12	55.19±2.06	ns
Acetovanillone	[9]	Floral, clove, vanilla	1000	26.77±1.06 a	28.86±0.72 a	22.98±0.06 b	22.48±0.82 b	***	167.04±6.55	156.98±16.90	153.86 ±11.36	157.90±2.48	ns
3-Hydroxy-4-phenyl-2-butanone	[2]	Creamy, sweet fruity	-	23.26±1.56 a	18.44±1.28 b	20.20±1.23 ab	18.77±0.64 b	*	29.06±3.16 a	20.97±1.61 b	19.76±0.82 b	17.48±0.95 b	***
∑ Benzenoids				3992.10 ±144.28 b	4033.34 ±0.51 ab	4226.21 ±25.23 ab	4541.69 ±243.13 a	*	8726.12 ±299.44	8192.77 ±1250.31	8102.57 ±280.81	7772.46 ±214.53	ns
Volatile phenols													
3,5-Diterr-butylphenol				31.15±0.15 ab	34.45±2.04 a	28.59±0.03 b	28.53±1.65 b	*	39.96±3.03	37.73±2.71	41.35±6.42	42.05±3.01	ns
4-Vinylguaiacol	[5]	Spicy, smoked, phenolic, curry	440	455.02 ±24.51 a	455.28 ±20.67 a	342.51±6.62 b	346.16 ±10.15 b	***	851.61 ±59.22 b	886.30±88.24 ab	924.81 ±35.04 ab	1004.99 ±24.53 a	*
Phenol	[2]	Sweet, tarry (phenol)	-	25.42±11.53	15.52±0.03	13.93±1.35	13.77±0.53	ns	20.61±4.24	24.14±3.24	22.45±0.77	22.04±2.99	ns
2,6-Dimethoxyphenol (Syringol)	[10]	Smoke, phenolic	570	71.38±23.40 a	48.73±1.55 a	34.15±1.34 a	33.38±1.56 a	*	50.00±10.32	52.67±4.95	49.12±3.44	50.61±5.03	ns
4-Vinylphenol	[5]	Clove, medicinal	770	455.74 ±35.31 a	324.22 ±16.57 b	324.41±0.85 b	364.86 ±42.47 ab	*	95.28±15.51	106.63±29.06 a	119.98 ±10.81	140.03 ±31.74	ns
∑ Volatile phenols				1038.69 ±54.95 a	878.20 ±40.79 b	743.58 ±10.14 b	786.70 ±39.53 b	***	1057.46 ±76.56 a	1107.47 ±127.11 a	1157.70 ±33.37 a	1259.73 ±61.74 a	*
Lactones													
Butyrolactone	[11]	Caramel, sweet	35	35.31±2.24 ab	43.14±3.60 a	23.08±2.96 c	32.59±3.79 b	**	16.98±3.65	21.93±9.82	29.95±4.56	60.04±33.11	ns
Total volatile compounds				60603.87 ±1597.57	61403.97 ±118.91	59343.86 ±628.00	60112.30 ±496.63	ns	83863.22 ±1068.20	80399.03 ±8712.79	85487.81 ±3068.92	84949.19 ±1047.22	ns

Odor threshold; § Values expressed in mg/L.

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences between different samples, for each variety and sampling point, according to Tukey's HSD test. "CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment periods, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment periods, 7, 14 and 21 days, respectively, for 'Arneis' wines.

Sensory descriptors were reported after comparison of literature and sources available online: [1] Fracassetti et al., 2020; [2] www.thegoodscentscompany.com; [3] Ferreira et al., 2000; [4] Scutaraşu et al., 2022; [5] Lambrechts & Pretorius, 2000; [6] Sanchez-Palomo et al., 2017; [7] Lavigne-Cruège et al., 1996; [8] Rutan et al., 2014; [9] Gambetta et al., 2014; [10] Lopez et al., 2002;[11] Sanchez-Palomo et al., 2010.

3.3 Impact of CLS treatment on wine sensory characteristics and correlation with instrumental data

'Arneis' and 'Cortese' wines have been evaluated through sensory analysis at the end of alcoholic fermentation (PAF) and one month after bottling (PWI). Regarding the first tasting stage, in 'Arneis' no differences were perceived in terms of mouthfeel, whereas AR14 received a lower score in aroma intensity ($p<0.05$) with respect to control (Table 4), which agreed with total free VOCs content (Table S6). As concerns the aromatic descriptors (Figure 4), *grapefruit* (58.3%), *jasmine, pear, and lemon* (all 41.7%) perceptions were recognized in 'Arneis' control wine. In general, the *lemon* descriptor was found in all the samples (41.7-50%). In contrast, *peach* was able to discriminate wines according to the Cochran's Q-test ($p=0.053$, Table S8), with AR07 sample showing the highest frequency (50%) in this wine group. In longer stabulated samples, *green apple* (67% and 50% for AR14 and AR21, respectively) and *rose* (41.7% for AR14 and AR21) were recognized. The aroma descriptors used predominantly by judges for AR21 were *pear* (75%), *lime and honey* (both 42%) (Cochran's Q-test p=0.019 for *honey*, Table S8).

At PAF, some significant differences were found in ‘Cortese’: CLS significantly increased the body attribute ($p<0.001$) and an increasing trend for the overall wine quality ($p<0.01$) was found (Table 4). At PWI polysaccharides content was investigated but neither ‘Arneis’ nor ‘Cortese’ wines reported significant differences among treatments (Tables 1 and 2). For the other mouthfeel and taste attributes, no statistical differences were found, indicating that the differences in polyphenolic content were negligible in terms of bitterness-astringency evaluation. Regarding the aromatic descriptors, *peach* (58.3%), *green apple* (58.3%), and *banana* (50%) hints were underlined in control. The last two descriptors were well represented in all the tasted ‘Cortese’ PAF samples (frequency above 41%). In contrast with ‘Arneis’, in ‘Cortese’ the *rose* descriptor was more frequent in control and CO07 with respect to the longer CLS samples, while *pear* (50%) and *honey* (33.3-50%) contributed mainly to CO14 and CO21 wines.

Table 4. Results of sensory Descriptive Analysis of wines mouthfeel (bitterness, astringency, acidity, body), aroma intensity, and overall wine quality after alcoholic fermentation (PAF) and one month after bottling (PWI) for Arneis (AR) and Cortese (CO) varieties.

Sample	Bitterness	Astringency	Acidity	Body	Aroma intensity	Overall wine quality
			Arneis (PAF)#			
AR-Control	3.71±0.54	1.75±0.58	6.53±0.43	5.17±0.54	7.29±0.31 a	6.27±0.68
AR07	3.72±0.62	1.90±0.67	6.48±0.37	4.10±0.51	6.90±0.31 ab	6.56±0.33
AR14	3.84±0.66	2.30±0.74	6.31±0.55	4.20±0.43	6.01±0.52 b	5.38±0.45
AR21	2.73±0.56	2.18±0.67	6.13±0.36	5.01±0.60	7.29±0.27 a	6.24±0.66
p value	0.285	0.667	0.876	0.211	0.015	0.474
Sign.	ns	ns	ns	ns	*	ns
			Arneis (PWI)			
AR-Control	3.38±0.77	1.99±0.91	4.76±0.18	4.47±0.57	6.34±1.13	5.45±1.96
AR07	3.58±0.35	1.79±0.53	5.02±0.43	4.66±0.61	7.03±0.14	5.26±0.82
AR14	3.39±0.74	2.03±0.07	4.34±0.39	4.74±0.08	6.04±0.04	5.98±0.36
AR21	3.16±0.04	1.73±0.65	4.99±0.32	5.64±0.35	6.35±0.19	5.91±1.33
p value	0.865	0.945	0.522	0.097	0.245	0.696
Sign	ns	ns	ns	ns	ns	ns
			Cortese (PAF)#			
CO-Control	2.33±0.51	2.13±0.78	6.18±0.42	3.02±0.48 b	5.83±0.67	5.03±0.55 b
CO07	2.29±0.61	2.08±0.75	5.78±0.53	4.88±0.62 a	6.24±0.53	6.71±0.58 ab
CO14	2.84±0.73	2.04±0.59	5.93±0.41	4.82±0.54 a	6.97±0.58	7.09±0.53 a
CO21	3.47±0.64	2.73±0.68	6.33±0.41	6.37±0.44 a	6.93±0.41	7.37±0.51 a
p value	0.321	0.536	0.587	0.0002	0.225	0.007
Sign.	ns	ns	ns	***	*ns*	**
			Cortese (PWI)			
CO-Control	2.99±1.38	2.99±0.14	6.26±0.86	3.66±0.43	5.21±1.10	4.31±0.85 b
CO07	2.39±0.58	1.96±0.80	6.59±1.68	4.11±0.71	6.03±0.41	5.01±1.63 ab
CO14	2.19±0.21	2.91±1.00	6.42±0.82	3.63±0.42	6.00±0.13	6.50±0.49 a
CO21	1.83±0.12	2.23±0.65	6.66±0.41	4.24±0.38	6.29±0.42	6.13±0.11 a
p value	0.255	0.233	0.893	0.618	0.334	0.003
Sign	ns	ns	ns	ns	ns	**

Note: #For samples after fermentation two-way ANOVA was performed with sample as fix effect and judges as random effect, and if statistical significance was found ($p < 0.05$), Tukey HSD was used for establishing significant differences among samples. Results are expressed as mean ± s/(n)$^{1/2}$, standard deviation; n, number of panellists) for PAF, whereas for PWI are expressed ad mean ± standard deviation of two independent sensory analysis sessions. "CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment periods, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment periods, 7, 14 and 21 days, respectively, for 'Arneis' wines.

At PWI, for each variety, the differences among samples decreased. 'Arneis' or 'Cortese' wines did not differ for mouthfeel descriptors, aroma intensity, and overall wine quality when considering the CLS treatment (Table 4). 'Arneis' stabulated samples showed no difference in total free VOCs content (Table 3) with respect to the control in agreement with sensory analysis. However, the perceived descriptors changed when

compared to PAF (Figure 4). In control samples there was an increase in frequency of descriptors like *rose* and *honey* (both 50%). The latter two descriptors were recognised also in AR07, with 50% and 45%, respectively. In fact, both descriptors were able to discriminate samples according with the Cochran's Q test (p=0.038 and p=0.064, for rose and honey, respectively) from longer stabulated samples. Also, in the AR07 sample, a contribution of some tropical fruits, particularly *pineapple* (45%), was found. In terms of descriptors *green apple*, *pear*, and *honey* were more perceived in AR14 (40%). Green apple was a descriptor more cited in AR14 and AR21 wines at both PAF and PWI when compared to control and AR07 samples.

In 'Cortese' samples, a significant difference persisted in overall *wine quality*, where CO14 and CO21 were preferred than control after bottling ($p<0.01$; Table 4). At PWI, the most used aroma descriptors for 'Cortese' were *green apple*, *pear, rose,* and *jasmine*. Particularly, *green apple* descriptor was more used for control (50%) and CO21 (57%), *pear* in CO14 and CO21 (50-57%), as well as *rose* (57.1% for CO14 and 42.9% for CO21). Furthermore, *jasmine* was used in the 35.7% of cases to describe the control and by 42.9% for CO14. Nevertheless, the total free VOCs of 'Cortese' wines had no significant difference among samples (Table 3), confirming the sensory results that showed no perceived differences in aroma intensity at wine tasting.

Although in this case the treatment did not affect neither the total concentration of VOCs nor the overall aroma intensity, some individual VOCs may have influenced the final *overall wine quality*. With this aim, a PCA was performed considering, for both varieties studied, the results of the wines after bottling (Figure 5). The first dimension accounted for the 41.6% of the explained variance whereas the second dimension for the

22.7%, with a total of 64.3%. Some trends can be underlined: control and short CLS (AR07 and CO07) samples are in the upper side of the graph, whereas the longest CLS samples (AR14, CO14 and AR21, CO21) are in the lower. The first dimension was positively correlated with four esters: ethyl 3-hydroxybutanoate, ethyl octanoate, isoamyl acetate, and hexyl acetate (R= 0.938, 0.920, 0.910, and 0.906, respectively, all p<0.01), instead it was negatively correlated with geraniol (R= –0.765) and ethyl phenyllactate (R= –0.770), both p<0.05. In fact, 'Arneis' esters were lower in AR14 and AR21 compared to control and AR07 wines (p<0.01). The presence of compounds, such as isoamyl acetate, 2-phenylethyl acetate, hexyl acetate or ethyl 3-hydroxybutanoate, in control and 7-day CLS samples conferred to those wines fruity and floral characters, like *pineapple, banana,* and *rose,* which were recognized by the judges during sensory analysis. In contrast, 14 and 21-day CLS samples were found in the opposite side of the graph, being characterised by significant higher contents of isoamyl alcohol and 2-phenylethanol, and in general, in higher alcohols. Overall, these two last compounds may have been related to *jasmine* aroma descriptor and, more generally, to the floral- sensation perceived in these wines. In addition, in 'Arneis' wines, geraniol content was significantly higher in AR21, although lower than its detection threshold.

Dimension 2 was positively correlated (p<0.05) mainly with benzyl alcohol, 3-methyl-1-pentanol, diethyl malate, homovanillic acid, and diethyl succinate (R> 0.8), and negatively with 1-hexanol (R= –0.753, p<0.05). C6-compounds may have differentiated the 'Cortese' wines by the CLS treatment, with CO14 and CO21 samples resulting in a lower content of this chemical class responsible for herbaceous hint. This could be linked to the higher overall wine quality scores (Table 4). Moreover,

the *rose* and *jasmine* aroma descriptors, mostly perceived in CO14 samples (Figure 4, Table S8), were associated with higher contents of linalool, even though not significantly, above its odour threshold. The similarity among the control and AR07 samples for 'Arneis' with the longer stabulated samples for 'Cortese' (CO14 and CO21) found in the PCA is mainly due to the opposite behaviour of volatile phenols (mainly, 4-vinylphenol and 4-vinylguaiacol) in the two varieties.

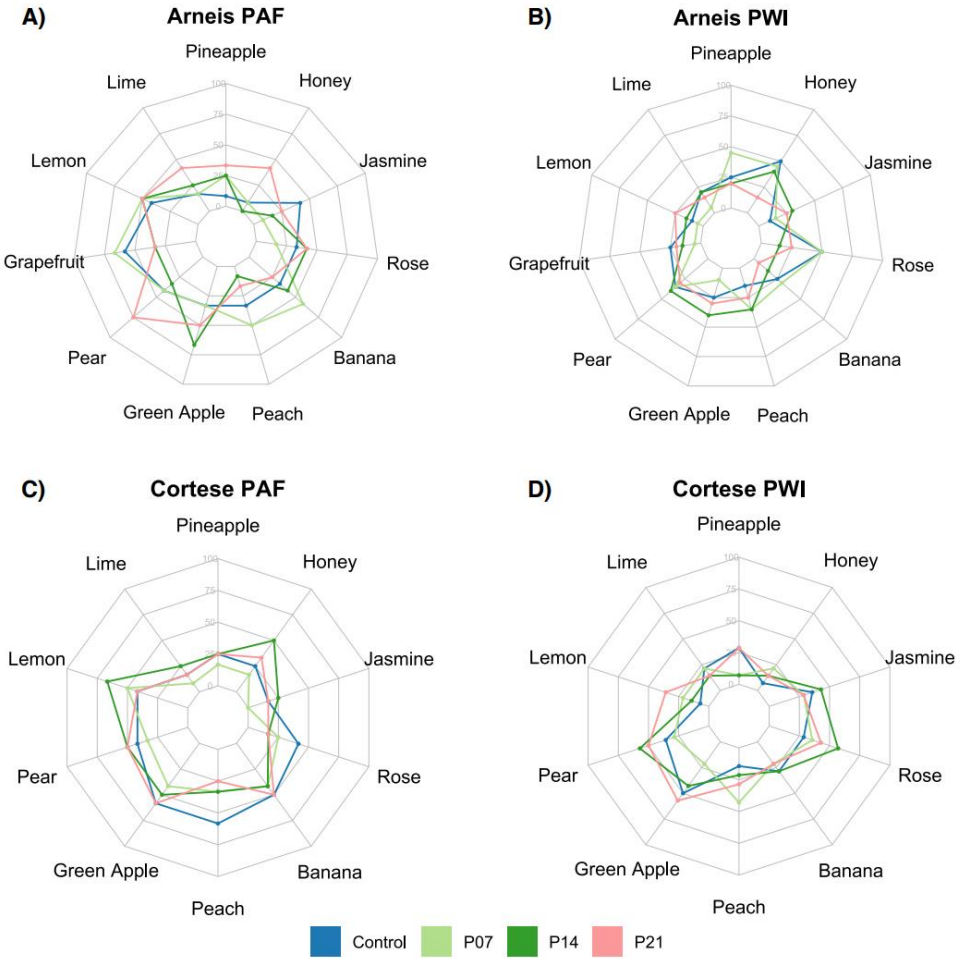

Figure 4. Sensory analysis results (percentage frequency) of aroma descriptors evaluated by Check-All-That-Apply method after the alcoholic fermentation (PAF) and one month after bottling (PWI). "Control", "P07", "P14" and "P21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for A) 'Arneis' after alcoholic fermentation, B) 'Arneis' one month after bottling, C) 'Cortese' after alcoholic fermentation, and D) 'Cortese' one month after bottling.

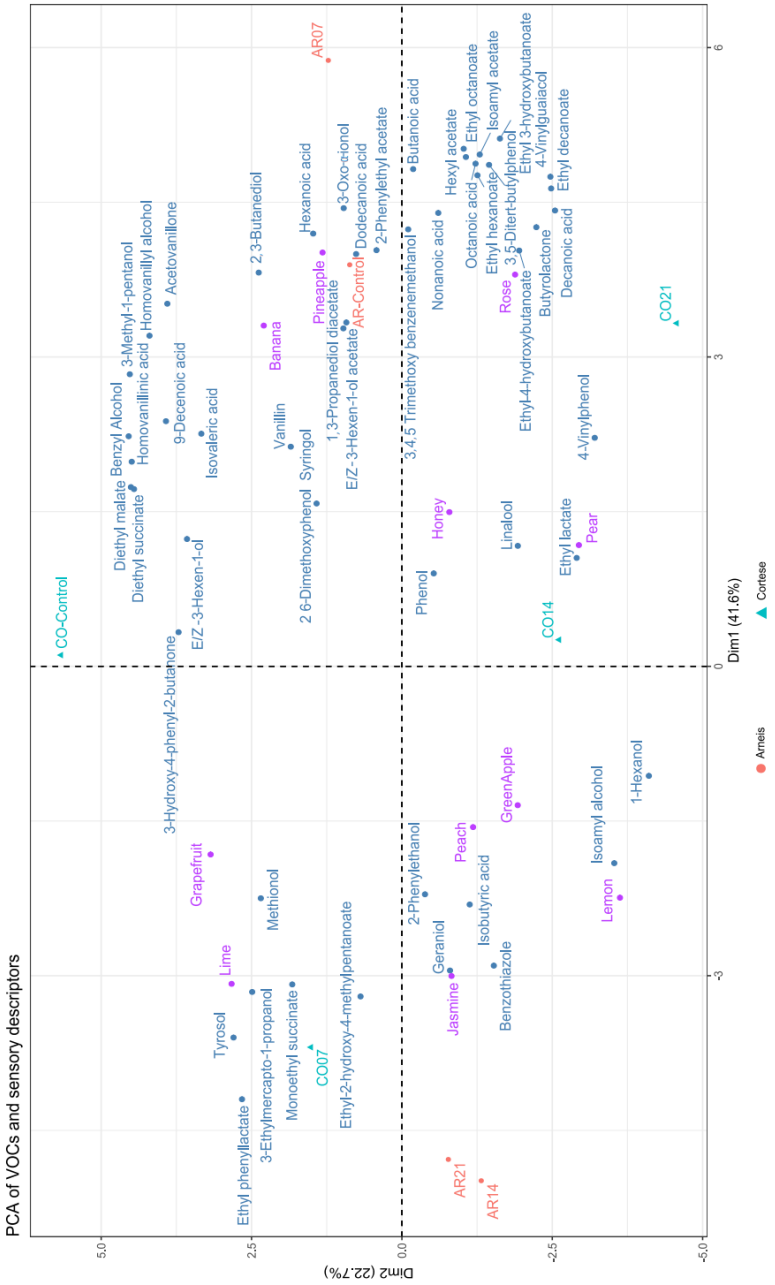

Figure 5. Principal Component Analysis of individual significant free VOCs (active variables, reported the 25 with higher contribution) and aroma sensory descriptors (supplementary variables). "CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths 7, 14 and 21 days, respectively, for 'Arneis' wines.

4. Conclusions

The cold liquid stabulation (CLS) technique gave contrasting results in the winemaking of ‘Arneis’ and ‘Cortese’ in terms of polyphenolic and aroma composition. Some chemical-physical parameters, such as total acidity, pH, and colour parameters of wines after one month of bottling changed similarly in the two varieties according to treatments, without affecting the related sensory features of mouthfeel and colour.

A major role of the variety on the effect of this oenological technique was highlighted for secondary metabolites. TPI increased in stabulated ‘Arneis’, while it decreased in ‘Cortese’, but these differences were limited and not perceivable by wine sensory analysis. Regarding VOCs, ‘Arneis’ non-treated control and a short CLS treatment (7 days) led to a higher accumulation of esters in wines whereas longer CLS (14 and 21 days) produced a major quantity of higher alcohols, and geraniol and benzenoids for the longest treatment. Instead, in ‘Cortese’, 14 and 21-day CLS led to wines with less C6-compounds but higher linalool after 14 days of CLS. Cortese overall wine quality rating increased in stabulated samples for 14 and 21 days.

This technique may be worth to be considered when starting from healthy white grapes but increased energy costs (due to refrigeration) and a continuous process control are necessary. Future research may concern the linkage between these results and the grape composition, as well an in-depth characterization of the solid residue – grape lees or *bourbes* – in terms of nitrogen-containing compounds, lipids, and polysaccharides, in connection with their extraction-adsorption phenomena.

Founding

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CRediT authorship contribution statement

Camilla De Paolis: Writing – original draft, Investigation, Formal analysis, Data curation. **Andrea Zava:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Maria Alessandra Paissoni:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Supervision, Writing – review & editing. **Susana Río Segade:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Giulia Motta:** Writing – review & editing, Formal analysis, Data curation. **Domen Škrab:** Writing – review & editing, Formal analysis, Data curation**. Sofia Beria d'Argentina:** Writing – review & editing, Formal analysis, Data curation. **Lorenzo Ferrero:** Writing – review & editing, Formal analysis, Data curation. **Simone Giacosa:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Vincenzo Gerbi:** Writing – review & editing, Supervision, Conceptualization. **Luca Rolle:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Table S1. Retention indexes calculated for each volatile compound using a C7-C30 n-alkanes certified reference material and reported in literature and the NIST database.

Volatile compound*	LRI (exp.)[#]	LRI (lit.)[§]	LRI (lit.)[¶]
Acetate esters			
Isoamyl acetate	1119	1115	-
(*E*/*Z*)-3-Hexen-1-ol acetate	1308	1313	-
1,3-Propanediol diacetate	1665	1660	-
2-Phenylethyl acetate	1815	1812	1811
Hexyl acetate	1286	1279	1270
Ethyl esters			
Ethyl hexanoate	1254	1240	1232
Ethyl octanoate	1445	1440	1440
Ethyl decanoate	1638	1638	1645
Ethyl 4-hydroxybutanoate	1804	1794	1800
Ethyl 3-hydroxybutanoate	1494	1501	1510
Ethyl 2-hydroxy-4-methylpentanoate	1536	1547	1547
Diethyl malate	2038	2060	2031
Ethyl lactate	1338	1340	-
Monoethyl succinate	2363	2368	2363
Diethyl succinate	1696	1672	1686
Ethyl phenyllactate	2247	2273	-
Higher alcohols			
3-Methyl-2-buten-1-ol	1328	1321	-
3-Methyl-3-buten-1-ol	1244	1254	-
1-Butanol	1147	1154	1140
1-Pentanol	1231	1258	1241
Isoamyl alcohol	1220	1225	1221
3-Methyl-1-pentanol	1319	1318	1316
2-Ethyl-1-hexanol	1515	1499	-
1-Octanol	1568	1569	-
1-Octen-3-ol	1474	1460	-
2,3-Butanediol	1547	1542	1543
2-Phenylethanol	1896	1914	1904
Volatile acids			
Isobutyric acid	1586	1580	1583
Butanoic acid	1623	1637	1638
Isovaleric acid	1685	1682	1682
Hexanoic acid	1855	1851	1848
Octanoic acid	2051	2067	2098
Nonanoic acid	2151	2174	2162

(continued on next page)

Table S1. (continued)

Decanoic acid	2269	2281	2279
9-Decenoic acid	2342	2348	2341
Dodecanoic acid	2498	2523	2489
Volatile sulphur compounds			
3-Ethylmercapto-1-propanol	1784	1802	1785
Methionol	1729	1714	1707
Benzothiazole	1959	1948	1956
C6 compound			
2-Hexenal	1210	1216	-
1-Hexanol	1367	1363	-
(*E*/Z)-2-Hexen-1-ol	1405	1394	-
(E/Z)-3-Hexen-1-ol	1390	1384	1386
Terpenes			
Linalool	1556	1547	1547
Geraniol	1841	1847	1844
Lilac alcohol	1830	-	-
(*E*/Z)-8-Hydroxygeraniol	2631	-	-
(*E*/Z)-pyran-Linalool oxide	1765	1732-56	-
Nerol	1753	1784	-
(*E*/Z)-8-Hydroxylinalool	2305	2251	2300
Geranic acid	2353	2347	-
Norisoprenoids			
3-Oxo-α-ionol	2644	2657	2641
3-Hydroxy-β-damascone	2563	2535	-
Benzenoids			
Benzenepropanol	2027	2058	-
Vanillin	2548	2560	-
Benzyl Alcohol	1864	1879	-
Homovanillinic acid	3057	3099	2992
Tyrosol	2980	2999	-
3,4,5-Trimethoxy-benzenemethanol	2720	-	-
α –Methylbenzyl alcohol	1795	1820	-
Methyl salicylate	1775	1753	-
Homovanillyl alcohol	2850	2830	-
Vanillyl alcohol	2801	2787	-
Acetovanillone	2654	2640	2650
2-Hydroxybenzeneethanol	2865	2839	-
3-Hydroxy-4-phenyl-2-butanone	2259	2259	-
Volatile phenols			
Eugenol	2164	2146	-
m-Cresol	2112	2081	-
3,5-Di*tert*-butylphenol	2330	2310	-

(continued on next page)

Table S1. (continued)

4-Vinylguaiacol	2192	2197	2200
Phenol	1991	2008	2000
2,6-Dimethoxyphenol (Syringol)	2284	2271	-
3,4,5-Trimethoxyphenol	3075	3060	-
p-Cresol	2096	2103	-
4-Vinylphenol	2409	2406	-
Lactones			
Butyrolactone	1647	1617	-

Note: *The volatile compounds are listed by chemical class. [#]Linear retention index determined using n-alkanes as external references. [§]Linear retention index reported in the NIST database for similar column type, stationary phase and separation conditions. [¶]Linear retention index reported in literature for similar column type and stationary phase (Boban et al., 2022).

Table S2. Aroma descriptors and selected reference standards for sensory analysis

Sensory descriptor	Standard
Aroma	
Pineapple	Pineapple pulp extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and commercial pineapple juice diluted 40 times in white wine
Lime	Lime peel extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and 20 times in white wine
Lemon	Lemon peel extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and 20 times in white wine
Grapefruit	Grapefruit peel extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and 20 times in white wine
Pear	Pear peel extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and commercial pear juice diluted 40 times in white wine
Green apple	Commercial green apple juice diluted 20 times in water and 10 times in white wine
Peach	Commercial peach juice diluted 10 times in white wine
Banana	Banana peel extract hydroalcoholic solution (40% of ethanol 96° Buongusto, Torriani) diluted 20 times in water and banana fruit (10 pieces, 10 g of weight) macerated 4 hours in white wine
Rose	Acqua alle Rose rosewater (Bolton Group S.r.l., Milan, Italy) in water and in white wine (5 drops in 200 mL)
Jasmine	Jasmine essential oil (4% Primavera Flora) in water and white wine (1 drop in 200 mL)
Honey	Commercial acacia honey in water and white wine (1 Tbsp in 200 mL)
Almond	Almond food additive (Paneangeli-Cameo, Desenzano del Garda, Italy) in white wine (10 drops in 200 mL)
Palate	
Bitterness	Caffeine (Sigma-Aldrich) 0.5 g/L in water and 1.2 g/L in white wine
Astringency	Tannic acid (Sigma-Aldrich) 1.0 g/L in water and 1.2 g/L in white wine
Body	Carboxymethylcellulose (Gioia group, COD.ART.SPCMC250, www.saporepuro.com) 1.5 g/L in white wine
Acidity	Tartaric acid (Sigma-Aldrich) 1.0 g/L in water and 1.2 g/L in white wine
Sweetness	White sugar (sucrose) 10.0 g/L in water

Table S3. Glycosylated Volatile Compounds (µg/L of 1-heptanol) of Arneis and Cortese juice after pressing.

		Arneis Juice	Cortese Juice
Chemical Class			
Higher alcohol	3-Methyl-2-buten-1-ol	66.31±8.46	47.1±3.98
	3-Methyl-3-buten-1-ol	62.73±13.82	32.84±2.84
	1-Butanol	25±8.26	94.69±10.87
	1-Pentanol	9.39±1.69	26.75±2.32
	Isoamyl alcohol	29.85±6.44	76.07±6.69
	2-Ethyl-1-hexanol	18.19±1.18	14.16±1.76
	1-Octanol	3.86±0.20	5.64±0.63
	1-Octen-3-ol	6.72±0.68	14.52±0.39
	2-Phenylethanol	741.24±30.54	555.40±10.57
	∑ Higher alcohols	936.28±18.51	867.16±16.41
Volatile acid	Butanoic acid	1.35±0.13	2.85±0.86
	Isovaleric acid	0.78±0.33	1.53±0.06
	Hexanoic acid	12.31±2.53	12.73±0.62
	Octanoic acid	2.89±0.58	2.93±0.20
	Nonanoic acid	6.91±1.00	5.40±0.49
	Decanoic acid	1.69±0.27	1.16±0.32
	Dodecanoic acid	31.84±4.59	13.96±2.07
	∑ Acids	57.76±1.28	40.55±1.85
C6 compound	1-Hexanol	48.51±4.26	128.27±4.08
	(*E*/*Z*)-2-Hexen-1-ol	9.32±0.68	9.67±0.59
	(*E*/*Z*)-3-Hexen-1-ol	13.58±1.20	29.16±2.25
	∑ C6 compounds	71.40±6.12	167.11±6.84
Volatile sulfur compound	Benzothiazole	43.90±3.42	0.84±0.16
Terpene	Linalool	0.00±0.00	41.32±0.72
	Geraniol	49.43±4.39	26.97±6.97
	Lilac alcohol	0.00±0.00	65.03±0.66
	(*E*/*Z*)-8-Hydroxygeraniol	19.58±1.04	27.62±1.86
	(*E*/*Z*)-pyran-Linalool oxide	7.78±0.74	9.15±1.55
	Nerol	7.48±0.16	15.46±1.39
	(*E*/*Z*)-8-Hydroxylinalool	98.47±6.35	263.64±11.02
	Geranic acid	90.41±5.44	17.70±0.71
	∑ Terpenes	273.16±17.39	466.90±3.89
Benzenoid	Benzenepropanol	1.81±0.10	4.96±0.15
	Vanillin	20.57±2.32	20.73±3.29
	Homovanillinic acid	268.06±27.72	142.02±18.01
	Tyrosol	293.19±20.48	73.75±10.82
	Benzyl alcohol	923.50±58.17	863.49±17.88
	3,4,5-Trimethoxy-benzenemethanol	29.92±1.88	28.74±1.16
	α –Methylbenzyl alcohol	5.67±0.39	2.98±0.13
	Methyl salicylate	15.00±0.82	33.53±0.59

(continued on next page)

Table S3. (continued)

	Homovanillyl alcohol	155.41±6.43	384.73±18.10
	Vanillyl alcohol	51.56±0.60	76.91±10.75
	Acetovanillone	42.92±3.49	119.95±3.26
	2-Hydroxybenzeneethanol	54.12±2.72	34.13±2.06
	∑ Benzenoids	1861.73±49.46	1785.92±69.18
Norisoprenoid	**3-Oxo-α-ionol**	417.65±26.45	155.93±6.42
	3-Hydroxy-β-damascone	112.73±8.54	39.72±1.45
	∑ Norisoprenoids	530.38±34.98	195.64±7.74
Volatile phenol	**Eugenol**	0.57±0.03	48.69±0.53
	m-Cresol	1.60±0.08	1.87±0.12
	3,5-Di*tert*-butylphenol	30.18±1.52	25.96±0.49
	4-Vinylguaiacol	528.73±68.39	808.62±83.64
	Phenol	10.78±0.25	24.27±4.48
	2,6-Dimethoxyphenol (Syringol)	3.79±0.50	16.86±4.47
	3,4,5-Trimethoxyphenol	59.82±4.57	39.62±1.21
	p-Cresol	4.36±0.27	2.54±0.03
	4-Vinylphenol	62.76±2.60	176.42±39.35
	∑ Volatile phenols	702.60±66.14	1144.84±117.98
Lactone	**Butyrolactone**	1.00±0.07	1.42±0.18
	Total glycosylated volatile compounds	4460.32±100.89	4668.13±178.92

Note: All data are expressed as average value ± standard deviation (n=3).

Table S4. Free Volatile Compounds (µg/L of 1-heptanol) of Arneis and Cortese control juice and CLS treated juice at the end of the stabulation (PS).

		Arneis					Cortese				
Chemical class	Compound	AR-Control	AR07	AR14	AR21	*Sign*	CO-Control	CO07	CO14	CO21	*Sig.*
Acetate ester	**Isoamyl acetate**	nd	nd	nd	nd		0.00±0.00	0.00±0.00	4.68±8.11	9.90±6.60	ns
Ethyl ester	**Ethyl hexanoate**	nd	nd	nd	nd		1.52±0.11	2.48±0.43	2.80±2.44	4.15±1.45	ns
	Ethyl octanoate	0.00±0.00 c	1.84±0.29 b	3.17±0.21 a	1.28±0.33 b	***	1.95±0.05	1.69±0.30	1.40±0.75	2.66±1.79	ns
	Ethyl decanoate	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	1.98±0.41 a	*	2.42±0.69 a	1.51±0.21 a	0.51±0.07 a	0.68±1.17 a	*
	Ethyl 4-hydroxybutanoate	nd	nd	nd	nd		2.12±0.46 a	1.99±0.09 ab	1.51±0.04 b	1.62±0.03 ab	*
	Diethyl succinate	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	ns	0.68±0.09 a	0.61±0.05 ab	0.51±0.04 b	0.52±0.04 b	*
	∑ Esters	0±0.00 c	1.84±0.29 b	3.17±0.21 a	3.26±0.66 a	***	8.69±1.41	8.29±0.98	11.40±11.17	19.53±6.89	ns
Higher alcohol	**3-Methyl-3-buten-1-ol**	1.23±1.04	1.74±0.47	1.21±0.03	1.16±0.02	ns	1.96±0.05 a	2.03±0.14 a	1.18±0.18 b	1.28±0.22 b	***
	1-Pentanol	4.81±2.63 a	1.26±0.06 a	1.39±0.19 a	1.46±0.10 a	*	nd	nd	nd	nd	
	Isoamyl alcohol	0.00±0.00 a	0.00±0.00 a	4.24±5.99 a	22.84±23.90 a	*	55.29±14.73	52.86±6.33	68.27±32.51	53.13±4.33	ns
	2-Ethyl-1-hexanol	23.51±0.90 a	13.38±2.03 b	5.35±2.14 c	0.00±0.00 d	***	nd	nd	nd	nd	
	2-Phenylethanol	45.59±2.06	43.44±0.67	48.47±7.85	53.98±3.99	ns	211.01±42.57	19.10±4.31	179.63±8.42	174.07±7.39	ns
	1-Octanol	3.35±0.37 a	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	***	nd	nd	nd	nd	
	1-Octen-3-ol	3.67±0.22 a	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	***	nd	nd	nd	nd	
	∑ Higher alcohols	82.15±3.70 a	59.81±2.38 a	60.67±15.83 a	79.44±27.99 a	*	268.26±57.25	245.99±2.18	249.08±41.00	228.48±11.36	ns
Volatile acid	**Butanoic acid**	5.5±0.83 a	1.42±0.19 b	1.67±0.09 b	1.67±0.11 b	*	3.17±0.75	3.18±0.25	2.58±0.23	2.28±0.42	ns
	Isovaleric acid	1.69±0.23	1.28±0.46	2.14±0.69	1.18±0.09	ns	4.46±1.41	4.65±0.40	4.81±1.75	3.49±0.38	ns
	Hexanoic acid	140.12±9.16 a	65.83±3.64 b	68.95±3.05 b	72.74±1.76 b	**	59.01±7.57	69.69±3.07	62.97±1.99	63.37±3.93	ns
	Octanoic acid	11.40±1.04	7.83±1.00	10.82±4.77	10.42±0.97	ns	11.59±3.10	13.81±0.94	15.10±0.79	18.59±3.55	ns
	Nonanoic acid	16.51±1.34 a	5.43±0.20 b	4.95±0.10 b	6.48±1.63 b	***	4.98±0.73 a	5.03±0.60 a	3.20±0.82 ab	2.64±0.81 b	*
	Decanoic acid	4.28±0.47	5.70±2.16	6.33±4.09	6.49±1.05	ns	4.43±0.80	3.73±0.15	3.79±0.54	5.72±3.56	ns
	Dodecanoic acid	2.14±0.36 c	13.67±1.30 b	14.33±1.45 ab	17.31±1.67 a	***	14.22±4.29	15.05±3.04	17.2±2.01	15.27±4.13	ns
	∑ Volatile acids	181.64±11.81 a	101.16±2.78 b	109.19±14.07 b	116.29±2.71 b	**	101.86±5.75	115.13±7.50	109.65±3.05	111.36±9.92	ns
C6 compound	**2-Hexenal**	311.61±183.08	363.61±7.36	328.02±30.54	312.73±8.20	ns	273.26±47.46 a	208.13±13.80 b	146.71±2.80 c	122.28±3.40 c	**
	1-Hexanol	196.63±31.92	211.31±1.97	217.95±7.94	216.03±5.61	ns.	207.62±17.62	207.19±20.24	201.71±3.61	205.65±4.86	ns
	(*E*/*Z*)-2-Hexen-1-ol	276.94±49.35	282.48±6.27	286.28±10.92	295.10±5.30	ns	458.57±59.85	439.89±18.33	431.66±7.09	446.29±2.71	ns
	(*E*/*Z*)-3-Hexen-1-ol	115.75±29.66	122.90±1.01	125.37±7.02	126.60±2.62	ns	115.60±19.38	108.09±2.84	104.68±3.23	106.20±2.45	ns

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Table S4. (continued)

	∑ C6 compounds	900.92±293.81	980.31±9.50	957.62±56.42	950.47±21.50	ns	1055.05±144.32	963.30±54.20	884.76±10.40	880.42±12.19	ns
Volatile sulphur compound	**Methionol**	nd	nd	nd	nd		0.21±0.06 a	0.24±0.01 a	0.00±0.00 b	0.00±0.00 b	***
	Benzothiazole	13.41±1.42 a	2.12±0.16 b	2.19±0.38 b	2.41±0.13 b	**	1.49±0.18 a	1.63±0.17 a	1.63±0.26 a	1.13±0.15 a	*
	∑ Volatile sulphur compounds	13.41±1.42 a	2.12±0.16 b	2.19±0.38 b	2.41±0.13 b	**	1.71±0.24 ab	1.87±0.18 a	1.63±0.26 ab	1.13±0.15 b	*
Terpene	**Linalool**	nd	nd	nd	nd		0.00±0.00 b	0.00±0.00 b	2.30±0.44 a	2.45±0.33 a	*
	Geraniol	nd	nd	nd	nd		3.81±0.94	3.24±0.23	3.09±0.14	2.88±0.24	ns
	∑ Terpenes	nd	nd	nd	nd		3.81±0.94 bc	3.24±0.23 c	5.40±0.35 a	5.33±0.52 ab	*
Norisoprenoid	**3-Oxo-α-ionol**	nd	nd	nd	nd		2.02±0.25	2.37±0.30	2.22±0.16	2.68±0.77	ns
Benzenoid	**Vanillin**	7.30±0.55 a	5.68±0.31 b	5.46±0.48 b	6.80±0.54 ab	**	7.38±1.10	6.85±0.07	6.59±0.20	6.09±0.65	ns
	Homovanillinic acid	3.11±0.37 c	8.09±0.77 b	8.75±0.20 ab	10.27±0.44 a	***	3.21±0.36	3.05±0.24	3.25±0.25	3.63±0.76	ns
	Tyrosol	12.32±1.66	10.27±1.65	12.17±3.04	12.46±0.78	ns	12.16±2.19	11.75±1.64	11.01±4.67	8.25±0.60	ns
	Benzyl alcohol	28.87±0.57	27.13±1.15	29.59±0.75	30.35±1.63	ns	26.93±5.35	25.99±1.45	22.19±0.41	21.12±0.76	ns
	Methyl salicylate	0.39±0.05	0.38±0.02	0.4±0.01	0.39±0.02	ns	0.28±0.04	0.29±0.04	0.28±0.03	0.28±0.02	ns
	Homovanillyl alcohol	11.15±1.15	9.42±0.12	11.22±2.31	10.46±0.33	ns	44.03±9.47	27.44±21.46	33.95±0.86	35.09±3.07	ns
	Acetovanillone	0.98±0.07	0.88±0.07	0.92±0.08	1.08±0.09	ns	3.73±0.26 b	5.50±0.54 a	5.74±0.21 a	6.36±0.53 a	**
	∑ Benzenoids	64.12±3.69	61.85±0.63	68.51±6.87	71.80±2.54	ns	97.72±17.96	80.87±23.22	83.01±3.79	80.82±5.83	ns
Volatile phenol	**m-Cresol**	1.13±0.10 b	1.71±0.07 a	1.66±0.20 a	1.54±0.09 a	**	1.71±0.29 a	1.67±0.03 a	1.21±0.01 b	1.10±0.03 b	***
	3,5-Di*tert*-butylphenol	26.16±2.53	30.47±3.24	28.05±1.61	34.97±6.49	ns	37.22±1.05 a	34.60±2.91 a	28.85±1.18 b	29.36±0.99 b	**
	4-Vinylguaiacol	0.40±0.10 d	11.46±1.14 c	22.10±1.78 b	28.10±1.97 b	**	17.42±6.56 c	45.12±6.78 b	53.73±4.64 ab	64.20±6.13 a	***
	Phenol	6.27±0.37	6.42±1.65	6.10±0.05	6.27±0.24	ns	7.85±1.59	7.17±0.38	5.54±1.02	7.32±0.56	ns
	2,6-Dimethoxyphenol (Syringol)	0.00±0.00 c	3.11±0.54 b	3.91±0.86 ab	4.90±0.67 a	*	1.01±0.20 b	1.69±0.30 a	1.89±0.12 a	1.96±0.10 a	**
	p-Cresol	1.33±0.07	1.45±0.03	1.40±0.21	1.29±0.07	ns	1.50±0.23 a	1.44±0.06 a	1.08±0.04 b	1.05±0.03 b	*
	4-Vinylphenol	2.58±0.83 c	33.31±8.92 c	76.44±12.32 b	120.95±22.59 a	*	11.07±4.74 b	31.95±7.60 ab	30.68±8.92 ab	35.27±5.56 a	*
	∑ Volatile phenols	37.87±3.13 c	87.93±8.54 b	139.64±17.02 b	198.02±29.37 a	***	77.77±1.15 b	123.63±17.30 a	122.98±12.66 a	140.27±11.01 a	**
Lactone	**Butyrolactone**	nd	nd	nd	nd		0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	0.47±0.04 a	***
	∑ Total free volatile compounds	1280.12±287.43	1295.03±12.25	1340.98±2.04	1421.69±33.05	ns	1616.89±217.26	1544.69±17.31	1470.14±57.95	1470.48±43.44	ns

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences between different samples, for each variety and sampling point, according to Tukey's HSD test. "CO- Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Arneis' wines.

Table S5. Glycosylated Volatile Compounds (µg/L of 1-heptanol) of Arneis and Cortese control juice and CLS treated juice at the end of the stabulation (PS).

		Arneis					Cortese				
		AR-Control	AR07	AR14	AR21	*Sign.*	CO-Control	CO07	CO14	CO21	*Sign.*
Chemical class	**Compound**										
Higher alcohol	**3-Methyl-2-buten-1-ol**	74.17±2.84	65.32±1.52	67.09±9.07	65.15±1.17	ns	41.74±4.84	40.94±1.71	37.82±3.86	39.79±1.55	ns
	3-Methyl-3-buten-1-ol	73.25±1.66	71.94±2.36	73.60±9.58	69.53±2.44	ns	27.99±2.95	26.96±1.68	24.08±2.83	28.04±0.09	ns
	1-Butanol	30.98±3.08	30.90±5.42	35.64±4.88	27.70±4.53	ns	74.73±9.50	74.03±4.79	64.93±10.33	76.63±1.87	ns
	1-Pentanol	10.68±0.40	10.88±0.23	10.65±1.38	10.58±0.79	ns	23.28±1.24	22.45±1.71	20.39±2.44	23.51±0.01	ns
	Isoamyl alcohol	35.32±0.81	29.03±4.61	34.06±5.04	28.34±4.55	ns	64.47±4.79	61.93±3.79	58.11±7.20	66.91±0.97	ns
	2-Ethyl-1-hexanol	17.42±0.36 a	8.46±0.93 c	12.71±2.77 b	7.83±0.37 c	***	12.68±0.48 a	14.00±2.20 a	0.00±0.00 b	0.00±0.00 b	*
	1-Octanol	3.61±0.13 a	2.72±0.15 bc	2.16±0.30 c	2.77±0.24 b	***	4.79±0.20 a	5.42±0.59 a	2.89±0.23 b	3.28±0.73 b	**
	1-Octen-3-ol	6.33±0.32 a	4.88±0.24 b	3.09±1.14 c	4.77±0.35 b	*	13.45±0.35	13.04±0.67	11.93±1.18	12.80±0.12	ns
	2-Phenylethanol	717.41±14.97 a	673.76±10.54 b	699.1±20.83 ab	676.73±10.48 b	*	521.23±27.85 b	532.55±8.64 b	618.67±29.34 a	573.98±16.48 ab	**
	∑ Higher alcohols	969.16±14.81 a	897.90±20.92bc	938.09±13.33ab	893.40±3.63 c	**	784.37±51.27	791.33±20.90	838.83±3.97	824.94±18.70	ns
Volatile acid	**Butanoic acid**	1.32±0.26	1.14±0.25	1.26±0.13	1.09±0.04	ns	1.45±0.01	1.99±0.14	5.00±2.86	3.00±0.37	ns
	Isovaleric acid	1.35±0.25 a	0.86±0.17 ab	0.75±0.14 b	0.82±0.12 b	*	1.54±0.14	1.52±0.17	1.87±0.30	1.96±0.01	ns
	Hexanoic acid	11.71±0.40 a	12.30±1.47 a	11.09±0.36 a	13.46±0.19 a	*	12.80±1.64 b	12.94±1.30 b	23.44±3.18 a	19.39±1.01 a	**
	Octanoic acid	2.47±0.14 ab	2.10±0.20 b	2.73±0.05 a	2.38±0.20 ab	*	2.50±0.44 b	2.53±0.25 b	6.85±0.74 a	5.61±0.43 a	***
	Nonanoic acid	5.71±0.22 a	3.43±0.39 b	4.26±0.32 b	3.69±0.67 b	**	4.04±0.64 ab	4.64±0.37 a	3.56±0.60 ab	2.97±0.04 b	*
	Decanoic acid	1.64±0.30 a	0.95±0.10 b	1.23±0.10 ab	0.84±0.14 b	**	0.92±0.08 b	1.16±0.10 ab	1.39±0.21 a	1.28±0.06 ab	*
	Dodecanoic acid	25.77±0.83 a	15.81±1.72 b	19.57±5.49 ab	18.01±2.79 b	*	13.35±0.49 b	18.34±3.32 b	42.23±9.18 a	41.82±3.78 a	**
	∑ Volatile acids	49.95±0.38 a	36.60±3.61 b	40.89±4.92 ab	40.30±3.50 b	**	36.59±2.62 b	43.13±3.10 b	84.34±8.75 a	76.03±2.08 a	***
C6 compound	**2-Hexenal**	nd	nd	nd	nd		1.04±0.42	1.44±0.49	1.03±0.21	1.85±0.13	ns
	1-Hexanol	51.84±0.73 a	47.69±1.18 ab	44.31±3.04 b	48.16±1.57 ab	*	120.30±3.82	117.09±5.08	108.44±6.25	117.43±1.40	ns
	(*E*/*Z*)-2-Hexen-1-ol	10.09±0.16	8.91±0.88	8.38±0.18	9.10±0.69	ns	7.68±0.53	8.49±1.73	10.00±1.81	10.14±1.30	ns
	(*E*/*Z*)-3-Hexen-1-ol	14.76±0.41 a	12.30±0.67 a	12.13±2.15 a	12.41±0.38 a	*	26.21±1.62	25.54±1.61	24.71±2.08	27.62±0.53	ns
	∑ C6 compounds	76.68±1.05 a	68.89±2.41 b	64.83±5.01 b	69.68±2.61 ab	*	155.22±4.54	152.56±8.28	144.19±9.87	157.03±0.30	ns
Volatile sulfur compound	**Methionol**	nd	nd	nd	nd		0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	0.27±0.04 a	***
Terpene	**Linalool**	nd	nd	nd	nd		39.02±0.99	37.18±1.90	40.66±2.32	40.33±0.10	ns
	Geraniol	43.66±1.93	41.48±6.25	28.86±0.19	40.40±7.01	ns	28.90±2.24 a	20.77±0.47 b	20.62±3.29 b	16.70±1.88 b	**

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Table S5. (continued)

	Lilac alcohol	nd	nd	nd	nd		59.12±2.34 b	60.82±2.90 b	73.44±6.28 a	65.37±0.91 ab	*
	(*E*/*Z*)-8-Hydroxygeraniol	17.20±0.86	16.46±0.74	15.58±1.60	16.78±0.49	ns	25.08±1.32 b	26.68±1.16 b	35.66±1.65 a	33.42±1.34 a	***
	(*E*/*Z*)-pyran-Linalool oxide	7.24±0.27 a	5.25±0.07 c	6.03±0.07 b	5.74±0.82 b	***	6.80±0.73 c	7.28±0.30 bc	10.69±0.90 ab	11.26±3.00 a	*
	Nerol	6.87±0.17	8.05±2.33	4.06±0.15	7.67±2.06	ns	16.20±1.26 a	13.61±0.51 b	15.27±0.40 ab	13.32±0.39 b	**
	(*E*/*Z*)-8-Hydroxylinalool	89.81±1.31	83.83±2.13	81.62±6.64	85.74±1.14	ns	246.22±8.26 b	246.09±13.60 b	286.68±13.12 a	257.53±13.86 ab	*
	Geranic acid	83.90±0.69 a	65.37±1.78 b	59.99±3.04 c	68.79±0.12 b	***	16.16±0.41c	18.1±0.90 b	23.31±0.11 a	21.80±0.73 a	**
	∑ Terpenes	248.69±4.49 a	220.44±5.31 b	196.14±11.38 c	225.12±8.38 b	***	437.49±11.46 b	430.52±11.09 b	506.32±15.16 a	459.73±13.40 b	***
Norisoprenoid	3-Oxo-α-ionol	389.89±9.50 a	290.96±9.32 b	299.29±15.56 b	301.40±7.55 b	***	141.75±3.36 c	160.51±8.31 b	197.86±6.32 a	184.80±2.41 a	***
	3-Hydroxy-β-damascone	107.27±2.48 a	90.72±4.61 b	96.11±4.67 b	107.07±2.47 a	**	37.97±0.69 d	46.43±1.64 c	61.67±3.32 a	54.82±1.99 b	***
	∑ Norisoprenoids	497.16±10.98 a	381.68±13.88 b	395.40±20.23 b	408.47±9.99 b	***	179.72±3.83 c	206.95±9.39 b	259.53±9.44 a	239.62±4.40 a	***
Benzenoid	Benzenepropanol	1.76±0.04 a	1.47±0.13 bc	1.23±0.11 c	1.60±0.08 ab	**	4.52±0.16	4.58±0.23	4.81±0.04	4.38±0.06	ns
	Vanillin	20.30±0.73 a	15.04±1.85 bc	12.35±1.87 c	17.44±0.57 ab	**	22.28±3.60 b	36.55±13.48 ab	48.82±3.14 a	48.53±1.46 a	**
	Homovanillinic acid	237.76±6.82 a	92.64±12.01 bc	75.10±6.09 c	108.14±8.25 b	***	134.29±4.86 c	142.47±32.62bc	207.61±8.15 a	192.62±3.20 ab	***
	Tyrosol	255.63±7.03 a	115.59±6.23 b	106.50±33.08 b	147.55±5.04 b	***	68.73±10.08 b	77.10±14.14 ab	103.69±6.80 a	98.90±3.72 ab	*
	Benzyl alcohol	922.43±31.15 b	958.31±33.03 b	1065.06±35.60a	941.28±39.23 b	*	838.68±71.99 b	845.41±45.43 b	994.36±35.22 a	930.11±38.41 ab	*
	3,4,5-Trimethoxy-benzenemethanol	27.50±0.71 a	16.61±0.91 bc	14.97±0.50 c	17.52±0.25 b	***	26.66±0.59 b	27.05±3.07 b	32.79±1.54 a	30.36±0.28 ab	**
	α –Methylbenzyl alcohol	5.33±0.09 a	3.70±0.10 c	4.15±0.03 b	3.82±0.07 c	***	2.60±0.19	2.68±0.17	2.95±0.09	2.93±0.09	ns
	Methyl salicylate	14.31±0.20 a	10.66±0.23 b	7.71±0.08 c	10.97±0.89 b	***	30.81±1.20	30.28±3.30	32.72±2.02	34.47±0.18	ns
	Homovanillyl alcohol	144.48±1.56 a	87.12±4.61 bc	84.61±3.32 c	93.68±1.12 b	***	339.96±47.89 b	334.39±12.74 b	448.51±18.42 a	410.55±6.71 ab	**
	Vanillyl alcohol	47.30±1.70 a	25.31±2.29 b	24.71±1.03 b	26.65±0.78 b	***	68.96±14.95	72.77±11.30	67.31±6.26	64.52±4.85	ns
	Acetovanillone	38.90±0.91 a	29.45±0.74 b	26.43±1.30 c	30.93±0.78 b	***	101.43±9.54 b	114.71±6.45 b	167.16±7.94 a	153.12±3.90 a	***
	2-Hydroxybenzeneethanol	50.17±0.23 a	29.66±1.84 b	28.43±1.27 b	31.49±0.13 b	***	29.44±4.46 b	30.37±0.99 b	38.44±1.24 a	35.26±1.63 ab	*
	∑ Benzenoids	1765.86±28.68 a	1385.56±22.03b	1451.26±84.06 b	1431.08±28.15 b	**	1668.37±166.81c	1717.88±46.40 bc	2149.17±82.44 a	2005.74±28.06 ab	**
Volatile phenol	m-Cresol	1.55±0.11	1.38±0.12	1.46±0.06	1.52±0.07	ns	1.65±0.13 b	1.72±0.14 b	2.03±0.09 a	1.97±0.02 ab	*
	Eugenol	0.51±0.03 c	0.64±0.05 ab	0.57±0.04 bc	0.74±0.05 a	**	44.60±0.98 b	47.44±2.67 b	53.73±2.82 a	48.94±1.00 ab	**
	3,5-Di*tert*-butylphenol	25.58±0.23 b	28.45±0.94 a	29.08±1.28 a	29.39±0.62 a	**	24.50±0.86 c	28.54±2.31 b	36.53±1.23 a	35.21±0.16 a	***
	4-Vinylguaiacol	728.77±88.57 a	436.24±158.02 b	645.89±52.64 ab	426.28±76.46 b	*	812.10±107.42 b	821.69±90.84 b	1097.16±116.43 a	1060.99±12.94 a	*
	Phenol	11.97±0.70	12.59±2.28	14.59±0.30	14.02±1.83	ns	19.01±4.63 b	19.81±1.60 b	38.33±7.00 a	35.26±1.15 a	**
	2,6-Dimethoxyphenol (Syringol)	5.48±0.56	7.03±2.21	7.04±1.21	9.00±1.86	ns	16.15±3.17 b	16.39±2.91 b	31.46±3.87 a	31.76±0.83 a	***
	3,4,5-Trimethoxyphenol	55.53±1.97 a	38.68±2.08 b	33.14±0.50 c	40.89±0.85 b	***	34.96±2.82 b	37.55±2.18 b	50.27±2.29 a	47.95±1.10 a	***

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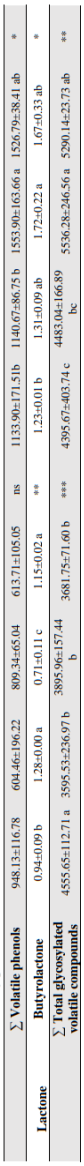
Table S5. (continued)



	p-Cresol	3.98±0.16 a	3.51±0.13 b	3.43±0.15 b	3.58±0.06 b	**	2.14±0.24	2.27±0.07	2.47±0.10	2.26±0.08	ns
	4-Vinylphenol	114.76±29.57	75.94±31.13	74.13±11.42	88.29±26.17	ns	178.79±54.11	165.26±9.25	241.92±46.58	262.45±23.34	ns
	∑ Volatile phenols	948.13±116.78	604.46±196.22	809.34±65.04	613.71±105.05	ns	1133.90±171.51b	1140.67±86.75 b	1553.90±163.66 a	1526.79±38.41 ab	*
Lactone	**Butyrolactone**	0.94±0.09 b	1.28±0.00 a	0.71±0.11 c	1.15±0.02 a	**	1.23±0.01 b	1.31±0.09 ab	1.72±0.22 a	1.67±0.33 ab	*
	∑ Total glycosylated volatile compounds	4555.65±112.71 a	3595.53±236.97 b	3895.96±157.44 b	3681.75±71.60 b	***	4395.67±403.74 c	4483.04±166.89 bc	5536.28±246.56 a	5290.14±23.73 ab	**

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences among different samples, for each variety and sampling point, according to Tukey's HSD test. "CO- Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Arneis' wines.

Table S6. Free Volatile Compounds (µg/L of 1-heptanol) of Arneis and Cortese control wines and CLS treated wine at the end of the alcoholic fermentation (PAF).

		Arneis					Cortese				
Chemical class	Compound	AR-Control	AR07	AR14	AR21	Sign.	CO-Control	CO07	CO14	CO21	Sign.
Acetate ester	Isoamyl acetate	4311.22±53.14 a	3794.16±109.80 b	3261.54±146.75c	3045.51±83.99 c	***	3710.86±108.20 a	3156.48±128.40b	3773.35±56.60 a	3915.69±57.49a	***
	(E/Z) 3-Hexen-1-ol acetate	184.06±6.86 b	207.09±5.52 a	139.07±0.57 c	143.98±3.72 c	***	71.15±6.29 a	60.05±1.92 b	73.51±4.53 a	66.59±2.14 ab	*
	1,3-Propanediol diacetate	86.25±4.71 a	86.14±3.64 a	75.79±1.03 ab	74.31±3.47 b	**	65.50±4.86 ab	54.39±0.74 b	66.98±6.50 a	71.24±2.51 a	**
	2-Phenylethyl acetate	1896.41±24.31 a	1618.23±19.30 b	1584.50±8.21 b	1565.96±40.45 b	***	1504.63±54.82 a	1203.93±42.06 b	1450.55±65.92 a	1413.90±39.34 a	***
	Hexyl acetate	648.09±9.48 a	626.06±15.07 a	489.80±11.78 b	456.34±6.95 b	***	361.06±13.56 a	299.74±8.64 b	376.80±13.17 a	360.82±10.80 a	**
Ethyl ester	Ethyl hexanoate	1346.90±10.22 a	1328.08±23.55 a	1081.89±38.78 c	1165.91±31.40 b	***	838.25±240.59	1106.74±15.22	1100.25±76.28	1039.91±71.13	ns
	Ethyl octanoate	1864.24±47.75 b	2012.38±52.51 a	1634.92±21.03 c	1744.96±59.26 bc	***	1544.48±25.05 a	1363.95±52.14 b	1573.79±34.28 a	1575.28±2.94 a	***
	Ethyl decanoate	654.82±43.24 b	827.14±28.35 a	669.83±23.60 b	731.31±30.51 b	**	640.46±34.29 a	475.70±54.15 b	678.59±31.67 ab	718.59±23.17 a	***
	Ethyl 4-hydroxybutanoate	1909.10±89.35 ab	2156.36±53.12 a	1616.96±122.76 b	2008.90±144.42 a	**	1097.58±49.02 a	844.93±36.62 b	1087.34±104.93 a	1122.71±48.83 a	**
	Ethyl 3-hydroxybutanoate	151.94±6.93 b	168.14±3.96 a	115.24±4.66 c	120.15±3.85 c	***	124.33±2.11 b	107.62±3.51 c	131.09±11.48 b	149.61±2.04 a	***
	Ethyl 2-hydroxy-4-methylpentanoate	30.53±2.51	33.58±4.27	33.72±2.84	37.91±4.72	ns	28.00±0.97 ab	29.11±1.74 ab	35.46±3.42 a	26.29±4.22 b	*
	Diethyl malate	14.81±0.79 b	11.64±0.13 c	12.73±0.60 bc	20.02±1.62 a	***	11.87±0.22 bc	19.00±1.57 a	12.92±1.55 b	9.57±0.29 c	***
	Ethyl lactate	508.64±14.06 a	585.76±36.34 a	588.64±43.05 a	589.74±30.47 a	*	519.65±12.61 c	709.79±53.51 a	652.42±58.59 ab	563.94±22.53 bc	**
	Monoethyl succinate	1872.14±57.79 ab	1740.71±63.19 b	1930.53±169.30 ab	2068.56±140.80 a	*	1777.22±62.85 b	2259.09±246.62a	1740.50±174.56 b	1507.53±56.56 b	*
	Diethyl succinate	145.15±11.60 ab	119.78±12.86 b	123.92±4.39 b	169.83±12.17 a	**	128.28±6.80 a	135.48±17.45 a	126.36±13.44 a	89.51±5.68 b	**
	Ethyl phenyllactate	388.59±11.03 a	73.83±2.99 d	109.63±0.81 c	144.09±8.32 b	***	113.37±4.99 a	101.32±11.94 a	102.72±7.49 a	77.68±3.24 b	**
	∑ Esters	16012.89±153.43 a	15389.10±154.84 a	13468.73±247.81 b	14087.49±357.65 b	***	12536.70±156.94 ab	11927.30±435.71 b	12982.64±338.03 a	12708.87±28.61 a	*
C6 compound	1-Hexanol	1628.60±24.96 a	1480.02±49.93 b	1653.39±52.39 a	1609.25±39.65 a	**	1003.00±44.14 a	1016.91±11.94 a	888.14±10.89 b	900.76±3.93 b	***
	(E/Z)-3-Hexen-1-ol	1217.61±23.75	1164.49±39.69	1177.59±40.05	1171.15±26.88	ns	358.11±7.78	335.52±4.35	332.85±26.16	343.16±6.88	ns
	∑ C6 compounds	2846.21±47.28 a	2644.50±89.05 b	2830.98±92.44 ab	2780.40±66.50 ab	*	1361.11±45.84 a	1352.43±13.07 a	1220.98±30.31 b	1243.91±4.16 b	***
Higher alcohol	Isoamyl alcohol	11045.95±112.72	9921.41±588.13	10355.02±762.66	10077.51±333.64	ns	8914.10±676.99 b	10287.27±421.39 a	9191.19±177.99 ab	9009.95±218.56 b	*
	3-Methyl-1-pentanol	207.58±11.15 b	246.65±2.04 a	179.56±2.15 c	170.88±8.42 c	***	246.28±12.52 a	206.96±2.53 b	188.10±1.77 c	166.13±4.24 d	***

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Table S6. (continued)

	2-Ethyl-1-hexanol	18.16±1.19 b	28.47±3.24 a	24.20±0.62 ab	20.92±1.64 b	**	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	ns
	1-Octanol	12.23±0.45 a	10.01±0.37 b	12.37±0.13 a	12.77±1.13 a	**	16.02±1.28	14.58±1.13	14.71±0.92	13.68±0.26	ns
	2,3-Butanediol	10.42±4.01	53.97±60.85	100.59±22.70	115.00±93.69	ns	56.37±29.01 ab	75.20±23.03 a	0.00±0.00 b	32.39±24.09 ab	*
	2-Phenylethanol	8698.79±295.61 a	6772.72±441.16 b	7906.76±738.85 ab	8986.23±305.99 a	**	7073.13±467.87 a	7158.35±92.34 a	6339.89±369.62 ab	6121.24±189.31 b	**
	∑ Higher alcohols	19993.13±373.79 a	17033.23±972.19 b	18758.50±1476.10 ab	19383.31±627.36 a	*	16305.90±1123.74 ab	17742.36±420.96 a	15733.89±454.28 b	15343.39±419.82 b	*
	Isobutyric acid	144.95±27.02 c	238.52±13.71 b	347.00±12.29 a	319.51±25.28 a	***	290.61±5.78 a	265.09±3.32 a	249.17±42.14 a	241.38±13.64 a	*
	Butanoic acid	135.85±20.16 c	281.48±23.58 a	224.77±1.54 b	221.10±6.48 b	***	254.07±10.17	218.53±10.40	218.07±39.17	243.72±19.98	ns
	Isovaleric acid	850.67±3.70	892.18±19.08	923.47±26.98	886.09±35.19	ns	863.42±40.47 ab	894.62±7.59 a	853.50±49.42 ab	806.21±5.19 b	***
	Hexanoic acid	3512.81±33.65 a	3018.88±113.76 b	3054.20±82.92 b	3423.97±33.64 a	***	3240.06±55.84 a	2844.54±66.95 b	2792.19±198.47 b	2825.38±40.17 b	**
Volatile acid	**Octanoic acid**	3984.59±28.62 a	3795.25±120.32 a	3244.96±146.86 b	3442.80±142.65 b	***	3205.67±135.34	3252.97±124.28	3383.36±120.13	3359.60±64.27	ns
	Nonanoic acid	14.85±2.61	11.61±1.65	12.09±0.55	14.98±1.72	ns	8.85±1.10 b	11.11±1.43 b	11.01±0.35 b	16.33±2.57 a	**
	Decanoic acid	1752.33±44.71 a	1403.50±53.74 b	1344.41±156.71 b	1193.10±75.62 b	**	1142.81±406.23	1183.95±45.42	1669.12±149.65	1691.49±145.26	ns
	9-Decenoic acid	95.56±17.80 ab	103.67±10.40 ab	67.22±13.60 b	105.35±3.71 a	*	38.11±9.49 a	18.61±1.83 bc	10.67±1.76 c	25.01±2.99 ab	**
	Dodecanoic acid	97.67±5.86 ab	89.64±8.69 b	107.73±3.54 ab	115.46±12.79 a	*	90.58±12.73 a	65.23±6.47 b	64.99±5.76 b	80.13±11.50 ab	*
	∑ Volatile acids	10589.28±105.08 a	9834.72±278.09 b	9325.85±413.62 b	9722.36±303.93 b	**	9134.17±502.01	8754.65±159.25	9252.09±375.49	9289.23±220.58	ns
	3-Ethylmercapto-1-propanol	18.60±0.48 c	20.19±0.29 c	24.26±1.19 b	27.22±0.62 a	**	27.01±0.38 a	18.91±1.81 b	20.53±2.27 b	18.31±0.90 b	***
Volatile sulfur compound	**Methionol**	131.46±9.30 c	146.94±5.73 c	184.91±2.44 b	217.65±11.29 a	***	135.30±3.36 a	91.98±4.45 b	93.30±9.72 b	97.22±5.65 b	***
	Benzothiazole	4.39±0.45 c	33.40±1.73 b	39.65±0.61 a	6.38±1.16 c	***	3.76±2.20	4.27±0.46	6.99±3.11	3.66±0.75	ns
	∑ Volatile sulfur compounds	154.44±9.70 c	200.52±4.09 b	248.82±4.25 a	251.25±11.95 a	***	166.07±3.62 a	115.17±6.61 b	120.82±14.17 b	119.19±6.86 b	***
Terpene	**Linalool**	nd	nd	nd	nd		28.82±2.34 bc	37.18±0.84 a	32.61±1.33 b	27.97±1.23 c	***
	Geraniol	8.79±0.17	9.92±1.05	10.46±0.78	10.93±1.08	ns	15.08±1.35	16.06±0.95	16.56±0.66	16.41±0.59	ns
	∑ Terpenes	8.79±0.17	9.92±1.05	10.46±0.78	10.93±1.08	ns	43.90±1.09 c	53.25±0.85 a	49.17±0.68 b	44.39±1.31 c	***
Norisoprenoid	**3-Oxo-α-ionol**	22.64±1.01 a	16.6±0.45 b	12.1±0.44 c	13.22±0.63 c	***	23.17±1.13 b	18.65±1.38 c	23.63±1.72 b	29.17±1.20 a	***
	Vanillin	3.07±0.42 ab	2.45±0.38 b	2.80±0.11 ab	3.32±0.03 a	*	4.09±0.08 b	3.78±0.16 b	4.90±0.76 ab	6.26±0.83 a	**
	Homovanillinic acid	28.47±1.32 ab	30.92±1.96 a	24.58±1.65 b	26.62±1.19 b	*	30.01±0.49 a	23.11±4.78 b	31.76±1.11 a	28.10±0.78 ab	*
	Tyrosol	4584.49±54.03 a	3729.95±385.10 b	4301.05±492.65 ab	3840.41±204.58 ab	*	3346.30±190.80 ab	3531.94±9.56 a	3050.64±249.80 b	2990.65±104.28 c	*
Benzenoid	**Benzyl alcohol**	21.74±1.69 b	23.40±0.81 ab	23.01±0.52 ab	25.55±1.40 a	*	24.89±0.81 a	23.45±2.33 a	27.56±1.44 a	27.67±1.92 a	*
	3,4,5-Trimethoxy-benzenemethanol	14.39±0.82 a	11.75±0.19 b	7.75±0.47 c	8.78±0.36 c	***	17.13±0.73 b	14.57±1.40 b	16.50±1.94 b	21.34±1.03 a	**
	Methyl salicylate	0.00±0.00 b	0.00±0.00 b	0.88±0.17 a	0.86±0.21 a	**	2.24±0.20 ab	1.09±0.36 c	2.42±0.23 a	1.49±0.52 bc	**

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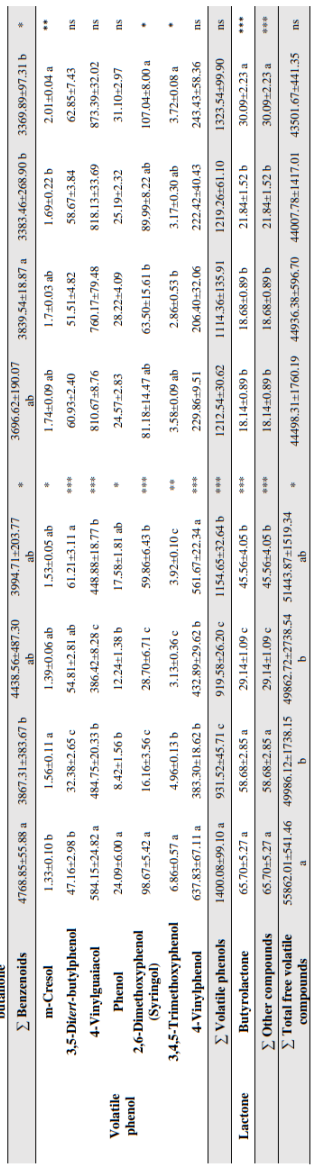
Table S6. (continued)



	Homovanillyl alcohol	16.96±1.20 a	15.86±0.70 a	13.05±0.37 b	13.69±0.12 b	**	60.66±1.07 ab	49.57±4.74 b	53.13±6.74 ab	62.77±3.60 a	*
	Acetovanillone	40.84±1.40 a	34.16±0.41 b	29.65±1.24 c	32.05±0.64 bc	***	173.07±5.44 ab	154.05±7.73 b	167.64±19.37 ab	191.25±1.87 a	**
	3-Hydroxy-4-phenyl-2-butanone	58.90±2.27 a	28.82±2.69 d	35.79±1.05 c	43.44±1.21 b	***	38.24±4.59 a	37.99±3.57 a	28.93±1.97 b	40.37±1.08 a	**
	∑ Benzenoids	4768.85±55.88 a	3867.31±383.67 b	4438.56±487.30 ab	3994.71±203.77 ab	*	3696.62±190.07 ab	3839.54±18.87 a	3383.46±268.90 b	3369.89±97.31 b	*
	m-Cresol	1.33±0.10 b	1.56±0.11 a	1.39±0.06 ab	1.53±0.05 ab	*	1.74±0.09 ab	1.7±0.03 ab	1.69±0.22 b	2.01±0.04 a	**
	3,5-Di*tert*-butylphenol	47.16±2.98 b	32.38±2.65 c	54.81±2.81 ab	61.21±3.11 a	***	60.93±2.40	51.51±4.82	58.67±3.84	62.85±7.43	ns
	4-Vinylguaiacol	584.15±24.82 a	484.75±20.33 b	386.42±8.28 c	448.88±18.77 b	***	810.67±8.76	760.17±79.48	818.13±33.69	873.39±32.02	ns
Volatile phenol	**Phenol**	24.09±6.00 a	8.42±1.56 b	12.24±1.38 b	17.58±1.81 ab	*	24.57±2.83	28.22±4.09	25.19±2.32	31.10±2.97	ns
	2,6-Dimethoxyphenol (Syringol)	98.67±5.42 a	16.16±3.56 c	28.70±6.71 c	59.86±6.43 b	***	81.18±14.47 ab	63.50±15.61 b	89.99±8.22 ab	107.04±8.00 a	*
	3,4,5-Trimethoxyphenol	6.86±0.57 a	4.96±0.13 b	3.13±0.36 c	3.92±0.10 c	**	3.58±0.09 ab	2.86±0.53 b	3.17±0.30 ab	3.72±0.08 a	*
	4-Vinylphenol	637.83±67.11 a	383.30±18.62 b	432.89±29.62 b	561.67±22.34 a	***	229.86±9.51	206.40±32.06	222.42±40.43	243.43±58.36	ns
	∑ Volatile phenols	1400.08±99.10 a	931.52±45.71 c	919.58±26.20 c	1154.65±32.64 b	***	1212.54±30.62	1114.36±135.91	1219.26±61.10	1323.54±99.90	ns
Lactone	**Butyrolactone**	65.70±5.27 a	58.68±2.85 a	29.14±1.09 c	45.56±4.05 b	***	18.14±0.89 b	18.68±0.89 b	21.84±1.52 b	30.09±2.23 a	***
	∑ Other compounds	65.70±5.27 a	58.68±2.85 a	29.14±1.09 c	45.56±4.05 b	***	18.14±0.89 b	18.68±0.89 b	21.84±1.52 b	30.09±2.23 a	***
	∑ Total free volatile compounds	55862.01±541.46 a	49986.12±1738.15 b	49862.72±2738.54 b	51443.87±1519.34 ab	*	44498.31±1760.19	44936.38±596.70	44007.78±1417.01	43501.67±441.35	ns

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences among different samples, for each variety and sampling point, according to Tukey's HSD test. "CO- Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Arneis' wines.

Table S7. Glycosylated Volatile Compounds (µg/L of 1-heptanol) of Arneis and Cortese control wines and CLS treated wine at the end of the alcoholic fermentation (PAF).

		Arneis					Cortese					
		AR-Control	AR07	AR14	AR21	*Sign.*	CO-Control	CO07	CO14	CO21	*Sign.*
Chemical class												
Ethyl ester	**Ethyl octanoate**	1.33±0.46	1.47±1.10	0.47±0.19	0.33±0.10	ns	0.86±0.14	0.85±0.17	0.44±0.31	0.47±0.15	ns
	Ethyl decanoate	1.13±0.57	1.67±1.24	0.53±0.14	0.70±0.10	ns	1.34±0.15	1.22±0.29	0.88±0.27	0.71±0.14	ns
	Ethyl 4-hydroxybutanoate	1.96±0.20 b	2.17±0.27 b	1.21±0.19 b	4.15±0.63 a	***	7.33±7.12	2.14±0.52	52.23±77.15	4.39±2.24	ns
	Monoethyl succinate	nd	nd	nd	nd		0.00±0.00	0.00±0.00	4.72±4.13	0.00±0.00	ns
	∑ Esters	4.41±1.19 a	5.31±2.50 a	2.21±0.13 a	5.18±0.77 a	*	9.52±7.27	4.24±0.91	58.26±81.72	5.58±2.24	ns
Higher alcohol	**3-Methyl-2-buten-1-ol**	52.47±3.49	48.69±5.40	43.04±5.52	41.72±1.46	ns	26.85±2.42	33.43±5.58	25.31±1.27	27.40±3.25	ns
	3-Methyl-3-buten-1-ol	54.08±4.38	52.34±5.78	44.14±4.83	43.53±2.09	ns	16.60±1.63	21.30±3.17	16.00±0.78	16.51±2.76	ns
	1-Butanol	14.46±0.98 ab	15.36±2.17 a	11.08±0.01 b	11.34±1.32 b	*	27.30±4.46	32.31±6.61	25.91±2.22	25.39±7.16	ns
	1-Pentanol	9.31±0.70	9.57±0.78	8.41±0.40	7.87±0.63	ns	18.08±1.49	21.02±1.35	17.45±0.91	17.30±3.49	ns
	Isoamyl alcohol	60.78±5.79 a	55.95±2.75 a	40.73±3.56 b	38.24±1.21 b	***	62.68±6.62	77.19±5.15	66.67±10.20	60.36±13.13	ns
	2-Ethyl-1-hexanol	12.76±1.34 b	29.58±2.58 a	28.80±0.91 a	13.73±1.09 b	***	5.27±0.80 b	4.50±0.20 b	4.55±0.59 b	7.36±0.17 a	**
	1-Octanol	3.12±0.26 a	2.41±0.34 b	2.16±0.03 b	3.15±0.14 a	**	2.88±0.20	2.87±0.53	2.54±0.25	3.67±0.83	ns
	1-Octen-3-ol	4.29±0.06 a	3.48±0.34 b	3.69±0.30 ab	4.27±0.30 a	*	10.78±0.67	10.74±0.73	10.42±0.28	11.83±0.10	ns
	2-Phenylethanol	724.72±8.69 a	703.56±20.87 ab	711.74±59.45 ab	627.88±30.13 b	*	617.11±131.11	568.10±11.96	1106.57±982.24	620.60±30.58	ns
	∑ Higher alcohols	936.00±7.32 a	920.95±33.46 a	893.79±74.42 ab	791.73±31.42 b	*	787.54±115.62	771.47±28.88	1275.41±994.62	790.44±1.35	ns
Volatile acid	**Butanoic acid**	0.83±0.05	0.79±0.39	0.69±0.19	0.97±0.21	ns	1.44±0.04 ab	1.83±0.17 a	1.22±0.21 b	1.46±0.15 ab	*
	Isovaleric acid	0.24±0.04	0.64±0.46	0.32±0.07	0.46±0.25	ns	1.05±0.09	1.18±0.29	1.84±1.57	1.49±0.30	ns
	Hexanoic acid	31.38±13.65 b	67.63±6.37 a	35.04±2.46 ab	31.87±19.02 b	*	50.92±2.56	60.45±7.11	92.78±54.65	66.00±5.72	ns
	Octanoic acid	27.37±4.27 b	53.92±15.27 a	31.93±0.50 ab	29.27±9.19 ab	*	24.98±1.84	22.43±2.05	47.33±22.42	27.39±2.65	ns
	Nonanoic acid	4.97±0.24	5.23±0.90	5.21±0.35	5.54±0.83	ns	4.46±0.95	5.17±1.88	5.03±0.42	3.72±1.42	ns
	Decanoic acid	4.98±1.27 b	6.52±1.75 ab	4.98±0.87 b	9.26±1.04 a	*	3.22±0.27	2.72±0.42	4.21±1.19	3.22±0.02	ns
	Dodecanoic acid	23.80±2.92 ab	30.10±2.21 a	32.33±8.01 a	19.29±2.10 b	*	20.23±3.77	21.19±2.37	19.98±0.77	21.74±5.69	ns
	∑ Acids	93.57±15.26 b	164.82±22.17 a	110.49±4.10 ab	96.65±30.81 b	*	106.30±6.87	114.97±13.21	172.39±78.97	125.02±15.35	ns
C6 compound	**1-Hexanol**	49.23±2.16	52.20±3.88	48.27±1.04	47.00±0.71	ns	106.77±4.29	110.42±2.11	100.04±2.32	105.88±10.71	ns

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Table S7. (continued)

	(*E*/*Z*)-2-Hexen-1-ol	9.57±0.37 a	9.57±2.61 a	8.61±0.97 a	7.44±0.29 a	*	7.96±0.27 b	9.66±0.79 a	8.31±0.31 ab	8.24±0.46 ab	*
	(*E*/*Z*)-3-Hexen-1-ol	13.35±0.51 a	14.16±1.32 a	11.97±1.79 ab	10.34±0.46 b	*	19.85±1.08 b	24.84±1.14 a	19.41±0.54 b	21.34±1.07 b	***
	∑ C6 compounds	72.15±2.74	75.93±7.39	68.85±1.72	64.78±0.38	ns	134.58±5.37 ab	144.92±3.45 a	127.76±2.99 b	135.45±12.25 ab	*
Volatile sulfur compound	Benzothiazole	0.93±0.15 c	46.72±0.67 a	35.49±1.97 b	0.97±0.15 c	***	1.65±0.19 a	0.98±0.13 b	0.91±0.12 b	0.85±0.37 b	**
Terpene	Linalool	nd	nd	nd	nd		22.80±2.04 ab	19.95±1.58 b	20.92±0.20 b	26.32±1.74 a	*
	Geraniol	37.68±0.74 a	35.70±0.79 b	31.21±0.62 c	37.09±0.17 ab	***	15.26±1.77	14.74±1.15	14.00±0.61	18.65±3.25	ns
	Lilac alcohol	nd	nd	nd	nd	ns	69.39±4.55	67.46±1.67	62.70±2.74	69.52±6.81	ns
	(*E*/*Z*)-8-Hydroxygeraniol	16.31±1.08	18.24±1.45	19.82±0.88	17.76±1.23	ns	28.71±2.55	28.69±0.40	27.08±1.45	29.20±2.87	ns
	(*E*/*Z*)-pyran-Linalool oxide	6.90±0.22 ab	7.59±0.54 a	7.50±0.22 a	6.27±0.21 b	**	6.00±1.58	6.45±0.31	6.61±0.26	5.67±0.19	ns
	Nerol	6.40±0.32 a	5.37±0.70 a	5.26±0.45 a	6.70±0.46 a	*	10.05±1.68	10.15±0.78	9.59±0.30	12.57±1.82	ns
	(*E*/*Z*)-8-Hydroxylinalool	81.20±2.62 bc	87.96±2.47 a	86.85±1.86 ab	79.69±1.38 c	**	232.98±16.15 ab	228.94±7.65 ab	201.63±8.70 b	243.22±19.16 a	*
	Geranic acid	63.72±4.73 b	82.49±1.31 a	76.66±0.97 ab	64.98±8.91 b	*	15.94±1.00	15.24±0.31	14.31±0.96	16.31±3.04	ns
	∑ Terpenes	212.22±5.00 b	237.35±3.76 a	227.29±3.75 ab	212.48±10.85 b	**	401.12±30.09	391.61±13.14	356.84±12.98	421.46±38.87	ns
Norisoprenoid	3-Oxo-α-ionol	329.55±4.45 b	390.32±11.64 a	389.44±10.01 a	332.75±12.61 b	***	162.54±12.38	153±1.94	155.13±7.76	166.09±14.52	ns
	3-Hydroxy-β-damascone	172.05±4.12 b	186.53±4.30 a	193.04±2.53 a	173.39±4.11 b	**	76.68±4.90	77.53±0.41	73.98±3.89	81.61±7.91	ns
	∑ Norisoprenoids	501.60±7.97 b	576.85±15.94 a	582.49±12.54 a	506.14±16.67 b	***	239.22±17.23	230.53±2.26	229.11±11.64	247.70±22.43	ns
Benzenoid	Benzenepropanol	1.00±0.16 a	1.42±0.06 a	1.41±0.11 a	1.13±0.23 a	*	4.87±0.37	4.61±0.15	4.22±0.17	4.84±0.46	ns
	Vanillin	8.01±0.59	8.67±1.10	7.75±0.07	6.56±0.74	ns	7.22±0.42	7.76±1.11	17.19±14.69	11.13±1.52	ns
	Homovanillinic acid	128.94±10.96 c	220.58±15.99 a	171.00±5.15 b	135.14±8.27 c	***	126.83±18.37	129.00±9.90	120.67±12.81	128.47±27.63	ns
	Tyrosol	109.20±26.93 b	205.37±59.38 a	100.11±8.66 b	210.01±7.14 a	*	450.21±337.41	96.13±53.98	761.51±274.50	353.19±43.48	ns
	Benzyl alcohol	876.20±8.20	806.98±66.31	829.09±116.34	737.54±39.51	ns	645.51±22.12 ab	773.48±53.00 a	618.64±12.16 b	783.40±91.83 a	**
	3,4,5-Trimethoxy-benzenemethanol	14.56±0.45 b	17.62±1.10 a	18.84±2.04 a	13.96±0.67 b	**	18.61±1.84 a	21.43±0.75 a	17.98±1.24 a	22.38±2.75 a	*
	α –Methylbenzyl alcohol	4.74±0.23 a	4.87±0.27 a	4.62±0.24 a	3.76±0.18 b	**	2.54±0.18 ab	2.69±0.09 a	2.23±0.04 b	2.61±0.14 a	*
	Methyl salicylate	12.73±0.67 a	9.53±0.52 bc	8.32±0.30 c	10.49±0.34 b	***	17.84±1.13 bc	21.00±1.10 b	16.62±0.70 c	27.86±3.17 a	*
	Homovanillyl alcohol	70.80±3.43 ab	85.64±9.70 a	73.81±10.84 ab	59.02±4.44 b	*	116.06±8.92	171.97±29.21	125.58±2.66	161.56±41.28	ns
	Vanillyl alcohol	6.67±0.72 b	10.35±1.74 a	7.31±1.22 ab	6.25±0.55 b	*	3.75±0.84 b	8.59±0.75 a	7.83±0.82 ab	10.82±3.71 a	*
	Acetovanillone	10.80±0.79 ab	10.78±0.78 ab	13.21±1.78 a	9.93±0.50 b	*	25.83±4.06	29.87±2.99	31.35±14.13	26.52±4.97	ns
	3-Hydroxy-4-phenyl-2-butanone	nd	nd	nd	nd		0.00±0.00	0.00±0.00	1.80±2.66	0.00±0.00	ns

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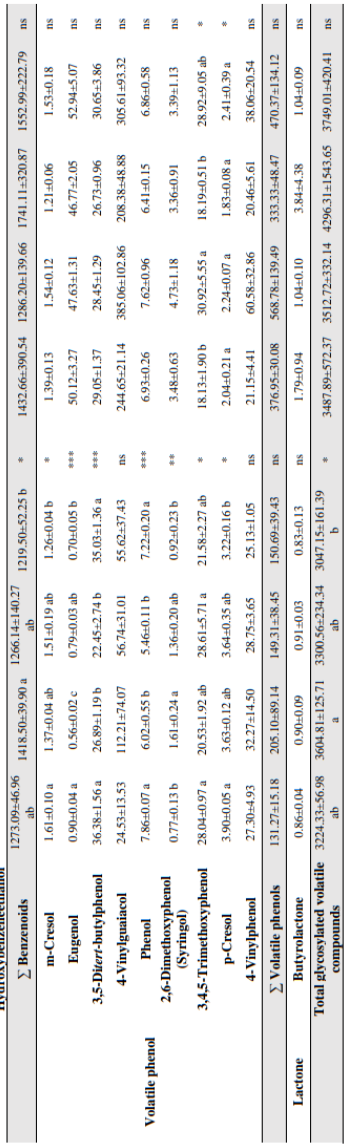
Table S7. (continued)



Volatile phenol	2-Hydroxybenzeneethanol	29.45±1.95 ab	36.69±3.54 a	30.66±3.96 ab	25.72±1.75 b	*	13.39±1.25	19.67±2.83	15.47±0.53	20.22±4.87	ns
	∑ Benzenoids	1273.09±46.96 ab	1418.50±39.90 a	1266.14±140.27 ab	1219.50±52.25 b	*	1432.66±390.54	1286.20±139.66	1741.11±320.87	1552.99±222.79	ns
	m-Cresol	1.61±0.10 a	1.37±0.04 ab	1.51±0.19 ab	1.26±0.04 b	*	1.39±0.13	1.54±0.12	1.21±0.06	1.53±0.18	ns
	Eugenol	0.90±0.04 a	0.56±0.02 c	0.79±0.03 ab	0.70±0.05 b	***	50.12±3.27	47.63±1.31	46.77±2.05	52.94±5.07	ns
	3,5-Di*ert*-butylphenol	36.38±1.56 a	26.89±1.19 b	22.45±2.74 b	35.03±1.36 a	***	29.05±1.37	28.45±1.29	26.73±0.96	30.65±3.86	ns
	4-Vinylguaiacol	24.53±13.53	112.21±74.07	56.74±31.01	55.62±37.43	ns	244.65±21.14	385.06±102.86	208.38±48.88	305.61±93.32	ns
	Phenol	7.86±0.07 a	6.02±0.55 b	5.46±0.11 b	7.22±0.20 a	***	6.93±0.26	7.62±0.96	6.41±0.15	6.86±0.58	ns
	2,6-Dimethoxyphenol (Syringol)	0.77±0.13 b	1.61±0.24 a	1.36±0.20 ab	0.92±0.23 b	**	3.48±0.63	4.73±1.18	3.36±0.91	3.39±1.13	ns
	3,4,5-Trimethoxyphenol	28.04±0.97 a	20.53±1.92 ab	28.61±5.71 a	21.58±2.27 ab	*	18.13±1.90 b	30.92±5.55 a	18.19±0.51 b	28.92±9.05 ab	*
	p-Cresol	3.90±0.05 a	3.63±0.12 ab	3.64±0.35 ab	3.22±0.16 b	*	2.04±0.21 a	2.24±0.07 a	1.83±0.08 a	2.41±0.39 a	*
	4-Vinylphenol	27.30±4.93	32.27±14.50	28.75±3.65	25.13±1.05	ns	21.15±4.41	60.58±32.86	20.46±5.61	38.06±20.54	ns
	∑ Volatile phenols	131.27±15.18	205.10±89.14	149.31±38.45	150.69±39.43	ns	376.95±30.08	568.78±139.49	333.33±48.47	470.37±134.12	ns
Lactone	Butyrolactone	0.86±0.04	0.90±0.09	0.91±0.03	0.83±0.13	ns	1.79±0.94	1.04±0.10	3.84±4.38	1.04±0.09	ns
	Total glycosylated volatile compounds	3224.33±56.98 ab	3604.81±125.71 a	3300.56±234.34 ab	3047.15±161.39 b	*	3487.89±572.37	3512.72±332.14	4296.31±1543.65	3749.01±420.41	ns

Note: 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, according to ANOVA test. Different lowercase letters within the same row refer to the existence of significant differences among different samples, for each variety and sampling point, according to Tukey's HSD test."CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Cortese' wines and "AR- Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths, 7, 14 and 21 days, respectively, for 'Arneis' wines.

Table S8. Frequency of descriptors of Arneis (AR) and Cortese (CO) wines at the end of the alcoholic fermentation (PAF) and one month after bottling (PWI).

			Descriptor frequency (%)											
			Pineapple	**Lime**	**Lemon**	**Grapefruit**	**Pear**	**Green apple**	**Peach**	**Banana**	**Rose**	**Jasmine**	**Honey**
Arneis	PAF	AR-Control	8.3	16.7	**41.7**	**58.3**	**41.7**	**33.3**	**33.3**	**33.3**	**33.3**	**41.7**	8.3
		AR07	**25.0**	16.7	**50.0**	**66.7**	**41.7**	**33.3**	**50.0**	**58.3**	16.7	8.3	8.3
		AR14	**25.0**	**25.0**	**50.0**	**33.3**	**33.3**	**66.7**	8.3	**41.7**	**41.7**	16.7	0.0
		AR21	**33.3**	**41.7**	**50.0**	**33.3**	**75.0**	**50.0**	16.7	**25.0**	**41.7**	**25.0**	**41.7**
		p-value	*0.549*	*0.464*	*0.962*	*0.147*	*0.248*	*0.248*	***0.053***	*0.280*	*0.506*	*0.142*	***0.019***
	PWI	AR-Control	**25.0**	**20.0**	10.0	**25.0**	**35.0**	**25.0**	15.0	**25.0**	**50.0**	10.0	**50.0**
		AR07	**45.0**	5.0	5.0	5.0	**35.0**	10.0	**35.0**	**30.0**	**50.0**	15.0	**45.0**
		AR14	**20.0**	**20.0**	15.0	15.0	**40.0**	**40.0**	**35.0**	15.0	15.0	**30.0**	**40.0**
		AR21	**20.0**	15.0	**25.0**	**20.0**	**30.0**	**30.0**	**25.0**	5.0	**25.0**	**25.0**	15.0
		p-value	*0.129*	*0.522*	*0.241*	*0.305*	*0.889*	*0.171*	*0.391*	*0.167*	***0.038***	*0.343*	***0.064***
Cortese	PAF	CO-Control	**25.0**	16.7	**41.7**		**41.7**	**58.3**	**58.3**	**50.0**	**41.7**	16.7	**25.0**
		CO07	16.7	8.3	**50.0**		**33.3**	**41.7**	**33.3**	**41.7**	**25.0**	0.0	16.7
		CO14	**25.0**	**25.0**	**66.7**		**50.0**	**50.0**	**33.3**	**41.7**	16.7	**25.0**	**50.0**
		CO21	**25.0**	16.7	**41.7**		**50.0**	**58.3**	**25.0**	**50.0**	16.7	16.7	**33.3**
		p-value	*0.942*	*0.944*	*0.440*		*0.666*	*0.786*	*0.245*	*0.934*	*0.463*	*0.425*	*0.241*
	PWI	CO-Control	**28.6**	**21.4**	7.1		**35.7**	**50.0**	14.3	**28.6**	**28.6**	**35.7**	7.1
		CO07	7.1	**21.4**	**21.4**		**28.6**	**21.4**	**42.9**	**21.4**	**35.7**	**28.6**	**21.4**
		CO14	7.1	14.3	14.3		**57.1**	**42.9**	**21.4**	**28.6**	**57.1**	**42.9**	14.3
		CO21	**28.6**	14.3	**35.7**		**50.0**	**57.1**	**28.6**	**21.4**	**42.9**	**28.6**	14.3
		p-value	*0.281*	*1.000*	*0.207*		*0.261*	***0.091***	*0.207*	*0.953*	*0.305*	*0.815*	*0.873*

Note: Values in bold represent frequency higher than 20% and p value according to the Cochran's Q test with *p* value < 0.1. "CO-Control", "CO07", "CO14" and "CO21" indicate the non-stabulated and the three treatment lengths, respectively 7, 14 and 21 days for 'Cortese' wines and "AR-Control", "AR07", "AR14" and "AR21" indicate the non-stabulated and the three treatment lengths, respectively 7, 14 and 21 days for 'Arneis' wines.

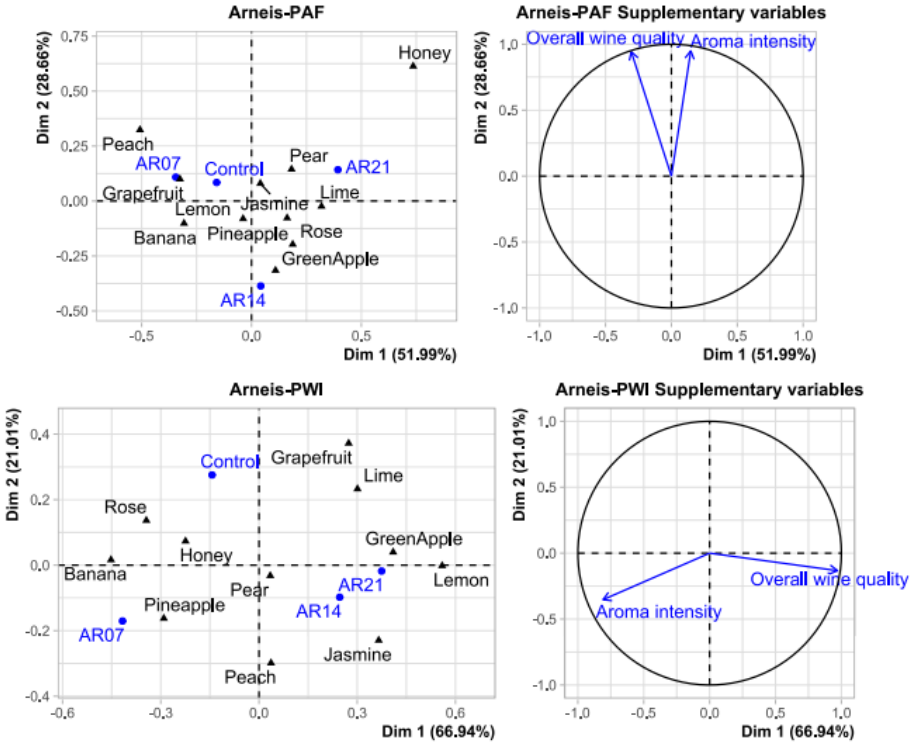


Figure S1. Correspondence Analysis of sensory analysis aroma descriptors at the end of the alcoholic fermentation (PAF) and one month after bottling (PWI) of Arneis wines.

Note: Only descriptors with frequency higher than 20% were used. “Control”, “AR07”, “AR14” and “AR21” indicate the non-stabulated and the three treatment lengths, 7, 14, and 21 days, respectively.

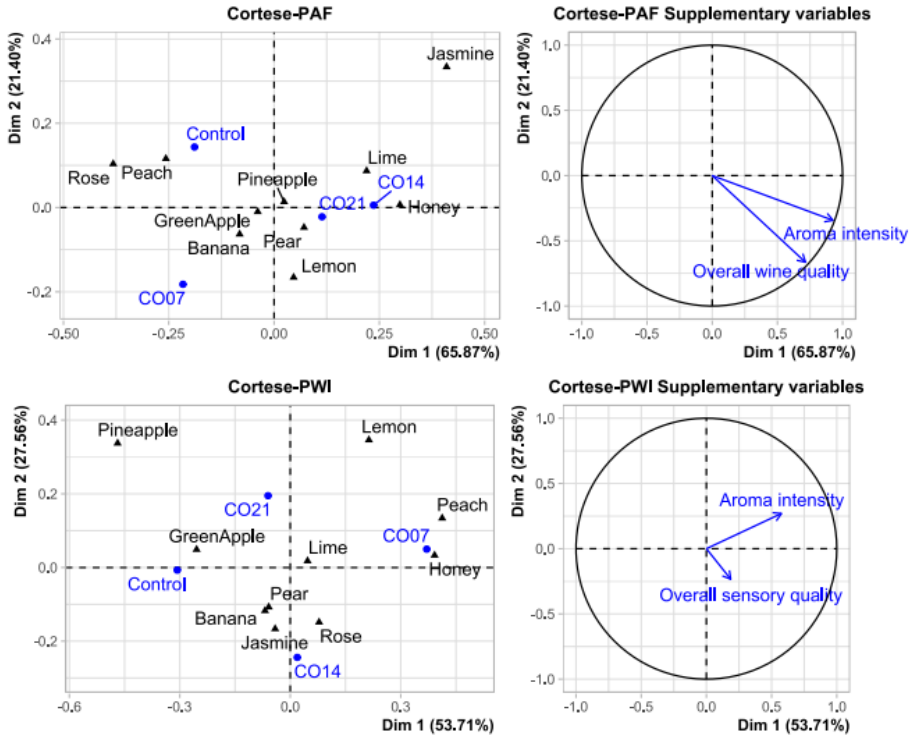


Figure S2. Correspondence Analysis of sensory analysis aroma descriptors at the end of the alcoholic fermentation (PAF) and one month after bottling (PWI) of Cortese wines.

Note: Only descriptors with frequency higher than 20% were used. “Control”, “CO07”, “CO14” and “CO21” indicate the non-stabulated and the three treatment lengths, 7, 14, and 21 days, respectively.

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Chapter 3

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

BACKGROUND: 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 present study aimed 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 from two Valtellina vineyards. During three consecutive vintages (2019, 2020, and 2021), three different technological binomials have been tested: early harvest/long withering (EL), medium-term harvest/medium-term withering (MM), and late harvest/short withering (LS).

RESULTS: 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. EL and MM 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.

CONCLUSION: Harvest time and withering length can be modulated according to the desired oenological objective, promoting the valorization of grape potentialities. The choice to harvest the grapes earlier and enhance

the withering length should be preferred to obtain wines with higher acidity and phenolic content, more suitable for long-ageing period.

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 winegrowing activities. It has a central role for the wine market for not only for economic aspects, but also cultural and social development reasons.[1-3] Therefore, it is 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 occurs no earlier than 1 December of the same year.[4]

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.[5-9]

The importance of the ripeness degree and the withering conditions on grape phenolic composition have been separately studied in recent years,[10-13] but, to the best of our knowledge, there is little information available in the 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.[14-16]

The attempt to obtain a better understanding on this topic represents a considerable challenge because of several other factors requiring consideration, such as the different climatic conditions of the year or the vineyard location and management.[17-19] Accordingly, a 3-year experimental plan (vintages 2019, 2020, and 2021) was designed to answer the question: 'what is the best time to harvest red grapes destined for 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 the withering process

Grape samples of cv. Nebbiolo (*V. 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): (A) the upper-valley vineyard, set in the western part (Villa di Tirano, 46°12'N, 10°8'E, 400 m asl) and (B) the lower-valley vineyard, 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, and 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, the grapes were

harvested every year 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, whereas LS was either targeted at 1% increment from MM or lower if the climate did not allow reaching this target. At each stage, approximately 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,[4] the withering lasted until 1 December of the same harvest year. Consequently, the length of the dehydration process depended on the harvest date, as shown in Fig. 1.

Harvest date and total days of withering are shown in the Supporting information (Table S1). The long withering process lasted approximately 70 days in total and, among the three different withering periods, there were around 10 days of difference.

Eight randomized 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.

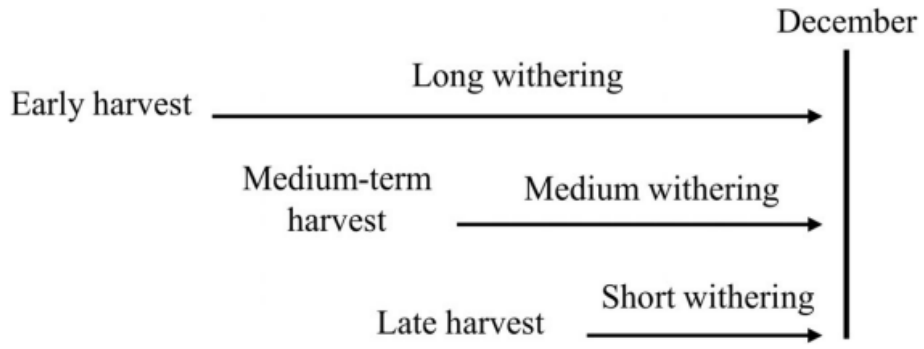

Figure 1. Experimental plan schematizing the length of the dehydration process for each binominal considered

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[20] 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 parameter 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 approximately 100 g of berries were collected and manually crushed for 2 min. The obtained grape must was centrifuged at 3000 × g for 15 min at 20 °C using a Hettich 32R centrifuge (Hettich, 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 pH meter (InoLab pH 730; WTW, Weilhelm, Germany) was used to measure pH by potentiometry, and total acidity (as g L−1 of tartaric acid) was determined by titration with sodium hydroxide 0.1 mol L−1 according to method OIV-MA-AS313-01.[21] 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, CA, USA) equipped with a refractive index and a UV detector.[22]

2.3.3 Extraction and determination of phenolic compounds from grape skin 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−1 tartaric acid, and 100 mg L−1 $Na_2S_2O_5$, adjusted to pH 3.20 with NaOH 1 mol L−1), following the proportions described by Mattivi et al. [23] 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 (20 × 6 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.[24,25] 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.[24] 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.[26]

2.4 Grape skin mechanical proprieties

Grape skin mechanical properties were evaluated using a TA. XTplus Universal Testing Machine (Stable Micro Systems, Godalming, UK). The Texture Analyzer was equipped with an HDP/90 platform, a SMS P/2N 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 (SMS P/2, diameter 2 mm) used for skin thickness evaluation (Sp_{sk}, μm), and a 5 kg load cell.[27] 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 R, 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 analysis of variance. A t-test was used to discriminate significant differences among fresh and withered grapes.

Multivariate analysis was performed through principal component analysis (PCA) to explore the association between the variables (grape chemical composition parameters) and groups (vineyards and treatment). Before conducting the PCA, data was normalized inside each year by using the Z-score transformation to exclude any variability caused by the vintage, as previously reported by Škrab et al. [28] The PCA was performed using R software and the package FactoMineR, and its results were

extracted and visualized using R packages factoextra and ggplot2, respectively.

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.[29] As shown in Fig. 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 3 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, Fig. 2b) show that 2019 was the hottest of the 3 years considered (18.4 versus 17.6 versus 17.6 °C of average yearly temperature for 2019, 2020, and 2021, respectively), 2020 was the wettest vintage (497 mm from August to October versus 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).

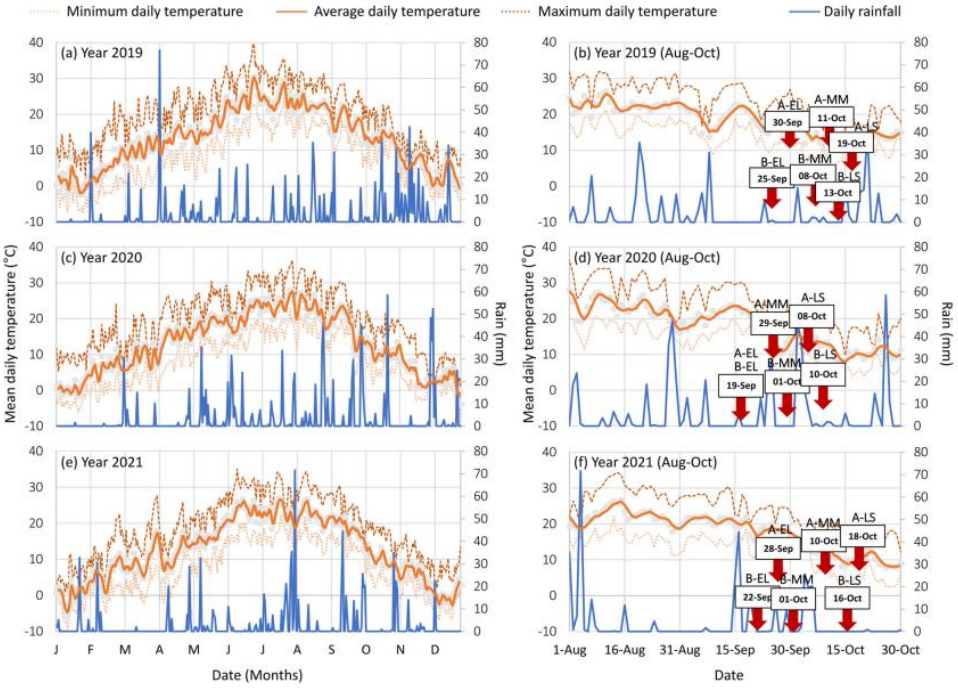

Figure 2. Minimum (dotted orange), maximum (dashed orange) and average (solid orange) daily temperature and rainfall (blue lines) or three consecutive harvest years studied (a, c, e), harvest times and weather conditions of the months near the harvest (b, d, f) from weather station located in Sondrio. Data from ARPA Lombardia[18]

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−1) with respect to the earliest ones (208–230 g L−1). The glucose/fructose ratio in fresh Nebbiolo grapes was almost 1, which is the typical ratio for ripe grapes,[30] 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.[31]

Total acidity (expressed as g L−1 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−1 for vineyard A and B, respectively. Malic and tartaric acids tend to decrease progressively with the ripening process in fresh grapes as a result of respiratory metabolism and dilution, respectively.[32,33] 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.[34,35]

The general effect on the ratio between juice sugar contents and total acidity values according to the harvest time is clearly visible: this ratio increases significantly from early to late harvest, reaching an average of approximately 30 points in the final harvest date (see Supporting information, Table S2).

Table 1. Standard parameters of fresh grapes

Harvest year	Ripeness parameter	Vineyard A				Vineyard B			
		EL	MM	LS	Significance	EL	MM	LS	Significance
2019	Reducing sugars (g L^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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	***

Note: All data are expressed as the mean ± SD (n=3). ***P<0.001, **P<0.01, and *P<0.05; ns, not significant. Different lowercase letters indicate significant differences among the three binomials tested for each vineyard studied according to the Tukey *b* test (P<0.05). A, upper-valley vineyard; B, lower-valley vineyard; EL, early harvest/long withering; MM, medium-term/medium withering; LS, late harvest/short withering; TA, total acidity; G/F ratio, glucose/fructose ratio.

3.2.2 Technological parameters of withered grapes

The average percentages of grape weight loss (WL%) for the 3 years and two vineyards at the end of the withering process were 19 ± 5, 16 ± 4, and 12 ± 3% 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 approximately 10 days in terms of withering length between EL and LS trials.

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 the withering period, the greater the percentage increase of sugars in withered grapes with respect to fresh ones as a result of a concentration effect, leading the EL thesis to be the richest in sugar content at the end of the process (247–292 g L−1, with a mean difference of +9.8 g L−1 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.[36-38] 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.[39,40]

The combined effect of sugars accumulation and weight loss during withering was also studied through the SIR-to-WLR (sugar increase rate as °Brix/day-to-weight loss percentage rate/day) parameter[6] : The general average of this parameter was found 0.26 °Brix/%, with a non-significant increase in EL samples with respect to MM and LS (see Supporting information, Table S2), indicating a possible dominance of the concentration effect with relation to other metabolic processes such as sugars respiration.[6,41] The vintage effect was not significant, but a growing tendency in 2020 data can be seen compared to 2019 and 2021 data (see Supporting information, Table S2).

As regards total acidity, the concentration effect because of 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−1 and + 0.85 g L−1 , 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 balanced sugar-to-acid ratio in withered grapes

(see Supporting information, Table S2), as previously hypothesized by Failla et al. [42] Furthermore, this ratio was also found to be significantly influenced by the vintage (see Supporting information, Table S2), as previously demonstrated.[43]

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 concentrations of malic and citric acids with respect to the other binomials studied for each year (except for 2020 vineyard B). Interestingly, the concentrations of citric and malic acids progressively increased from fresh to withered grapes, presumably because of a positive balance between catabolism and concentration effect (t-test, $P < 0.01$ with respect to fresh and withered citric acid values, whereas the difference was not statistically significant for malic acid). By contrast, a decreasing trend in the concentration of tartaric acid was observed from fresh to withered grapes. Rösti et al. [44] explained the drop in tartaric acid observed during Merlot and Syrah winegrapes dehydration as consequence of precipitations occurred already inside the berries, probably because of a loss of compartmentation over the process. Nevertheless, the ratio between juice malic and tartaric acid seemed more influenced by the vintage rather than the harvest date, both on fresh and withered grapes (see Supporting information, Table S2), especially for year 2020 fresh grapes that reported the lowest values.

A small amount of glycerol has been detected only in withered grapes (from 0.05 to 2.14 g L−1), more prominently in the grapes from 2019 vintage. The increase of the glycerol content as a result of the withering process has often been observed in the literature.[45,46] Indeed, during dehydration, grape cells under hyperosmotic stress for the increasing sugar concentration appear to react to stress by increasing the

intracellular glycerol.[47] However, the differences in glycerol contents from EL to LS observed after withering were not statistically significant.

Table 2. Standard parameters of withered grapes

Harvest year	Ripeness parameter	Vineyard A				Vineyard B			
		EL	MM	LS	Significance	EL	MM	LS	Significance
2019	Reducing sugars (g L^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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})	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^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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^{-1})	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^{-1})	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^{-1} 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^{-1})	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^{-1})	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^{-1})	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^{-1})	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

Note: All data are expressed as mean ± SD (n = 3). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; ns, not significant. Different lowercase letters indicate significant differences among the three binomials tested for each vineyard studied according to the 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.

3.3 Grape skin mechanical proprieties

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.[48] 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.[49,50] Moreover, it has been demonstrated that the berry skin hardness at harvest affects the dehydration kinetics.[51]

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 the literature on Nebbiolo grapes from Piedmont region (around the range of 0.23–0.55 N), probably because of the influence of the Valtellina mountainous growing area, as previously found for Carema mountainous growing area compared to the La Morra and Barbaresco hill areas.[52] 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.[53,54] However, among the three harvest points (early, medium, and 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.[25,55] 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 occurred in the first days of October, therefore before the A-MM, A-LS, and B-LS sampling points (Fig. 2). Indeed, water availability appears to influence the grape skin physical features, especially in the period before the harvest.[56] 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 that previously reported in the literature.[57]

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 different level of WL reached appeared 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.[57] 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 3 years of experiments (197–262 μm) were generally higher than those present in literature for Nebbiolo grapes from other regions.[52,58] 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. [36] 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 (t-test, $P < 0.001$ for both parameters) (Table 3). In 2021, this tendency has been less remarkable than the previous vintages probably for the higher variability of grape samples (t-test, $P = 0.09$ 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.[48] The results of phenolic compounds presented in the next section confirm this hypothesis.

Table 3. Fresh and withered grape skins mechanical proprieties

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	

Note: All data are expressed as the mean ± SD (n=30). ***P<0.001, **P<0.01 and *P<0.05; ns, not significant with respect to the differences among the three binomials

studied (†) and between fresh and withered grapes (‡) according to analysis of variance and a t-test. Different lowercase letters in the same column indicate significant differences (†) according to the 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; F_{sk}, berry skins break force; W_{sk}, berry skins break energy; E_{Sk}, berry skin resistance against deformation; Sp_{sk}, berry skin thickness.

3.4 Extractable phenolic composition of fresh and withered grapes

Here, the results obtained from the analysis of seed and skin extracts using wine-like solution for both fresh and dehydrated samples are 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), total flavonoids (TF), and condensed tannins (MCP) in seeds appeared to show a decreasing trend by leaving the grapes on the plant longer by an averaged value of the 3 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.[59,60] 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 to oxidation reactions,[61] 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.[62,63] This tendency has been observed in all the three vintages considered, although the differences were not always

statistically significant because of sample variability.

The impact of the grape's ripeness degree observed in fresh grapes increased considerably after withering because of 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 seed 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 with respect to producing wines destined for long ageing periods, as is the case for *Sforzato di Valtellina* DOCG wines.

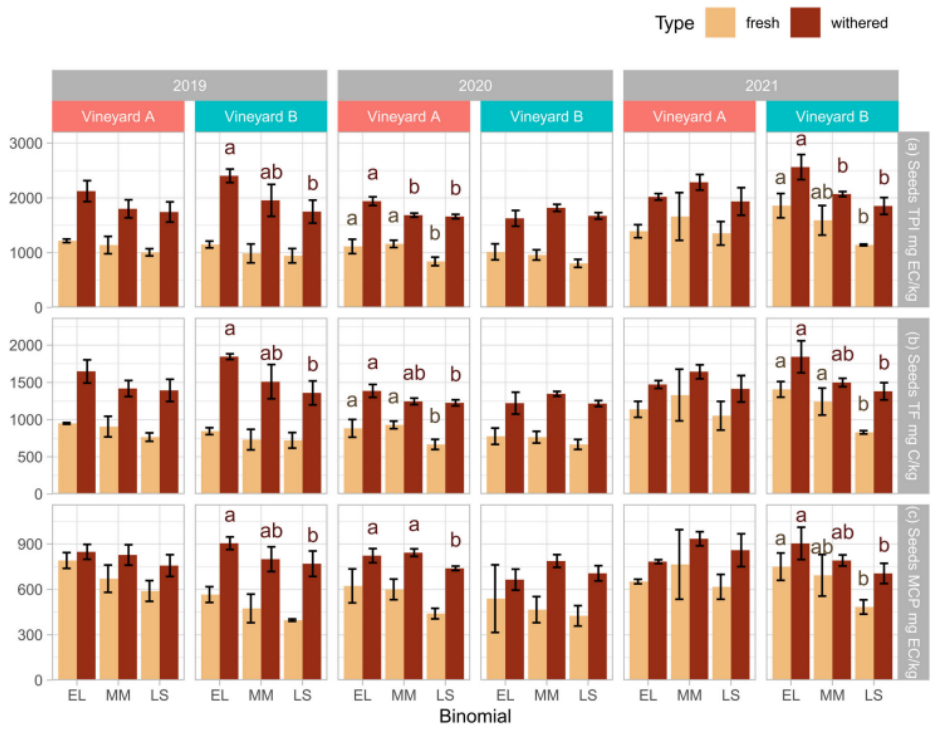

Figure 3. Phenolic profile of fresh and withered grape seeds. TPI, extractable total phenolic compounds (a); TF, extractable total flavonoid compounds (b); MCP,

extractable condensed tannins determined by methyl cellulose assay (c). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; ns, not significant. Different lowercase letters indicate significant differences among the three binomials tested for each vineyard and year studied according to the 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 to be less influenced by the harvest period compared to those of seeds, although their contents expressed on grape weight generally increased after withering, as a result of a balance between concentration and degradation effects (Fig. 4 a, b). In general, the increase from fresh to withered for EL samples was less evident for grape skins than for seeds, probably because the skin phenolic compounds are more subjected to biotic and abiotic stress, mitigating the concentration effect.[64,65] Previous studies[66-68] have reported that a long withering time and greater water loss could determine a significant loss of phenolic compounds as a result of oxidation and senescence metabolism. Nevertheless, the cool temperatures that occur during the period when the natural withering is performed in Valtellina probably delayed the water loss stress, as hypothesized by Bellincontro et al.[66] This results in a final increase in polyphenols, in accordance with the results of Panceri et al.[69] on Merlot and Cabernet sauvignon grapes under controlled withering conditions. Indeed, Nicoletti et al.,[70] when investigating Nebbiolo grapes destined for the production of *Sfursat* and subjected to different withering rate under controlled temperatures, observed an increase in skins polyphenols (at 10 and 20% WL) after dehydration at 10 °C.

Extractable anthocyanins (TA) expressed as the malvidin-3-O-

glucoside equivalent on berry weight (mg kg^{-1} berries) (Fig. 4d) 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^{-1} berries), as well in 2021 from early to medium harvest (from 312 to 329 mg kg^{-1} berries). Their ratio with respect to juice sugars showed significant differences only among vintages (see Supporting information, Table S2). 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 not stable during the vintages or common for the two vineyards evaluated. Hence, considering the risks involved (climate, loss of product, etc.,) leaving the grapes on the vine longer does not appear to be justified in terms of any real gain in anthocyanin compounds. Moreover, during the first 2 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 above. Instead, in 2021, the high variability likely induced by the dry season of the vintage makes it difficult to highlight the trend observed in the previous years. This confirms the greater variability in the grape composition observed in dry

years.[71] Therefore, among the compounds analyzed, anthocyanin was the compound 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 c), 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^{-1} berries) to MM (650 mg kg^{-1} berries), as observed by Ó-Marques et al. [60] on Cabernet sauvignon and Tinta Roriz varieties with the progress of maturation, but followed by a decrease in LS point to 580 mg kg^{-1} berries. However, for MCP, the withering length appeared 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 enhancement among EL and LS comprised between +29 and +114 mg kg^{-1} grapes), although not always significant, indicating an important impact of the concentration effect over degradation during withering. Condensed tannin content has been previously reported to be less affected by dehydration than other phenolic compounds on a dry weight basis.[72] The main changes are connected with structural modification, as observed previously in dehydrated Nebbiolo grapes at 10% and 20% weight loss,[73] which is in agreement with the present study.

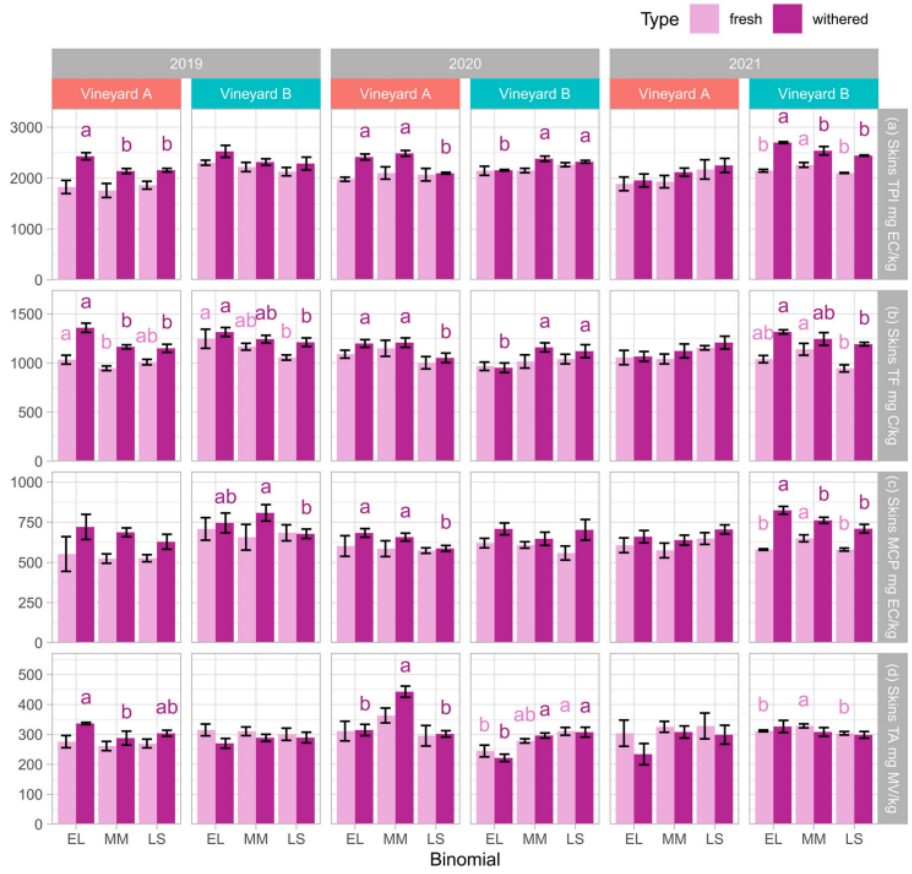

Figure 4. Phenolic profile of fresh and withered grape skins. TPI, extractable total phenolic compounds (a); TF, extractable total flavonoid compounds (b); MCP, extractable condensed tannins determined by methyl cellulose assay (c); TA, extractable total anthocyanins (d). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; ns, not significant. Different lowercase letters letters indicate significant differences among the three binomials tested for each vineyard and year studied according to the 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.

3.5 Multivariate evaluation of data

By performing a preliminary PCA on the original data, the effect of vintage was predominant over all other variables (data not shown); therefore, to assess the real impact of the effect of vineyard and thesis, the data were

normalized inside each vintage. Because of the strong differences between fresh and dehydrated grapes, these two matrices were studied separately. The biplots demonstrating the characterization of our samples and the correlation among variables are shown in Fig. 5. In fresh grapes (Fig. 5a, c), the first two PCs explained 58.8% of the variance of the samples, with a PC1 contribution of 39.4%. Concerning withered grapes (Fig. 5 b, d), the first two PCs accounted for 35.9% and 21.5%, respectively, explaining 57.4% of the total variance.

As expected, in fresh grapes, the juice pH correlated positively with sugars and negatively with the total acidity parameter. On the other hand, in withered grapes, pH was no longer correlated with sugars level. The phenolic parameters of the skins (Skins TA and Skins MCP) were close and positively correlated in both fresh and withered grapes, whereas Seeds MCP was found to be poorly correlated with these parameters in both cases. These results show that the extraction of phenolic substances from the different berry components did not appear to influence each other.

Interestingly, malic acid and Seeds MCP were found to be correlated, especially in fresh grapes: one hypothesis concerning this behaviour may involve ripeness evolution through sampling points, with a decreasing tendency of malic acid content in juice (Table 1) and the extractable condensed tannins from seeds (Fig. 3 c). In Fig. 5(a) (fresh grapes) and Fig. 5(b) (dehydrated grapes), it is possible to observe that vineyards A and B are well separated on each PC2. In general, grapesfrom vineyard A were characterized by higher values of malic acid and Seeds MCP, whereas grapes from vineyard B were more associated with Skins TA, Skins MCP, and tartaric acid. It is worth noting that differences between vineyards were kept also at the end of the withering process (Fig. 5 b), with sugars and glycerol contents also contributing to the

differentiation. The biplots in Fig. 5(c) (fresh grapes) and Fig. 5(d) (dehydrated grapes) reported the binomial harvest date/length of withering effect. In fresh grapes (Fig. 5 c), the three binomials show differences mostly related to the acid–sugar composition of the grapes. It is interesting to note that the groups were separated on each PC1, following the harvest date order on which they were picked. In particular, LS is characterized by high pH and sugar values, EL by high total acidity, malic acid and also Seeds MCP values, and the MM sample group stands in the middle. Therefore, the late harvest date resulted in grapes with a higher sugar content and higher pH value. In withered grapes (Fig. 5 d), it is possible to observe that treatments (binomials) are still well separated. As previously noted, EL showed a good correlation with high sugar content, confirming that long withering periods give a higher sugar increase. On the other hand, high pH values characterize the LS thesis, showing that the combination of late harvest date and short withering period contributes to lower the juice total acidity and to increase the pH values.

Considering these results, we may confirm that different combinations of harvest date and withering length result in different grape composition and that the effect of the treatment can be observed across different vintages.

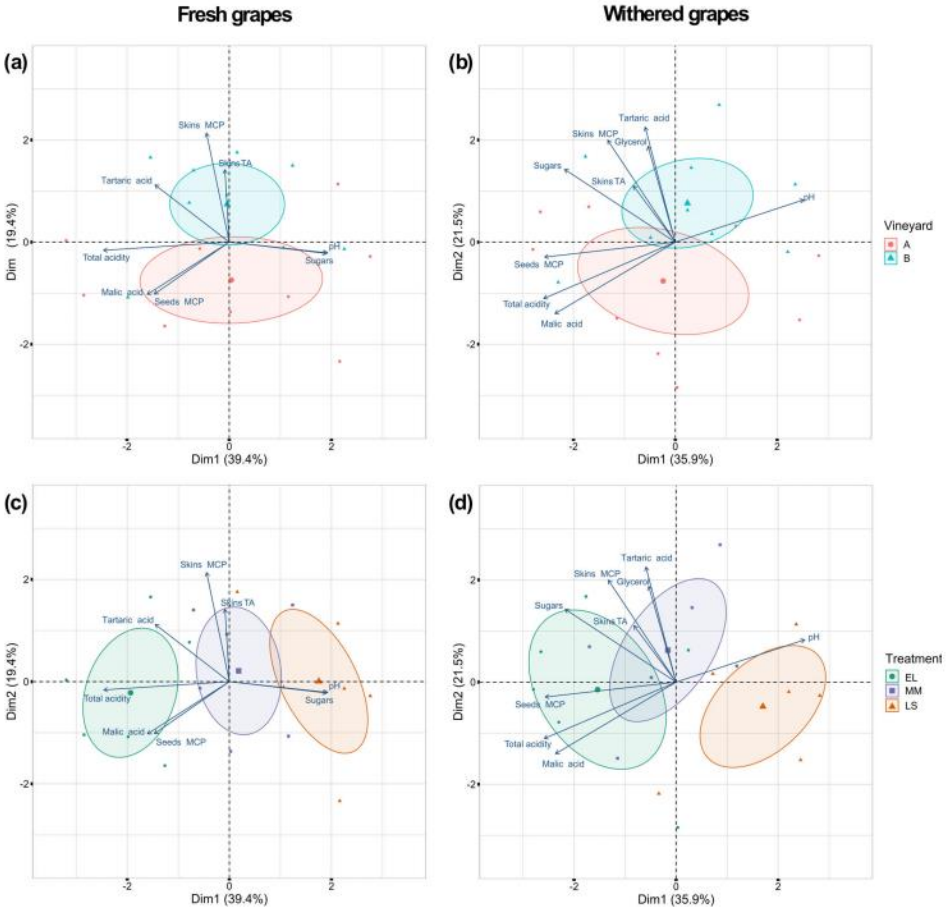

Figure 5. Biplots of the Principal component analyses (PCAs) performed on fresh (a, c) and withered grapes (b, d) examining the effect of vineyards (a, b) and treatments (c, d). The observations shown in the biplots represent the mean of three replicates of grapes from the same vineyard and vintages and subjected to the same treatment. The arrows indicate the strength and direction of the relationship between the variables and the principal components.

4. Conclusions

During the three consecutive vintages, all the analyzed parameters were affected by the close interaction between the harvest time and the withering length. The most evident changes were observed in technological parameters. The grapes harvested earlier and subjected to

long-withering process were found not only to be richer in sugars, but also showed higher total acidity and lower pH values, presenting a balanced sugar-to-acid ratio. By contrast, the combination of late harvest date and short withering period contributes to lowering the juice total acidity and increasing the pH. These observations confirm not only the great importance of ripeness degree for grapes destined for dehydration, but also the importance of considering the withering process. Mechanical properties were affected by the combined effect of the studied variables: the skin stiffness (E_{sk}) was 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. Nevertheless, the same parameters showed some different trends between the two vineyards tested.

Seed extractable polyphenols showed a decreasing trend when leaving the grapes on the plant longer, whereas the concentration effect considerably enhanced 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 resulted in withered grapes characterized by higher amounts of extractable phenolic compounds, particularly for seeds and skins condensed tannins. For anthocyanins, harvest time appeared to be more of an influence than withering length regarding final extractable content, even if a sustained variability among vintages and vineyards was present.

The great weather differences among the three vintages allowed a common trend to be highlighted in very different situations, although further studies are needed to better clarify the impact of the climate conditions. Our results suggest that the choice with respect to anticipating

harvest time for Nebbiolo grapes destined for withering should be preferred in view of the production of reinforced wines destined for long wine ageing, as a result of higher phenolic compounds contents, higher acidity, and lower pH, as well as for grape health reasons, aiming to avoid possible adverse climate and pests.

Author contributors

GS, MAP, CDP, DŠ, AZ, GM, LF and SBDA were responsible for formal analysis. GS, SG, MAP and SRS were responsible for investigations. GS, CDP, DŠ, AZ, GM, LF and SBDA were responsible for data curation. GS and LF were responsible for visualization. GS was responsible for writing the original draft. SG, MAP, SRS, VG and LR were responsible for conceptualization. SG and MAP were responsible for methodology. SG and VG were responsible for project administration. SG, MAP, SRS, CDP, DŠ, AZ, GM, LF, SBDA, VG and LR were responsible for reviewing and editing. SRS, VG and LR were responsible for supervision. VG and LR were responsible for funding acquisition.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting information

Table S1. Harvest time and total days of withering for each vineyard studied (A-B) and correspondent binomials: early harvest/long withering (EL), medium-term harvest/medium withering (MM), and late harvest/short withering (LS)

Binomial	Vineyard	Harvest time			Total days of withering		
		2019	2020	2021	2019	2020	2021
EL	A	30-Sep	19-Sep	22-Sep	62	73	70
	B	25-Sep	19-Sep	28-Sep	67	73	64
MM	A	11-Oct	29-Sep	10-Oct	51	63	52
	B	8-Oct	1-Oct	1-Oct	54	61	61
LS	A	19-Oct	8-Oct	18-Oct	43	54	44
	B	13-Oct	10-Oct	16-Oct	49	52	46

Table S2. Ratios calculated based on compositional parameters: SIR-to-WLR ratio, juice sugar-to-acids ratio, malic-to-tartaric acid ratio, anthocyanins-to-sugars ratio, tannins (Skins MTC)-to-sugar ratio.

Year	Vineyard	Binomial	SIR-to-WLR †	Sugars-to-Total acidity ‡		Malic-to-Tartaric acid ‡		Anthocyanins-to-Sugars §		Anthocyanins-to-Tannins #	
			-	Fresh	Withered	Fresh	Withered	Fresh	Withered	Fresh	Withered
2019	A	EL	0.33	22.58	30.72	0.46	0.53	1.20	1.16	0.51	0.47
2019	A	MM	0.26	29.63	37.41	0.38	0.45	1.07	0.99	0.50	0.42
2019	A	LS	0.14	31.37	34.30	0.56	0.54	1.05	1.10	0.51	0.49
2019	B	EL	0.23	23.81	32.01	0.44	0.58	1.37	0.93	0.45	0.36
2019	B	MM	0.20	26.06	31.15	0.43	0.43	1.29	1.04	0.48	0.36
2019	B	LS	0.20	26.81	34.42	0.40	0.43	1.26	1.04	0.44	0.43
2020	A	EL	0.27	24.69	32.99	0.35	0.42	1.44	1.13	0.52	0.46
2020	A	MM	0.20	29.90	39.71	0.33	0.46	1.56	1.59	0.62	0.67
2020	A	LS	0.45	34.78	45.64	0.21	0.41	1.32	1.14	0.51	0.51
2020	B	EL	0.44	29.31	36.86	0.23	0.33	1.11	0.81	0.40	0.31
2020	B	MM	0.31	29.61	36.51	0.23	0.34	1.23	1.08	0.46	0.46
2020	B	LS	0.39	32.28	38.04	0.22	0.34	1.37	1.15	0.56	0.44
2021	A	EL	0.21	20.91	24.42	0.57	0.63	1.46	0.95	0.51	0.35
2021	A	MM	0.18	24.88	27.27	0.51	0.54	1.44	1.21	0.57	0.48
2021	A	LS	0.09	27.68	25.96	0.46	0.64	1.39	1.20	0.51	0.42
2021	B	EL	0.20	21.30	27.80	0.47	0.56	1.44	1.24	0.54	0.40
2021	B	MM	0.35	25.91	32.83	0.40	0.45	1.45	1.14	0.51	0.40
2021	B	LS	0.19	27.00	30.11	0.38	0.40	1.32	1.20	0.52	0.42
Average±standard deviation data by year, vineyard, binomial											
2019			0.23±0.06	26.71±3.35	**33.34±2.53 ab**	**0.45±0.06 a**	**0.49±0.07 ab**	**1.21±0.13 b**	1.04±0.08	0.48±0.03	0.42±0.05
2020			0.34±0.10	30.10±3.37	**38.29±4.23 a**	**0.26±0.06 b**	**0.38±0.05 b**	**1.34±0.16 ab**	1.15±0.25	0.51±0.08	0.48±0.12
2021			0.20±0.08	24.61±2.88	**28.06±3.01 b**	**0.47±0.07 a**	**0.54±0.10 a**	**1.42±0.05 a**	1.16±0.11	0.52±0.02	0.41±0.04
	A		0.24±0.11	27.38±4.48	33.16±6.96	0.42±0.12	0.51±0.08	1.33±0.18	1.16±0.18	0.53±0.04	**0.48±0.09 a**
	B		0.28±0.10	26.90±3.26	33.30±3.42	0.36±0.10	0.43±0.09	1.32±0.11	1.07±0.14	0.48±0.05	**0.40±0.05 b**
		EL	0.28±0.09	**23.77±3.07 b**	30.80±4.31	0.42±0.12	0.51±0.11	1.34±0.15	1.04±0.17	0.49±0.05	0.39±0.06
		MM	0.25±0.07	**27.66±2.29 ab**	34.15±4.59	0.38±0.09	0.44±0.06	1.34±0.18	1.18±0.22	0.52±0.06	0.47±0.11
		LS	0.24±0.15	**29.99±3.30 a**	34.75±6.76	0.37±0.14	0.46±0.11	1.28±0.12	1.14±0.06	0.51±0.04	0.45±0.04

All ratios are calculated from Tables 1, 2 and Figure 4 averaged data, except for SIR-to-WLR (Mencarelli and Bellincontro, 2013; https://doi.org/10.1002/9781118569184.ch3). SIR = rate of sugar increase (total soluble solids in °Brix/day); WLR = weight loss rate (%/day). † °Brix/%. ‡ g/g. § mg/g. # mg/mg; only tannins extracted from skins (Skins MCP) were considered. For each factor (year, vineyard, binomial) and parameter, different letters among rows indicate significant differences (Tukey HSD post-hoc; $p<0.05$).

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Experimental section – PART B

Genetic traceability in monovarietal wines

Chapter 4

TaqMan® and HRM approaches for SNP genotyping in genetic traceability of musts and wines

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ABSTRACT

The fight against fraud in the wine sector requires continuous improvements and validations of new technologies applicable to musts and wines. Starting from published data from the Vitis18kSNP array, a series of new specific single nucleotide polymorphism (SNP) markers have been identified for some important north-western Italian cultivars, such as Barbera, Dolcetto and Arneis (*Vitis vinifera* L.), used in the production of high-quality wines under Protected Denomination of Origin. A pair of new SNP markers for each grape variety were selected and validated using two real-time PCR techniques: TaqMan® genotyping assays and high-resolution melting analysis (HRM). The TaqMan® assay has proven to be more reliable and repeatable than HRM analysis because despite being an economical and versatile technique for the detection of different types of genomic mutations (SNPs, insertions or deletions), HRM has shown limitations in the presence of poor-quality DNA extracted from musts and wines. TaqMan® assays have successfully identified Barbera, Dolcetto and Arneis in their respective musts and experimental wines, and with good efficiency in commercial wines. Marked differences between genotypes were observed, varietal identification in Dolcetto-based musts/wines was more efficient than that in Arneis-based wines. Therefore, the TaqMan® assay has considerable potential for varietal identification in wines and the procedure described in the present work can be easily adapted to all wines with adequate setup of DNA extraction methods that should be adapted to different wines.

Keywords: Genetic traceability, Grapevine, Musts, Wines, SNPs

1. Introduction

Authenticity, safety, and traceability of high commercial value wines produced under Protected Denomination of Origin (PDO) are major concerns for markets and consumers since wine is susceptible to fraud, adulteration and mislabeling. The addition of water, glycerol, alcohol, dyes, sweeteners, flavourings, unapproved sugar additions, and acidity modifications (Schlesier et al., 2009), as well as the use of wines from grape varieties different from those allowed, are examples of adulteration that can change the value of a high-quality wine. In this scenario, the European Union has created specific rules for wine traceability to protect both consumers and winemakers (Regulation EC No. 1151/2012 and subsequent amendments).

A wide range of analytical approaches for wine traceability and authentication, such as chemical analysis of proteins, volatile compounds, amino acids, polyphenols, anthocyanins, and minerals have been developed and evaluated (Moreno Arribas et al., 1999; Versari et al., 2014). However, because chemical compositions can be modified by winemaking procedures, farming techniques and environmental factors, these approaches are not always accurate (Villano et al., 2017). Since DNA is a stable molecule, DNA-based techniques are considered more reliable for food authentication (Martins-Lopes et al., 2013; Scarano and Rao, 2014; Catalano et al., 2016). Single sequence repeats (SSRs) are considered the most common markers for grapevine fingerprinting and are characterized by locus-specific polymorphism. As a result, many studies have used SSRs for grape identification in mono- and multi-varietal musts and wines (Faria et al., 2000; Siret et al., 2002; Boccacci et al., 2012; Pereira et al., 2012; Recupero et al., 2013; Catalano et al., 2016; Zambianchi et al., 2021, 2022). However, when considering SSR

genotyping in wine, polymerase chain reaction (PCR) amplification results are often inaccurate due to DNA degradation during alcoholic fermentation (low quality and quantity) and for the presence of PCR inhibitors such as polyphenols, proteins and polysaccharides (Vignani et al., 2019).

Single nucleotide polymorphisms (SNPs) have been proposed as new molecular markers for grapevine fingerprinting and have become a valid alternative to SSR genotyping in musts and wines (Cabezas et al., 2011). SNPs are the most abundant markers in the genome, purely biallelic with a low mutation rate, and can be detected and amplified by PCR in low-quality fragmented DNA (Cabezas et al., 2011; Catalano et al., 2016; Fanelli et al., 2021). Unlike SSR markers, many SNPs are needed for varietal identification; however, the progressive reduction in sequencing and data analysis over the years has allowed the identification of several mutations and polymorphisms between different grapevine cultivars (Emanuelli et al., 2013; Mercati et al., 2016; Gambino et al., 2017; De Lorenzis et al., 2019). Recent reports have explored the potential of SNP genotyping for must and wine traceability using qPCR coupled with a specific TaqMan® assay protocol (Catalano et al., 2016; Boccacci et al., 2020; Gambino et al., 2022; Song et al., 2024) or high-resolution melting (HRM) technology (Pereira and Martins-Lopes, 2015; Pereira et al., 2017,2018). The SNP TaqMan® genotyping assay provides significant technological advantages, including a single enzymatic step, flexible primer location in the region surrounding the SNP site, high sensitivity and specificity in DNA detection using labelled probes, and reduced analysis time (Boccacci et al., 2020). HRM is a versatile post-PCR method that can be used for the identification of SNPs, SSRs and other mutations, such as insertions and deletions (INDELs), and it can be used in the field of food

traceability (Mackay et al., 2008; Xanthopoulou et al., 2014; Merkouropoulos et al., 2016; Villano et al., 2017; Pereira et al., 2017; Barrias et al., 2023). HRM technology entails qPCR amplification in the presence of a saturation dye and subsequent melting of the amplicons by progressively increasing the temperature. The unique shape of the melting curve depends on the length, sequence, GC content and melting temperature of the amplicon (Simko, 2016). Primer design, PCR reagents, DNA (quality and quantity), amplicon length, multiple SNPs in the same DNA region and dye selection are all important factors that influence the success of HRM genotyping assays (Pereira et al., 2017). In wine, HRM has been performed on amplicons containing at least four SNPs and one INDEL in the same DNA region (Pereira et al., 2017). Nevertheless, there are no studies in the literature wherein HRM has been applied to a single SNP for varietal identification in wines.

Starting from previous experience in our laboratory in which an efficient traceability method in Nebbiolo (*Vitis vinifera* L.) wines based on SNPs and TaqMan® probes was developed (Boccacci et al., 2020; Gambino et al., 2022; Song et al., 2024), we extended and implemented this procedure to other important grapevine genotypes used to produce high-quality wines. Barbera and Dolcetto (*V. vinifera*) are two of the most important traditionally cultivated varieties in Piedmont (northwest Italy) and are the major and third regional black grapes, respectively, that contribute to fine PDO wines. Arneis (*V. vinifera*), an Italian autochthonous white grape cultivar grown mainly in Piedmont, is used to produce homonymous white wines. The wines derived from these genotypes are an excellent starting point for the development of a genetic traceability system that can be extended to different wines.

The aim of this study was to develop and validate effective assays for the genetic traceability of musts and wines typical of north-western Italy produced from Barbera, Dolcetto and Arneis grapes. We identified cultivar-specific SNPs starting from available databases produced using the Vitis18kSNP array (https://urgi.versailles.inra.fr/Species/Vitis/GrapeReSeq_Illumina_20K). This array, containing more than 18,000 SNPs, was produced using sequencing data from different genotypes of *V. vinifera* and other *Vitis* species and it has already been used to study the genetic relationships between cultivated and wild grapevine germplasm (Laucou et al., 2018; De Lorenzis et al., 2019). In addition, we implemented rapid SNP assays for varietal authentication in wine by comparing TaqMan® and HRM approaches to establish the best SNP-based traceability procedure. The proposed and validated protocol can be easily adapted and used for varietal identification in any wine.

2. Methods

2.1. Plant material and SNP identification

DNA from Barbera, Dolcetto and Arneis was extracted from young leaves using a NucleoSpin® Plant Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. In addition to ampelographic observations, the plants were genotyped using six SSR markers (This et al., 2004; Ruffa et al., 2016) to confirm the identity of the three cultivars.

We collected and processed all SNP data available in the literature by analysing several grapevine genotypes using the Vitis18kSNP array (Illumina, Inc., San Diego, CA, USA). In addition to SNP data obtained in

cultivars typical of northwestern Italy (Boccacci et al., 2020; Raimondi et al., 2020), we processed the data from six available SNP databases (Laucou et al., 2018; De Lorenzis et al., 2019; D'Onofrio et al., 2021; D'Onofrio, 2020; Crespan et al., 2020; Crespan et al., 2021) for a total number of 1857 genotypes (Table S1). The SNP data were subjected to several filtering steps following the previous indications (Boccacci et al., 2020): i) development of an overall database without redundant genotypes; ii) removal of SNPs with missing data even in a single genotype; iii) removal of SNPs with heterozygous allelic profile and selection of SNPs with a homozygous allelic profile in the cultivar of interest; iv) selection of SNPs with a "homozygous alternative" allelic profile to the cultivar of interest in other cultivars, in order to facilitate varietal identification in unknown wine samples. The two best SNPs respecting these parameters specific for Barbera, Dolcetto and Arneis were validated by PCR amplification and Sanger sequencing, as reported by Boccacci et al. (2020). The primers used are reported in Table S2.

2.2. Experimental vinification and commercial wines

A total of 100 kg of grapes for each true-to-type Arneis, Barbera and Dolcetto cultivars were used to produce the experimental wines.

Arneis grapes, after harvesting, were placed in a thermo-controlled room at 10°C overnight to use refrigerated grapes. After this cool time these were destemmed and crushed in a TEMA de-stemmer–crusher (Enoveneta, Piazzola Sul Brenta, Italy). Then the crushed grapes were pressed using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy), reaching the maximum pressure of 1.0 bar. After mashing, a 24-h cold static clarification was carried out on grape juice, using 2 g/hL of

pectolytic enzyme (Lallzyme cuvée blanc, Lallemand Inc., Montreal, Canada). Successively must was racked and finally inoculated for the alcoholic fermentation, with 20 g/hL of *Saccharomyces cerevisiae* active dry yeast (Fermol Chardonnay, AEB Group, San Polo, Italy). During the alcoholic fermentation, the temperature (18/±1 °C) and the sugar decrease were daily controlled and two additions (one at the beginning and the second at 1/3 of fermentation process) of 20 g/hL of nutrients (Fermaid E, Lallemand Inc.) corresponding to a total increase of 56 mg/L yeast assimilable nitrogen (YAN) were done. At the end of alcoholic fermentation, the wine was racked and 50 mg/L of SO_2 added.

Barbera and Dolcetto grapes were destemmed and crushed (Enoveneta, Piazzola Sul Brenta, Italy). The mash was placed into a CO_2 saturated inox tank and inoculated for the alcoholic fermentation with 20 g/ hL of *Saccharomyces cerevisiae* active dry yeast (Lalvin BRL 97, Lallemand Inc.). During the alcoholic fermentation, the temperature (26/ ±1 °C) and the sugar decrease were daily controlled. As in the white vinification two addition of nutrients (Fermaid E, Lallemand Inc.) have been done. Moreover, two punch-down per day were carried out in the first three days and subsequently two pumping-over were done until the end of maceration, which lasted 8 days. Later, the pomace cap was pressed (Velo SpA, Altivole, Italy) and the pressed wine was added to the free-run part. Then 1 g/hL of lactic bacteria *Oenococcus oeni* VP41 MBR ML (Lallemand Inc.) was added to inoculate the malolactic fermentation. At the end, the wines were racked and 50 mg/L of SO_2 added. The wines, both red and white, were stored at 0 °C for two weeks for cold stabilization and finally filtered and bottled.

The red wine samples (500 mL) were collected in five different steps: after crushing (M1), at the end of maceration (M2), after alcoholic

fermentation (M3), after the malolactic fermentation (M4), and once bottled (W). Instead, Arneis samples were only collected in three steps: on the juice after mashing (M1), at the end of alcoholic fermentation (M3) and after bottling (W). This because in the white winemaking usually there is no maceration, and the malolactic fermentation is not performed. For all samples, 50 mL aliquots were stored at −20 °C until DNA extraction.

Commercial wines from Barbera (Barbera d' Alba, 2020, 2021 and 2022), Dolcetto (Dolcetto d' Alba, 2021 and 2022) and Arneis (Roero Arneis 2022) grapes were provided by Enocontrol Scarl (Alba, Italy). Before DNA isolation, each wine was homogenised by inverting the bottle, and 50 mL aliquots were stored at – 20 °C until DNA extraction.

2.3. DNA extraction and quantification

Total DNA extraction from each must and wine was performed following the Cetyltrimethylammonium Bromide (CTAB)-based protocol described by Gambino et al. (2022). Briefly, 50 mL of musts and wines frozen at – 20 °C for at least 15 days was centrifuged (4000 g, 1 h, 4 °C). About 200–300 mg of each must pellet and the wine pellet collected from 50 mL were dissolved in 5 mL of buffer containing 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1 M Tris–HCl (pH 8.0), 3% CTAB, 1% β-mercaptoethanol and incubated for 1 h at 65 °C. A volume of 5 mL of chloroform:isoamyl alcohol (C:I 24:1) was added and centrifuged at 8000 g for 10 min at 4 °C. The supernatant with 0.1 volume of prewarmed (65 °C) 10% CTAB was purified with 1 volume of C:I. Two volumes of cold ethanol were added to the supernatant and stored overnight at − 25 °C. The precipitated DNA (10,000 g, 30 min, 4 °C) was suspended in Tris-EDTA (TE) buffer (250 μL) and incubated with the addition of proteinase K (20 μL, 20 mg/mL)

for 30 min at 48 °C. The sample was purified with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged (11,000 g, 15 min, 4 °C). Two volumes of cold ethanol and 2.5 mol/L of ammonium acetate were added to the supernatant and stored for 2 h at − 25 °C. The pellet obtained after centrifugation (20,000 g, 30 min, 4 °C) was washed with 500 µL 70% ethanol and dissolved in 100 µL sterile water. Final purification was performed with the NucleoSpin® Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The final elution was performed in 45 µL PE buffer. Total DNA was preliminary evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by determining the spectrophotometric absorbance of the samples at 230, 260 and 280 nm. Grapevine DNA was measured by qPCR amplification of the 9-cis-epoxycarotenoid dioxygenase gene (*VvNCED2*) using the TaqMan® FAM-labelled probe and methods reported by Savazzini and Martinelli (2006). The qPCR reaction was performed with a reaction volume of 10 µL, containing 2.5 µL of extracted DNA, 5 µL of TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.3 µM of each primer and 0.2 µM of the FAM probe. Amplification, carried out using a CFX96 Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was performed as follows: initial denaturation step at 95 °C for 10 min, 55 cycles of 95 °C for 15 s, and 60 °C for 1 min. The grapevine DNA concentration was determined by plotting the Ct values obtained from the DNA of the samples with the standard curve of the *VvNCED2* produced with serial dilutions of DNA extracted from leaves. All samples were analysed in triplicate.

2.4. SNP genotyping protocols and data analysis

We compared two genotyping protocols (TaqMan® probes and HRM analysis) to identify the cultivar-specific SNPs in musts and wine of Barbera, Dolcetto and Arneis. For each cultivar-specific SNP, primers and TaqMan® probes were designed using Primer Express version 3.0 (Thermo Fisher Scientific) (Table S3). The amplification reaction was performed in triplicate in a reaction volume of 10 μL, containing 2.5 μL of DNA, 5 μL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific) and 0.25 μL of 40X TaqMan® SNP Genotyping Assay mix (containing pre-mixed forward and reverse primers, VIC probe and FAM probe). The amplification profile used was the same as reported for the *VvNCED2* probe. The threshold position was automatically calculated using Bio-Rad CFX Manager 3.1 software (Bio-Rad Laboratories, Inc.), and allelic discrimination plots were performed using the CFX96 Detection System.

In the HRM protocol, we used the same primers reported for TaqMan® assays (Table S3) and the amplification reaction was carried out in triplicate with a total volume of 10 μL, containing 2.5 μL of DNA, 5 μL MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific) and 0.2 μM of each primer. Amplification, using a CFX96 Detection System, was performed as follow: initial denaturation step at 95 °C for 10 min, 55 cycles of 95 °C for 30 s, 60 °C (58 °C for SNP_87) for 30 s and 72 °C for 30 s. Melting curves were generated over a 60–95 °C range with an increment of 0.1 °C every 5 s. During the incremental melting step, fluorescence data were continuously acquired and analysed using High-Resolution Melt Software v3.0.1 (Bio-Rad Laboratories, Inc.).

PCR inhibitors in the extracted DNA were determined by adding TaqMan® Exogenous Internal Positive Control (EIPC) reagents (Thermo

Fisher Scientific) to the qPCR mix. The amplification reaction in a final volume of 10 µL contained 2.5 µL genomic DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.2 µL EIPC DNA and 1 µL EIPC mix (containing pre-mixed forward, reverse primers and VIC probe specific for EIPC). The amplification profile used was the same as reported above for TaqMan® assays. PCR inhibition was calculated from a calibration curve with serial dilution of EIPC, assuming 100% amplification efficiency of EIPC in samples containing DNA extracted from leaves.

Statistical analyses were carried out using a one-way analysis of variance (SPSS Version 22). The differences among the treatments were indicated with different letters using Tukey's post-hoc test at p-value ≤ 0.05.

3. Results and discussion

3.1. Identification of cultivar-specific SNPs

In recent years, in addition to SSRs, which have historically been considered the markers of choice for cultivar identification in grapevine (This et al., 2004; http://www.eu-vitis.de/index.php; http://www.vivc. de), SNPs are becoming highly performing markers and are widely used in grapevine genotyping studies (Myles et al., 2010; Laucou et al., 2018; Raimondi et al., 2020; D'Onofrio et al., 2021). Due to their characteristics, SNPs have been very useful in varietal identification in musts and wine for genetic traceability studies (Catalano et al., 2016; Pereira et al., 2017; Boccacci et al., 2020; Carrara et al., 2023). However, for SNP detection in wine, it is not effective to use arrays, such as the Vitis18kSNP array

(Illumina) or high throughput sequencing, due to the low quality of the extracted DNA, while high-performance qPCR-based detection systems, which work effectively even with traces of DNA, are recommended. Accordingly, the identification of a minimum number of specific and unique SNP markers (ideally 1 or 2) for each cultivar is a fundamental prerequisite for the practical application of SNPs in molecular traceability in the wine field.

Based on seven studies (Laucou et al., 2018; De Lorenzis et al., 2019; D'Onofrio et al., 2021; D'Onofrio, 2020; Crespan et al., 2020; Crespan et al., 2021; Raimondi et al., 2020) that published SNP profiles of many grapevine genotypes using the Vitis18kSNP array, we produced an overall database to identify cultivar-specific SNPs. A total of 1857 accessions analysed in these seven works corresponded to 1493 unique non-redundant genotypes. Among them, 4408 of the 18,071 SNP markers analysed with the Vitis18kSNP array failed or showed an unclear hybridisation signal and were thus discarded. The remaining 13, 663 SNPs were further filtered for each cultivar. For example, we selected SNPs that were homozygous and without polymorphisms within the Arneis accession present in the database but none of these SNPs were specific to Arneis. Therefore, we selected SNPs showing a profile homozygous alternative to Arneis in the largest number of non-Arneis cultivars, because they are potentially more discriminating in SNP genotyping assays (Boccacci et al., 2020). Within this group of markers, two SNPs located on different chromosomes were therefore extrapolated (Table S1). The combination of which was unique in Arneis (SNP_16408, SNP_6647) and sufficient to uniquely identify Arneis among the 1493 genotypes present in the overall SNP database. Following the same procedure, we identified two SNP markers whose allelic combination was specific for Barbera

(SNP_15726 and SNP_3356); the same was done for Dolcetto (SNP_1722 and SNP_87) (Table S1).

The six selected SNPs were validated by Sanger sequencing confirming the hybridisation results (Fig. S1). In addition, for each cultivar, clone selections officially registered in the Italian National Register of Grape Varieties (http://catalogoviti.politicheagricole.it/catalogo.php) were collected from nurseries, and we confirmed that all accessions had the same allelic profiles reported in the SNP database, suggesting that these SNPs were very robust markers specific for Arneis, Barbera or Dolcetto. This cultivar-specific SNP selection methodology, first used in Nebbiolo (Boccacci et al., 2020), has now been successfully confirmed for three other varieties, and can be extended very easily to any genotype analysed with the Vitis18kSNP array. This approach should be more effective for identifying 1 or 2 cultivar-specific SNPs that can be analysed in wine using qPCR techniques, compared, for example, to identifying mutations in genes such as UDP-Glucose:Flavonoid 3-O-Glucosyltransferase (Pereira and Martins-Lopes, 2015) or flavanone 3-hydroxylase and the leucoanthocyanidin dioxygenase gene (Gomes et al., 2018), which would require a large unavailable database and would be complex to produce considering the high number of grapevine genotypes. The specificity of the SNPs identified in the output of the Vitis18kSNP array was validated by the large number of unique genotypes analysed thus far in seven previously published studies (1493), which included all major wine varieties that could potentially be used for fraudulent blends. However, if this method of identifying varietal SNPs is useful for targeted analyses of wines, it is not particularly suitable for traditional grapevine fingerprinting,

as it is more appropriate to use arrays (Myles et al., 2010; Laucou et al., 2018) or a selection of more polymorphic SNPs (Cabezas et al., 2011).

3.2. Genotyping assays using HRM and TaqMan® approaches

High-efficiency analysis of SNPs can occur using two qPCR approaches: HRM and TaqMan® probes. HRM represents a simple and low-cost technique for the identification of genomic mutations (SNPs, INDELs), and it has been successfully applied for the authenticity of olive oil and wine (Pereira et al., 2017,2018). Compared to the TaqMan® technique, HRM is more versatile for detecting different mutations (including INDELs) and is more suitable for difficult genomic regions, such as repeat sequences in nucleotides surrounding SNPs, where it is difficult to design TaqMan® probes. However, in previous studies (Pereira et al., 2017, 2018), HRM was applied to amplicons containing several SNPs and INDELS in each fragment, facilitating the genotype identification using the difference in melting curves. In our experiment, with only one SNP available in each amplicon, the efficiency of the HRM technique should be evaluated and compared with the TaqMan® approach which proved to be very efficient for the detection of a single SNP in must and wine (Catalano et al., 2016; Boccacci et al., 2020; Gambino et al., 2022; Song et al., 2024).

The six SNP loci for Arneis, Barbera and Dolcetto were analysed by HRM using specific primers (Table S3) on DNA extracted from leaves of true-to-type plants. The results confirmed the efficiency of HRM in SNP genotyping and cultivar specificity, with only five out of six selected SNPs (Fig. S2). SNP_16408 (specific for Arneis) showed some problems in the melting difference plot with incorrect distinction between homozygous

and heterozygous genotypes (Fig. S2). It is likely that this genomic region is not optimal for accurate melting curve analysis, as it presents some problems (Fig. S3), and the marker is not efficient for genotyping with HRM. In addition, to assess the limits of the technique for detecting blends, we produced an artificial mix of genomic DNA, increasing the levels of non-target DNA in the target DNA (Fig. 1). Non-Barbera DNA was mixed with Barbera DNA (from 0.1% to 50% v/v of contamination), and SNP_15726 and SNP_3356 were analysed using HRM. The same procedure was carried out for SNPs specific to Arneis (excluding SNP_16408) and Dolcetto. Data obtained from HRM showed that the detection limit of non-specific cultivars in the DNA mixture was 20% for SNP_87, 10% for SNP_6647, and 5% for SNP_1722, SNP_15726 and SNP_3356 (Fig. 1).

The same primers used for detection in HRM of the six selected SNPs were applied in the TaqMan® approach in addition to specific probes labelled with FAM or VIC fluorescent dyes (Table S3). The TaqMan® discrimination plots confirmed the efficiency of the technique for SNP detection (Fig. S4) for all six SNP loci, including SNP_16408, showing a problem in HRM. The detection limit of non-specific cultivars in the artificial DNA mixture was 1% for SNP_1722 and 5% for the other five SNP genotyping assays (Fig. 2 and Fig. S5). Our data support the potentiality and sensitivity of SNP genotyping using TaqMan® probes, confirming a detection limit of 1% in the discrimination of DNA in extract mixtures for SNP_1722, as previously reported for SNPs specific to Nebbiolo (Boccacci et al., 2020), which is the lowest level described in the literature to date (Catalano et al., 2016; Siret et al., 2002). For the other five SNPs, the detection limits stand at 5%, but at levels higher than those referable, for example, to SSR markers. These data also highlight that the

efficiency of the technique is closely linked to the genomic region in which the SNP marker is located, the surrounding sequences and the possibility of designing excellent quality primers and probes influence the performance of the TaqMan® approach and consequently not all SNPs may be suitable for varietal identification in complex matrices such as musts and wines. In addition, HRM analyses appear less performing compared to TaqMan® probes in the same SNP loci and using the same primers, with a clear reduction in the limit of detection for non-specific DNA in a mixture (in 4 out of 6 SNPs) and with incorrect distinction between homozygous and heterozygous genotypes of SNP_16408.

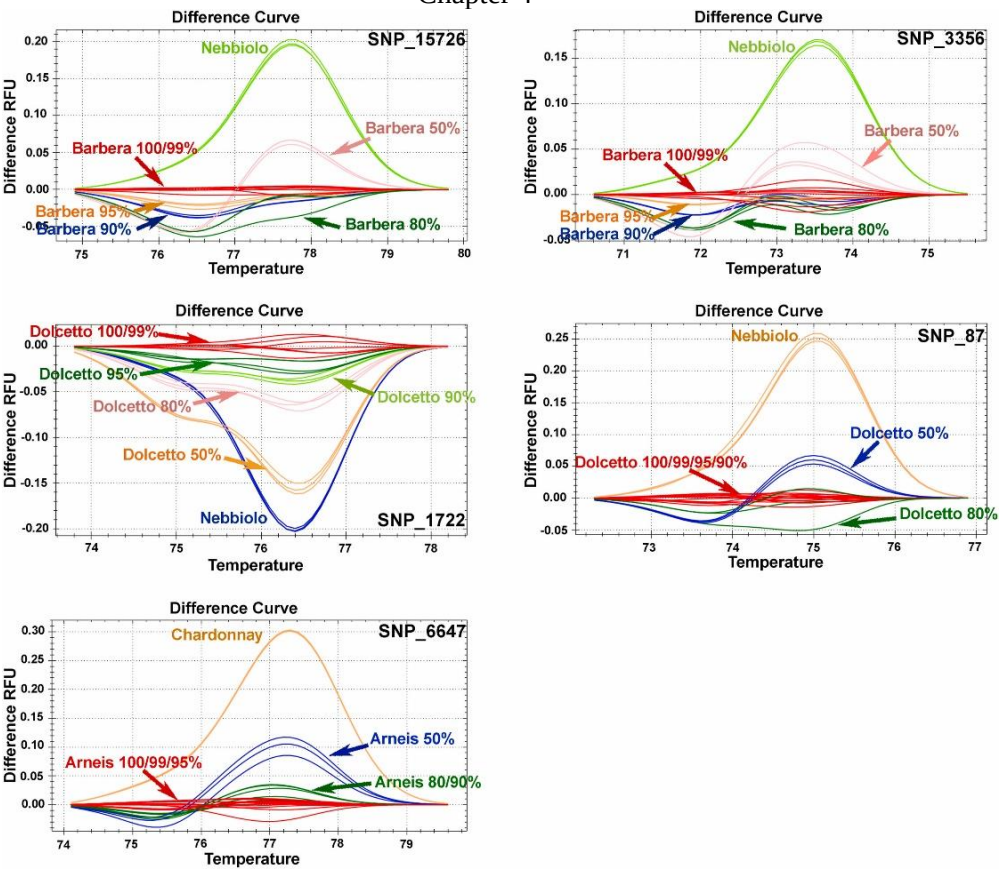

Figure 1. Detection limit of HRM genotyping assays in mixtures of DNA extracted from leaves. Samples were grouped based on the melting curve shape shown in the difference plots. SNP_3356, SNP_15726 (Barbera-specific), SNP_6647 (Arneis-specific), SNP_1722 and SNP_87 (Dolcetto-specific). Increasing levels of non-target DNA (1–50%) were mixed with the target DNA. For SNP_3356 and SNP_15726, Nebbiolo was used as the non-target and Barbera as the target genotype; for SNP_1722 and SNP_87, Nebbiolo was used as the non-target and Dolcetto as the target genotype; for SNP_6647, Chardonnay was used as the non-target and Arneis as the target genotype. The detection limit was determined using triplicates of each sample. Genotypes were assigned using a cut-off confidence value of 95%.

3.3. SNP genotyping in experimental musts and wines

Experimental musts/wines of Arneis, Barbera and Dolcetto obtained from true-to-type grapes were collected from five time points for red varieties (mashing_M1, end of maceration_M2, end of AF_M3, end of MLF_M4 and wine_W), considered the most impacting time points for DNA extraction during the winemaking process, as previously observed in Nebbiolo (Boccacci et al., 2020), and from three time points for Arneis (mashing_M1, end of AF_M3, wine_W). DNA from all samples was extracted using a CTAB-based method that has proven to be very effective in Nebbiolo wines (Gambino et al., 2022), and DNA was obtained from all time points with decreasing quantity and quality from musts to finished wine, as expected (Table 1). Considering the well-known problems in the spectrophotometry quantification of DNA extracted from musts/wines (Savazzini and Martinelli, 2006; Vignani et al., 2019; Boccacci et al., 2020; Gambino et al., 2022), due to the presence of yeast contamination, DNA degradation and the interference of other compounds, such as phenol used in DNA purification, the *VvNCED2* TaqMan® probe (Fig. S6) was used for a more specific quantification of grapevine DNA (Savazzini and Martinelli, 2006; Boccacci et al., 2020; Gambino et al., 2022). In musts/wines of Dolcetto and Barbera, the grapevine DNA varies from 3978 pg/µL in M1 to 0.28 pg/µL in W with comparable values between the two varieties (Table 1). The recovery of grapevine DNA after malolactic fermentation (M4) was significantly reduced, confirming the data reported in Nebbiolo (Boccacci et al., 2020). In musts/wines of Arneis, the M3 values were much lower than in Dolcetto and Barbera at the same collection time point, by a factor of 100, as well as in experimental wine by about 10 times (Table 1), but at this collection time point, the data were less significant, given that we are very close to the

detection limit of the *VvNCED2*-based technique for wines (Boccacci et al., 2020). This difference in DNA extractability from red wines, such as Barbera and Dolcetto, to white wine such as Arneis, may be linked to the different mashing and clarification operation to which the different wines are subjected.

The extracted DNA was then analysed with the six selected SNPs using HRM and TaqMan® approaches. Each SNP was analysed in all types of wines and musts; for SNP_3356 and SNP_15726 (Barbera-specific), the Barbera musts/wines represented the target controls and the Dolcetto and Arneis musts/wines represented the non-target controls. For SNP_1722 and SNP_87 (Dolcetto-specific), the Dolcetto musts/wines represented the target controls and Barbera and Arneis musts/wines represented the non-target controls, and similarly for Arneis-specific SNP_6647 and SNP_16408. For the univocal varietal identification of a must/wine, at least 1 replicate of both cultivar-specific SNPs must be amplified correctly. Using TaqMan® probes, SNP_3356 and SNP_15726 correctly identified Barbera experimental musts/wine at all collection time points, as well as SNP_1722 and SNP_87 in all Dolcetto experimental musts and wines. In some M4 and W technical replicates no amplification was observed (Table 2, Fig. 3). These data confirmed the results obtained in Nebbiolo musts (Boccacci et al., 2020). After malolactic fermentation, TaqMan® genotyping assays showed some amplification problems, probably attributed to the small amount of recovered grapevine DNA. However, with at least two technical replications for sample, it was possible to correctly determine the grapevine genotype from the experimental musts and wine of Dolcetto and Barbera (Table 2). SNP_16408 and SNP_6647 correctly identified experimental Arneis musts at M1 and M3, while no amplification or incorrect allelic calls were

observed in wine (Table 2). These amplification problems in Arneis wine are probably linked to the low yield of extracted DNA, as reported for the *VvNCED2* gene. As demonstrated in previous works (Gambino et al., 2022; Song et al., 2024), TaqMan® assays using DNA concentrations lower than 0.5 pg/mL of starting wine are not reliable and may result in a lack of amplification or incorrect allelic identification (Table 1). Overall, considering all target and non-target musts and wine, the SNP_15726, SNP_1722 and SNP_87 assays showed the highest percentages of correct allelic identification (88.4%). Furthermore, the six SNP TaqMan® assays showed a higher percentage of correct allelic calls in the experimental Dolcetto musts and wines (93.3%) than in Barbera musts/wines (86.6%), and significantly higher than in Arneis musts/wines (63.9%) (Table 2). These data demonstrate how the genotype effect can be important in determining the effectiveness of a molecular assay for varietal identification in wines, even in experimental wines vinified in the same winery with the same oenological procedures as in Dolcetto and Barbera. The strong reduction observed in Arneis wines was probably linked to a fermentation without solid parts (skins, seeds and in some case also stalks) and the use of adjuvants, mainly pectolytic enzyme or bentonite, gelatine and silica sol (Gambino et al., 2022).

The same DNA samples were then analysed with five SNPs using the HRM method; SNP_16408 was excluded due to the problem in the melting curve reported above (Fig. S2). The HRM method did not perform as well as the TaqMan® probes. In musts/wine of Barbera and Dolcetto, the allelic calls were optimal only in M1 and M2. In M3, after alcoholic fermentation, the first amplification problems were observed, and at M4 and W it was not possible to correctly identify the experimental musts/wines. Furthermore, in musts/wine of Arneis, some problems were

observed at M1, as well as at M3 and W (Table 2, Fig. 4, Fig. S7). The HRM analyses in the experimental musts and wines highlighted two types of problems, lack of PCR amplification or incorrect amplification, producing non-specific melting curves with melting peaks even 5–6 °C different from those expected for the three possible allelic combinations at each SNP locus (Fig. S8). These differences in melting peaks cannot be attributed to unknown allelic variants, as the nucleotide sequences of the cultivars used in this experiment are well known in the SNP loci considered and no other allelic variants were observed.

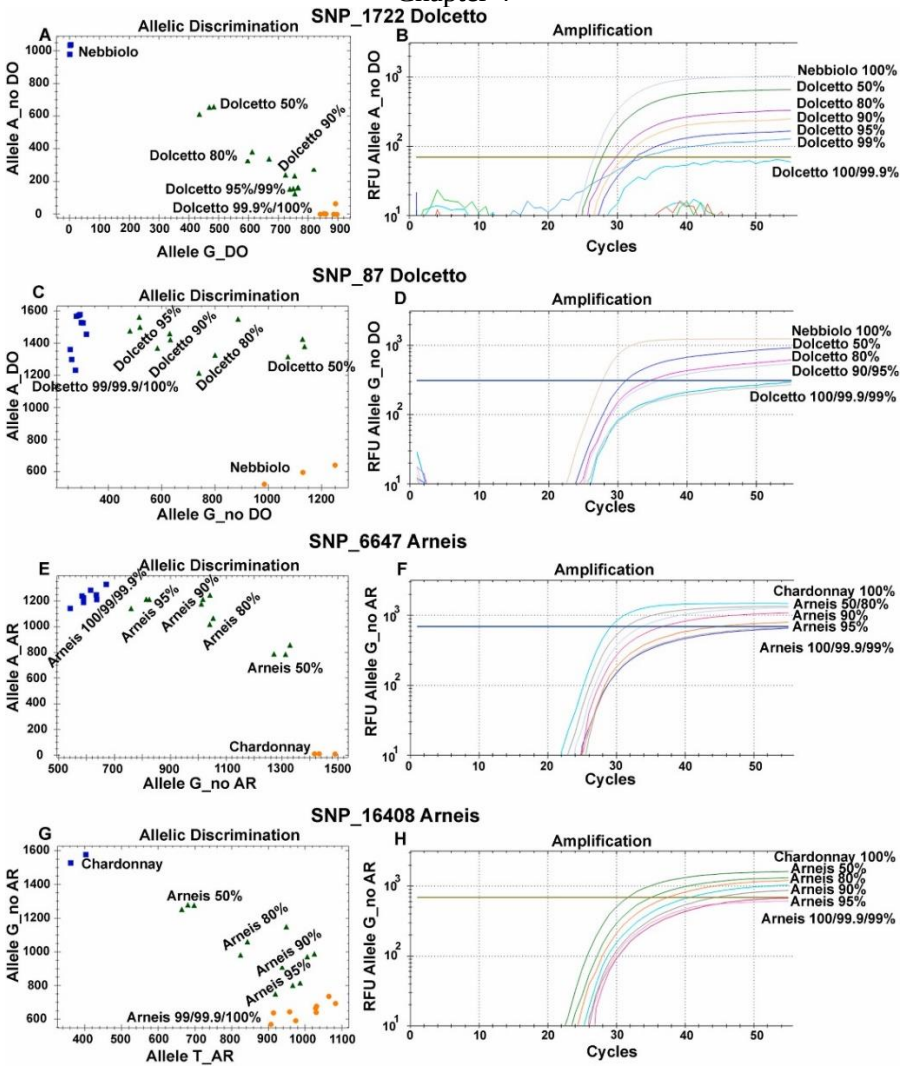

Figure 2. Detection limit of TaqMan® SNP_1722, SNP_87, SNP_6647 and SNP_16408 genotyping assays in mixtures of DNA extracted from leaves. Allelic discrimination plots (A, C, E, G) and relative fluorescence unit (RFU) of TaqMan® probes for non-specific alleles (B, D, F, H). Nebbiolo and Chardonnay DNA were used as non-specific genotypes in the SNP assays for Dolcetto (A–D) and Arneis (E–H), respectively. Increasing levels of Nebbiolo DNA (from 0.1 to 50%) were mixed with Dolcetto DNA (A–D). The yellow line (B) and blue line in the amplification plot (D) indicate the RFU level of 100% Dolcetto, above which it was possible to detect contamination of non-Dolcetto DNA. Increasing levels of Chardonnay DNA (from 0.1 to 50%) were mixed with Arneis DNA (E–H). The blue line (F) and the yellow line in the amplification plot (H) indicates the RFU level of 100% Arneis, above which it was possible to detect contamination of non-Arneis DNA. For each SNP assay, the detection limits of non-specific DNA mixed in Dolcetto (A–D) or Arneis (E–H) DNA were determined using three replicates of each sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Samples producing amplicons with non-specific melting peaks were therefore excluded from subsequent analyses so as not to alter the analyses of the melting curves of samples that amplified correctly. As shown with the artificial mix of genomic DNA extracted from the leaf (Figs. 1 and 2), the HRM technique was less performing than the TaqMan® approach. Furthermore, in samples with small quantities of DNA and contaminated by secondary metabolites present in musts and wines, several non-specific amplifications were observed in the HRM which compromised the reliability of the assays. However, HRM has proven effective in detecting grape DNA in the must and wine from Portuguese varieties (Pereira et al., 2017). This could be linked to the analysis of different wines, the use of loci with multiple mutations in the same amplicon and a longer work of fine-tuning the assay. In amplicons with a single SNP mutation, the TaqMan® approach does not require a complex setup and guarantees greater sensitivity and specificity than HRM due to the use of FAM/VIC-labelled probes, which increases the reliability of the technique for SNP marker detection in complex matrices such as musts and wines.

Table 1. DNA quantity and quality extracted from Barbera (_B), Dolcetto (_D) and Arneis (_A) musts (M) and wines (W) collected during five and three (for Arneis) experimental winemaking steps, and from commercial wines. Purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*. Data are means ± SDs of three replicates and are expressed as ng/µl of DNA eluted from the NucleoSpin® Plant Kit.

Must/Wine	Description	Spectrophotometric quantification			*VvNCED2* quantification
		DNA yield (ng/µl)	A_{260}:A_{280}	A_{260}:A_{230}	DNA yield (ng/µl)
M1_B	mashing	322.65 ± 44.49	2.08 ± 0.02	1.91 ± 0.11	3978.20 ± 678.45
M2_B	end maceration	391.15 ± 99.20	2.16 ± 0.01	2.39 ± 0.05	519.02 ± 5.04
M3_B	after AF*	9.38 ± 4.69	1.83 ± 0.14	0.91 ± 0.23	15.50 ± 6.05
M4_B	after MLF**	5.55 ± 0.49	1.90 ± 0.21	0.60 ± 0.05	0.28 ± 0.12
W_B	wine	4.40 ± 0.48	1.83 ± 0.18	0.67 ± 0.02	0.39 ± 0.55
M1_D	mashing	264.30 ± 55.14	2.07 ± 0.04	1.78 ± 0.24	3649.80 ± 451.2
M2_D	end maceration	130.90 ± 65.93	2.08 ± 0.03	1.74 ± 0.06	1134.61 ± 359.77
M3_D	after AF*	4.20 ± 0.14	1.64 ± 0.06	0.72 ± 0.04	5.88 ± 2.86
M4_D	after MLF**	7.30 ± 0.42	1.68 ± 0.09	0.89 ± 0.08	1.87 ± 1.15
W_D	wine	3.80 ± 0.28	1.54 ± 0.07	0.72 ± 0.02	0.34 ± 0.14
M1_A	mashing	377.40 ± 17.96	2.10± 0.01	2.12 ± 0.02	6829.81 ± 2683.5
M3_A	after AF*	6.25 ± 0.78	1.77 ± 0.10	0.61 ± 0.08	0.18 ± 0.25
W_A	wine	4.15 ± 0.21	1.70± 0.27	0.60± 0.01	0.04 ± 0.01
Commercial wines	Barbera 2020	9.10 ± 3.82	1.47 ± 0.04	0.46 ± 0.42	0.06 ± 0.11
	Barbera 2021	5.05 ± 0.49	1.60 ± 0.08	0.45 ± 0.23	-
	Barbera 2022_a	3.92 ± 0.37	1.88 ± 0.00	0.46 ± 0.03	1.06 ± 0.36
	Barbera 2022_b	3.15 ± 0.11	1.90 ± 0.22	0.63 ± 0.07	1.56 ± 0.43
	Dolcetto 2021_a	4.75 ± 0.07	1.50 ± 0.04	0.66 ± 0.00	0.69 ± 0.25
	Dolcetto 2021_b	6.20 ± 0.52	1.60 ± 0.17	0.63 ± 0.20	0.14 ± 0.20
	Dolcetto 2022_a	42.20 ± 0.42	1.93 ± 0.03	1.92 ± 0.06	7.64 ± 2.45
	Dolcetto 2022_b	9.17 ± 0.16	1.96 ± 0.00	1.10 ± 0.01	-
	Dolcetto 2022_c	32.03± 6.80	1.94 ± 0.02	0.79 ± 0.15	-
	Arneis 2022_a	5.80 ± 0.95	1.69 ± 0.03	0.76 ± 0.02	-
	Arneis 2022_b	5.40 ± 0.28	1.52 ± 0.13	0.72 ± 0.05	-
	Arneis 2022_c	504.28 ± 125.40	2.17 ± 0.02	2.34 ± 0.02	0.14 ± 0.11

*AF= alcoholic fermentation
**MLF=malolactic fermentation

Table 2. Allelic profiles of genotyping assays from Barbera (_B), Dolcetto (_D) and Arneis (_A) musts (M) and wines (W) collected during five and 639 three (for Arneis) experimental winemaking steps. SNPs specific for Barbera (SNP_15726, SNP_3356), Dolcetto (SNP_1722, SNP_87) and Arneis (SNP_16408, SNP_6647) were analysed using high resolution melting (HRM) and TaqMan® 640 probes. Lower-case letters in the allelic profile denote an 641 incorrect call of the genotyping assay; "-" indicates a sample without amplification or incorrect amplification in HRM. For each sample, two independent 642 extractions were analysed (R1, R2).

		TaqMan® probes										High Resolution Melting									
		Barbera				Dolcetto				Arneis		Barbera				Dolcetto				Arneis	
Must/ Wine	Description	SNP_15726		SNP_3356		SNP_1722		SNP_87		SNP_6647		SNP_16408	SNP_15726		SNP_3356		SNP_1722		SNP_87		SNP_6647		SNP_16408
		Alleles		Alleles		Alleles		Alleles		Alleles		Alleles	Alleles		Alleles		Alleles		Alleles		Alleles		Alleles
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
M1_B	mashing	**AA**	**AA**	**AA**	**AA**	AA	AA	GG	GG	AG	AG	GT	GT	**AA**	**AA**	**AA**	AA	AA	GG	GG	aa	AG	-	-
M2_B	end maceration	**AA**	**AA**	**AA**	**AA**	AA	AA	GG	GG	AG	AG	GT	GT	**AA**	**AA**	**AA**	AA	AA	GG	GG	AG	AG	-	-
M3_B	after AF*	**AA**	**AA**	**AA**	**AA**	AA	AA	GG	GG	AG	AG	GT	GT	**AA**	**AA**	**AA**	AA	AA	GG	-	aa	aa	-	-
M4_B	after MLF**	**AA**	**AA**	**AA**	-	AA	gg	GG	-	gg	AG	tt	GT	**AA**	-	-	-	-	-	-	-	-	-	-
W_B	wine	**AA**	**AA**	-	**AA**	AA	AA	GG	GG	AG	gg	GT	tt	**ee**	**ee**	**AA**	-	-	-	-	-	-	-	-
M1_D	mashing	AC	AC	AA	AA	**GG**	**GG**	**AA**	**AA**	GG	GG	GG	GG	aa	-	AA	AA	**GG**	**GG**	**AA**	**AA**	GG	GG	-
M2_D	end maceration	AC	AC	AA	AA	**GG**	**GG**	**AA**	**AA**	GG	GG	GG	GG	AC	aa	AA	-	**GG**	**GG**	**AA**	**AA**	GG	GG	-
M3_D	after AF*	AC	AC	AA	AA	**GG**	**GG**	**AA**	**AA**	GG	GG	GG	GG	-	AC	-	AA	-	-	**AA**	**AA**	aa	aa	-
M4_D	after MLF**	-	AC	AA	AA	**GG**	**GG**	**AA**	**AA**	GG	GG	GG	GG	ee	-	-	AA	-	-	**AA**	**AA**	-	-	-
W_D	wine	AC	ee	AA	ag	-	**GG**	**AA**	**AA**	GG	GG	GG	GG	aa	-	-	-	-	-	**AA**	**AA**	-	GG	-
M1_A	mashing	CC	CC	AG	AG	AA	AA	GG	GG	**AA**	**AA**	**TT**	**TT**	ac	CC	aa	aa	AA	AA	GG	GG	**AA**	**AA**	-
M3_A	after AF*	CC	CC	-	AG	AA	AA	GG	-	**AA**	-	**TT**	**TT**	CC	-	-	gg	-	-	GG	-	-	-	-
W_A	wine	CC	-	-	gg	-	-	GG	-	**gg**	-	-	**gg**	-	-	-	-	-	-	-	-	**AA**	-	-

*AF= alcoholic fermentation
**MLF=malolactic fermentation

3.4. SNP genotyping in commercial wines

SNP genotyping assays were tested in commercial wines of Barbera (1-, 2- and 3-years old), Dolcetto (1- and 2-years old) and Arneis (1-year old). The yield of DNA extracted from all wines was consistent with the previous data reported for the CTAB-based method (Boccacci et al., 2020; Gambino et al., 2022), and the DNA quality determined using the spectrophotometric ratio A260/A280 was higher than that reported for Nebbiolo wines (Boccacci et al., 2020) (Table 1). Quantification of grapevine DNA with the *VvNCED2* TaqMan® probe confirmed the low levels of DNA in the samples extracted from the wines. For 5 out of 12 commercial wines no amplifications were observed, suggesting that the DNA yield was below the detection limit of the assays. Only from the commercial wine Dolcetto 2022 we obtain a high DNA yield and very high A260/A280 and A260/A230 ratios (Table 1). Using TaqMan® probes, SNP_3356 and SNP_15726 (Barbera-specific) correctly identified two Barbera commercial wines from the 2022 vintage, in at least one replicate, while amplification problems were observed in Barbera bottled in 2020 and 2021 (Table 3). The 2021 and 2022 Dolcetto commercial wines were correctly identified by SNP_1722 and SNP_87, but only for Dolcetto2021_b did we not observe any amplification. For Dolcetto2022_a, which showed a very high DNA yield and good quality (Table 1), all six SNPs amplified correctly with unexpected results for a commercial wine (Table 3). The DNA extracted from the commercial wines of Arneis showed amplification problems in Arneis-specific SNP_16408 and SNP_6647; only Arneis 2022_c was correctly identified (Table 3). The SNP genotyping assays based on the TaqMan® probe have shown limitations for the authentication of some commercial wines, as previously reported (Catalano et al., 2016; Boccacci et al., 2020). We

determined whether the DNA extracts contained PCR inhibitors by adding an exogenous internal positive control (EIPC). By setting the amplification efficiency to 100% in the controls containing optimal quality DNA extracted from leaves, the amplification efficiency of all commercial wines ranged between 98 and 105%, with no statistical differences from the control, demonstrating that were no PCR inhibitors in the DNA extract. As reported above for experimental musts and wines, a clear difference was observed in the efficiency of the TaqMan® assays based on the type of wine. Four of five commercial Dolcetto wines, two of four commercial Barbera wines and one of three Arneis wines were correctly identified (Table 3). The data from Dolcetto and Barbera wines confirmed that TaqMan® genotyping assays were more effective and sensitive than traditional SSR markers (Baleiras-Couto and Eiras-Dias, 2006; Boccacci et al., 2012; Recupero et al., 2013) for grape identification in commercial wines. DNA extraction from musts and wines produced from Dolcetto grapes appears to be more efficient, guaranteeing greater DNA recovery and better quality which facilitates the genetic traceability of these products. DNA extraction from Arneis musts and wines presents more problems, which could be overcome by developing a more efficient extraction method specific to these wines. The effect of age on the molecular traceability of wine was potentially observed in Barbera; wines bottled in 2022 showed better results than those bottled in 2020 and 2021, although it was not possible to clearly demonstrate this aspect in our experimental plan, in which different wines from different producers were analysed. In fact, wines of the same vintage from different producers showed a great variability of results, as observed for Dolcetto and Arneis (Table 3). As demonstrated for Nebbiolo wines, the effect of the oenological processes used during wine production (processing aids,

additives, filtration) has a greater impact than the age of the wine in varietal identification through molecular markers (Gambino et al., 2022; Song et al., 2024).

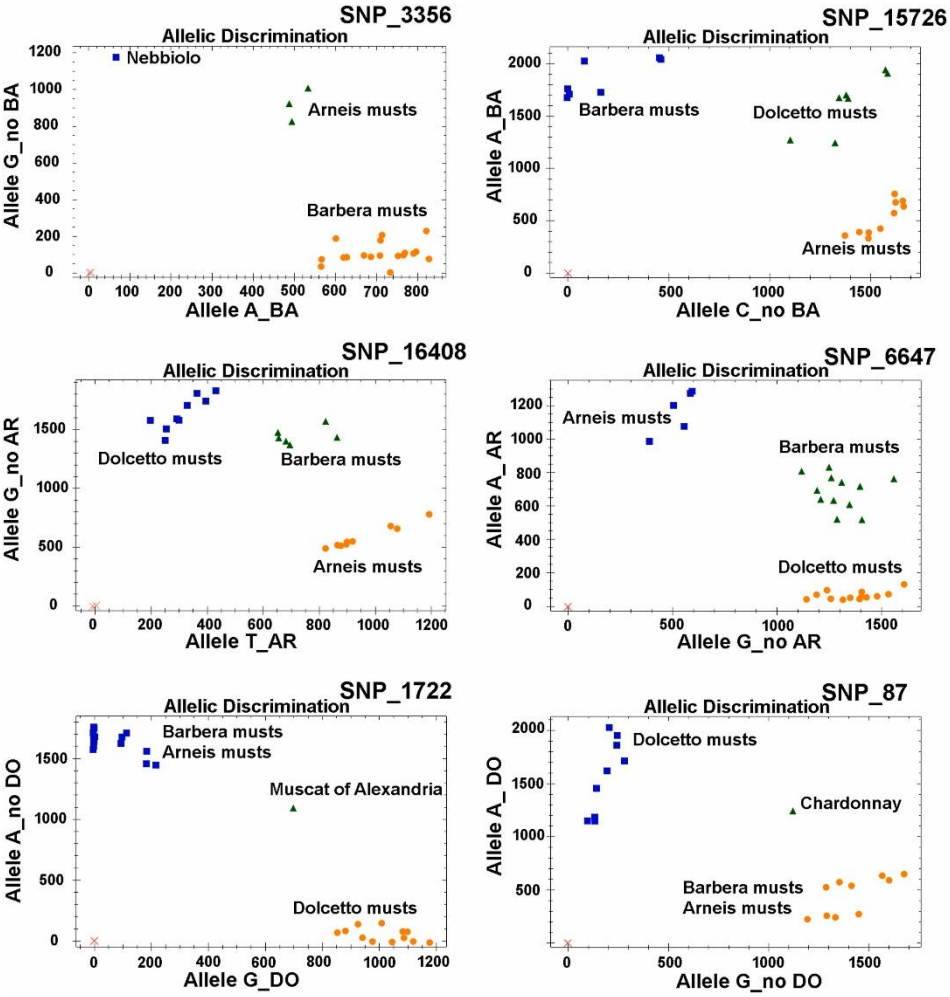

Figure 3. Output of TaqMan® SNP_3356, SNP_15726 (Barbera-specific), SNP_16408, SNP_6647 (Arneis-specific), SNP_1722 and SNP_87 (Dolcetto-specific) genotyping assays. Blue squares and orange points correspond to homozygous genotypes; green triangles are controls for heterozygous genotypes. DNA was extracted from experimental musts and wines of Barbera, Dolcetto and Arneis (details in Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The DNA extracted from the commercial wines was then analysed with the HRM method using five SNPs, as reported above for the experimental musts and wines. The problems highlighted with musts after alcoholic fermentation and in experimental wines also recurred in commercial wines. The HRM technique with these markers did not prove suitable for varietal traceability in wines; in fact, no commercial wines of Barbera, Dolcetto or Arneis were correctly identified (Table 3).

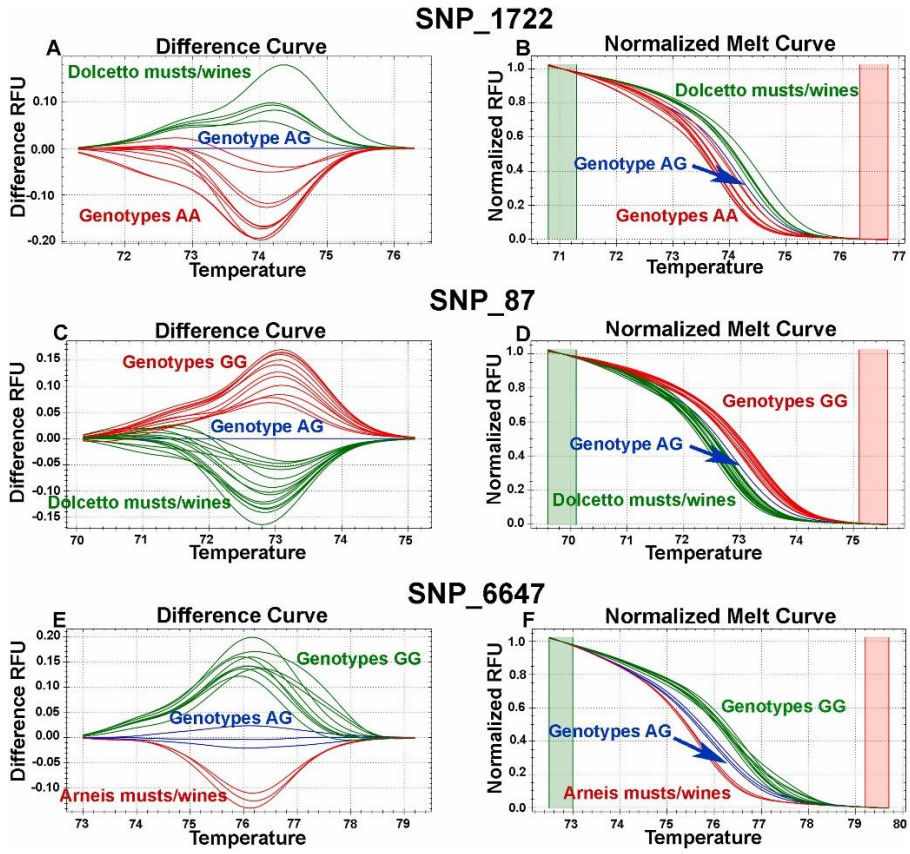

Figure 4. HRM analysis of SNP_1722, SNP_87 and SNP_6647. Normalised melting curves (B, D, F) and difference plots (A, C, E) correspond to two representations of the same data obtained for the three SNPs. Samples from Dolcetto (A–D) and Arneis (E–F) experimental musts and wines were grouped based on the shape of the melting curve into three distinct groups corresponding to the three expected allelic profiles. Genotypes were assigned using a cut-off confidence value of 95%.

Table 3. Allelic profiles of genotyping assays from Barbera, Dolcetto and Arneis commercial wines. SNPs specific for Barbera (SNP_15726, SNP_3356), Dolcetto (SNP_1722, SNP_87) and Arneis (SNP_16408, SNP_6647) were analysed using high resolution melting (HRM) and TaqMan® 651 probes. Lower652 case letters in the allelic profile denote an incorrect call of the genotyping assay; "-" indicates a sample without amplification or incorrect amplification 653 in HRM. For each sample, two independent extractions were analysed (R1, R2).

Wine	TaqMan® probes										High Resolution Melting									
	Barbera				Dolcetto				Arneis		Barbera				Dolcetto				Arneis	
	SNP_15726		SNP_3356		SNP_1722		SNP_87		SNP_6647	SNP_16408	SNP_15726		SNP_3356		SNP_1722		SNP_87		SNP_6647	SNP_16408
	Alleles		Alleles		Alleles		Alleles		Alleles	Alleles	Alleles		Alleles		Alleles		Alleles		Alleles	Alleles
	R1	R2	R1	R2	R1	R2	R1	R2	R1 R2	R1 R2	R1	R2	R1	R2	R1	R2	R1	R2	R1 R2	R1 R2
Barbera 2020	-	-	**AA**	-	-	AA	-	GG	- -	- -	-	-	-	-	-	-	-	-	- -	- -
Barbera 2021	**cc**	-	**gg**	-	-	AA	-	-	- -	- -	-	-	-	-	-	-	-	-	- -	- -
Barbera 2022_a	-	**AA**	-	**AA**	-	-	-	-	- gg	- gg	-	-	-	-	-	-	-	-	- -	- -
Barbera 2022_b	**AA**	-	**AA**	**AA**	AA	-	GG	-	AG -	tt -	-	-	-	**gg**	-	-	-	-	- -	- -
Dolcetto 2021_a	cc	aa	AA	-	-	**GG**	-	**AA**	aa ag	- -	-	-	-	-	-	-	-	-	- GG	- -
Dolcetto 2021_b	cc	-	-	-	-	-	-	-	- -	GG GG	-	-	ag	-	-	-	**AA**	-	- -	- -
Dolcetto 2022_a	AC	AC	AA	AA	**GG**	**GG**	**AA**	**AA**	GG GG	GG GG	aa	-	AA	AA	**aa**	**aa**	AA	**AA**	GG GG	- -
Dolcetto 2022_b	-	-	-	-	-	**GG**	**AA**	-	GG -	GG -	-	-	AA	gg	-	-	-	-	- -	- -
Dolcetto 2022_c	cc	-	-	AA	**GG**	-	**AA**	-	- -	GG GG	-	-	-	-	-	**aa**	**AA**	**gg**	- -	- -
Arneis 2022_a	-	CC	gg	-	AA	-	-	-	**AA** -	**gg gg**	-	-	AG	-	-	-	GG	-	- -	- -
Arneis 2022_b	aa	aa	-	aa	AA	-	-	GG	- **gg**	- **gg**	ac	-	-	-	-	-	-		- -	- -
Arneis 2022_c	CC	CC	-	AG	AA	AA	GG	-	**AA** -	- **TT**	-	-	aa	-	-	AA	GG	-	- -	- -

4. Conclusion

We identified and validated six new SNP markers specific for three important grapevine genotypes from north-western Italy, Barbera, Dolcetto and Arneis, used in the production of high-quality wines. These SNPs have been successfully used for varietal authentication in musts and wines produced from these genotypes, using TaqMan® assay and HRM analysis for SNP genotyping. The protocol described in this work for the identification of grapes in must and wine can be easily applied to any wine since SNP data are available for many unique genotypes (1493), which include the main wine varieties that can potentially be used for fraudulent mixes. TaqMan® probes represent the most robust method for identifying SNPs and are more efficient than the HRM approach, which has achieved poor results in detecting single SNPs in musts and wines with many non-specific amplifications. The technique probably requires a more laborious set-up than TaqMan® probes and higher quality DNA, but HRM can nevertheless be useful in the presence of more complex mutations, such as INDELS or multiple SNPs in neighbouring sequences, where TaqMan® probes cannot be used (Pereira et al., 2017). The data from the present work showed that grapevine genotypes, the winemaking process and the SNP locus can influence the efficiency of TaqMan® genotyping assays for the correct detection of grapes in wine. However, these variables become decisive for the success of the assay only by analysing low quantities of grape DNA typical of the extracts of some wines; therefore, further efforts are necessary to improve the extraction methods and adapt them to different wines.

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CRediT authorship contribution statement

Amedeo Moine: Investigation, Formal analysis, Data curation, Writing – review & editing. **Paolo Boccacci:** Conceptualization, Supervision, Investigation, Formal analysis, Data curation, Validation, Writing – review & editing. **Camilla De Paolis:** Investigation, Formal analysis, Data curation, Validation. **Luca Rolle:** Conceptualization, Supervision, Writing – review & editing. **Giorgio Gambino:** Conceptualization, Supervision, Funding acquisition, Methodology, Visualization, Writing – original draft.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

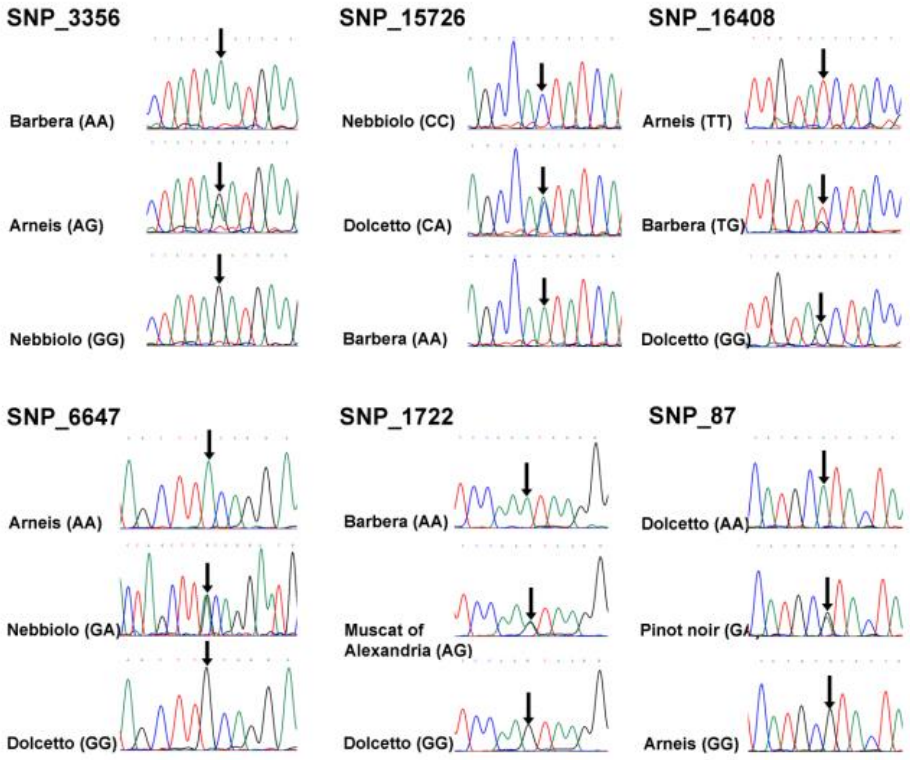

Figure S1. Output of Sanger validation of six SNPs specific for Barbera (SNP_3356, SNP_15726), Arneis (SNP_16408, SNP_6647) and Dolcetto (SNP_1722, SNP_87) selected in this work. The arrows indicate the allelic variants of the SNPs.

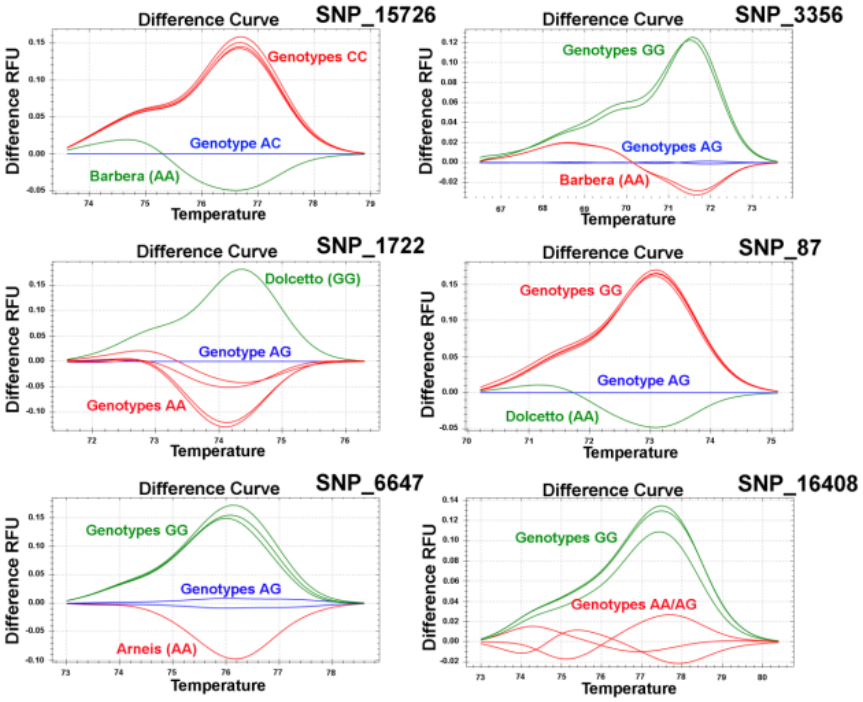

Figure S2. High-resolution DNA melting curve differences for the amplicons produced from SNP_3356, SNP_15726 (Barbera-specific), SNP_16408, SNP_6647 (Arneis-specific), SNP_1722 and SNP_87 (Dolcetto-specific). For SNP_16408, HRM analysis did not distinguish heterozygous (AG) from homozygous (GG) genotypes. All DNA was extracted from leaves. Genotypes were assigned using a cut-off confidence value of 95%.

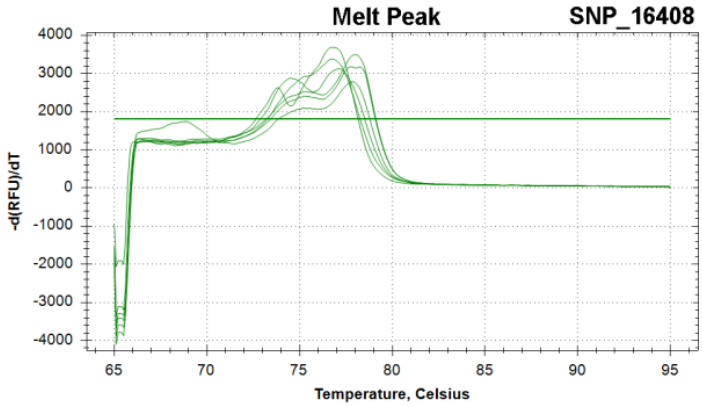

Figure S3. Melting curves of amplicons from locus SNP_16408. The melting curves appear with multiple nonspecific peaks and the locus is not suitable for HRM analysis, as shown in Figure S2.

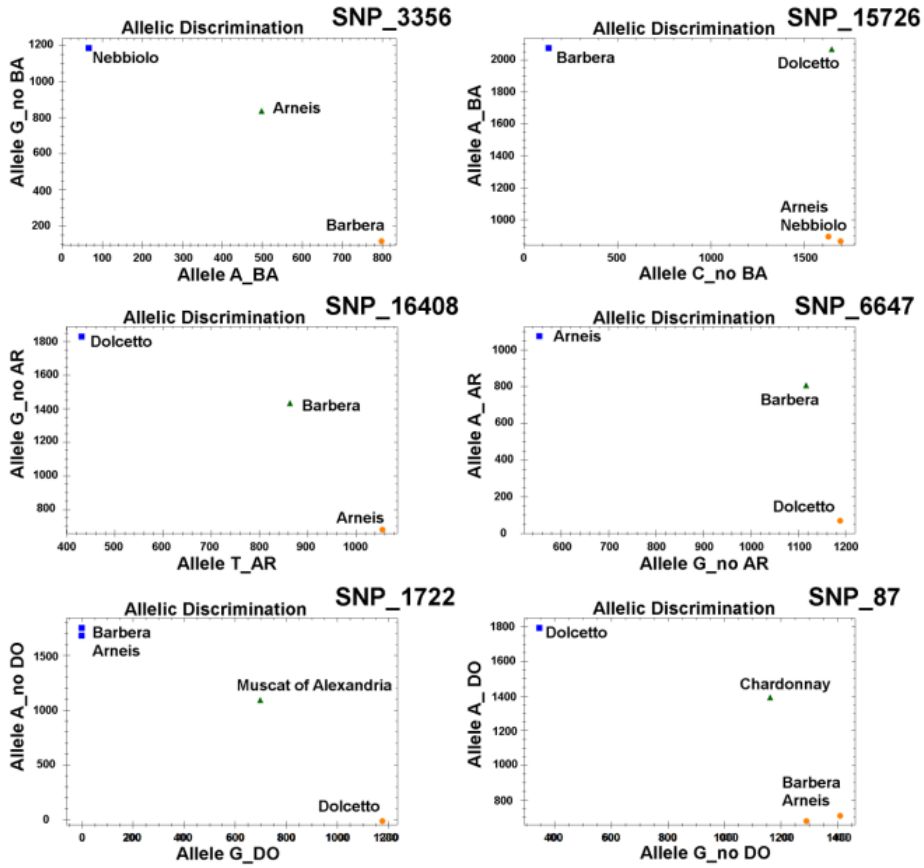

Figure S4. Output of TaqMan® SNP_3356, SNP_15726 (Barbera-specific), SNP_16408, SNP_6647 (Arneis-specific), SNP_1722 and SNP_87 (Dolcetto-specific) genotyping assays. Blue squares and orange points correspond to positive controls for homozygous genotypes; green triangles are positive controls for heterozygous genotypes. DNA was extracted from leaves.

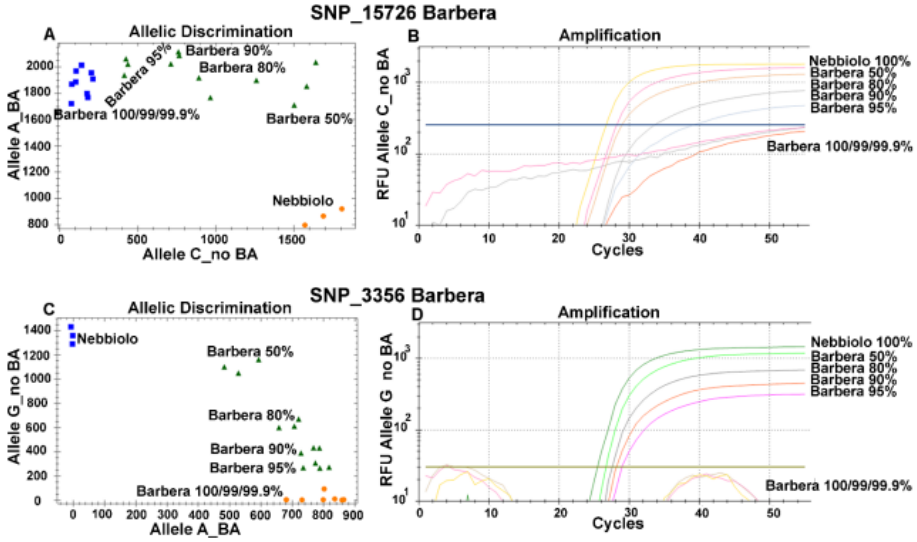

Figure S5. Detection limit of TaqMan® SNP_15726 and SNP_3356 genotyping assays in mixtures of DNA extracted from leaves. Allelic discrimination plots (A, C) and relative fluorescence unit (RFU) of the TaqMan® probes specific for non-Barbera alleles (B, D). Increasing levels of Nebbiolo DNA (from 0.1–50%) were mixed with Barbera DNA. The blue line (B) and yellow line in the amplification plot (D) indicate the RFU level of 100% Barbera, above which it was possible to detect contamination of non-Barbera DNA. For each SNP assay, the detection limit of 5% of non-Barbera DNA mixed in Barbera DNA was determined using three replicates of each sample.

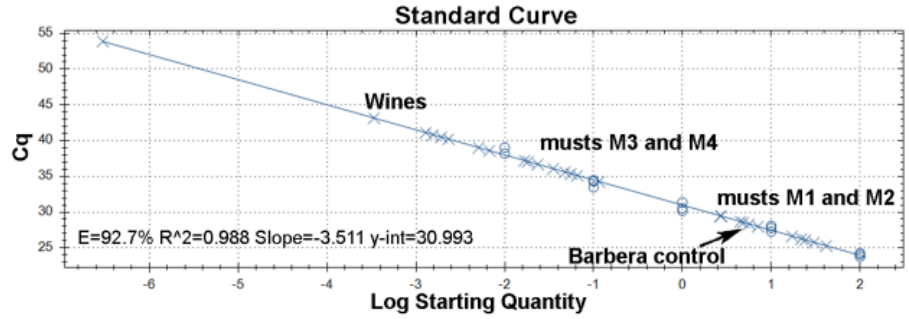

Figure S6. Standard curve of *VvNCED2* TaqMan® probe used to quantify grapevine DNA present in the extracts from experimental musts and wines from Barbera, Dolcetto and Arneis. DNA from Barbera leaves was used as calibrator for the standard curve.

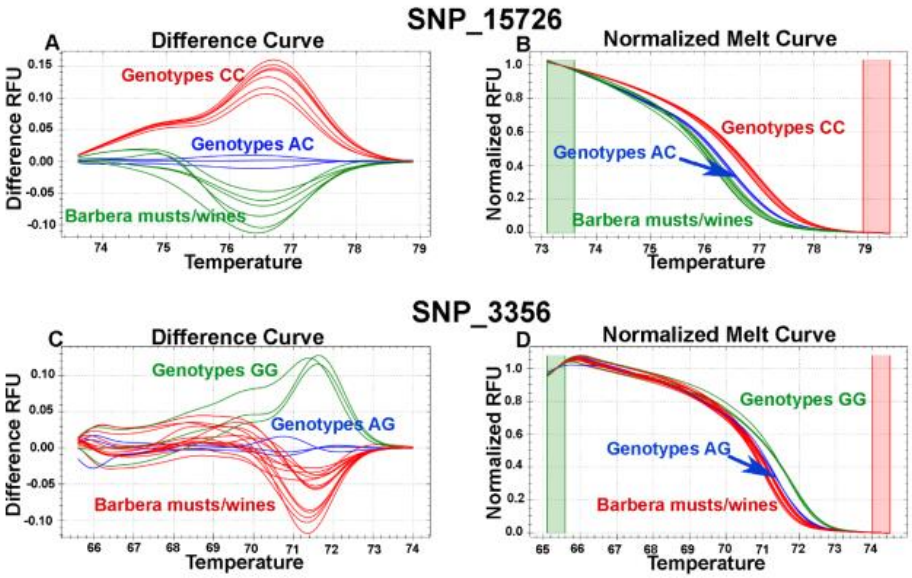

Figure S7. HRM analysis of SNP_15726 and SNP_3356. Normalised melting curves (B, D) and difference plots (A, C) correspond to two representations of the same data obtained for SNP_15726 and SNP_3356. Samples from Barbera musts and wines were grouped based on the shape of the melting curve into three distinct groups corresponding to the three expected allelic profiles. Genotypes were assigned using a cut-off confidence value of 95%.

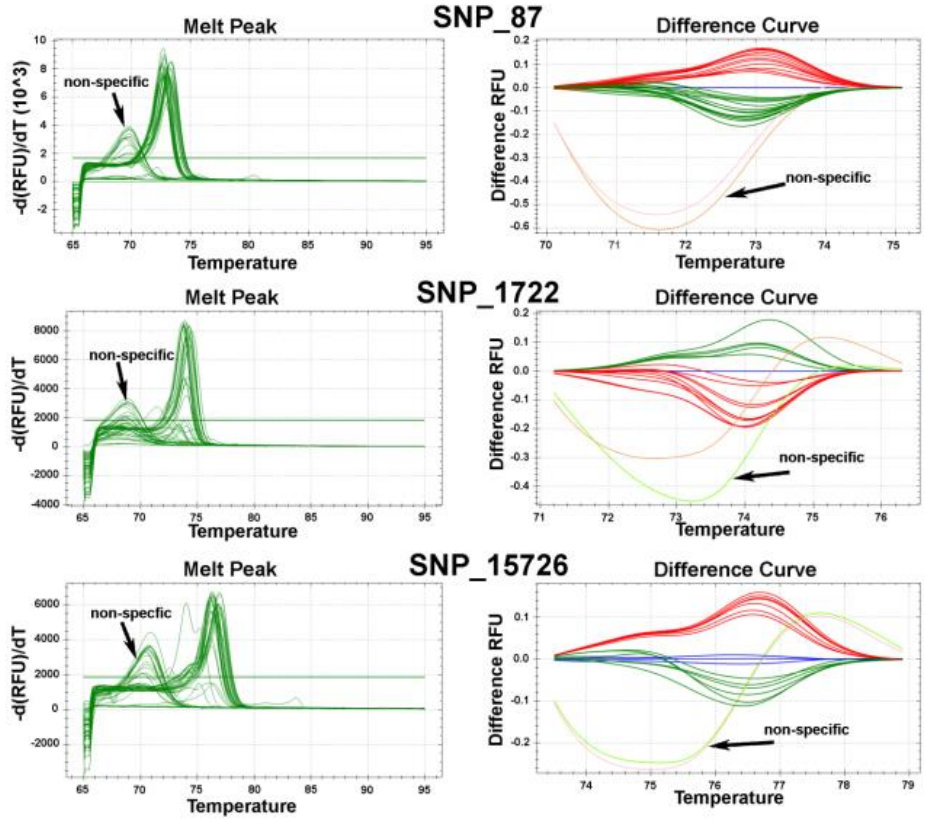

Figure S8. HRM analysis of SNP_87, SNP_1722 and SNP_15726. For each SNP locus, melting curves and difference plots obtained by amplifying the musts and wines of Barbera and Dolcetto were reported. In each analysis, nonspecific amplifications (nonspecific melting peaks) of DNA from musts and wines produced nonspecific melting curves, different from the curves associated with the 3 allelic combinations expected for each locus. Samples with non-specific melt peaks were removed from the analysis. Genotypes were assigned using a cut-off confidence value of 95%

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Chapter 5

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 (*Denominazione di Origine Controllata e Garantita)* wines, such as Barolo, Barbaresco, 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.

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	–

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). 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-SO_2 were quantified by titration after the extraction using a Solfotech apparatus (Exacta + Optech Labcenter Spa, San Prospero, Italy) according to the OIV-MAAS323-04A 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) (A420 + A520 + A620) and hue (A420/A520) 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.

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 4000g 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 manufacturer'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 8000g 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 10,000g 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 11,000g 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 22,000g 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 premixed 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 analysis

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 González- 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 anthocyanins and tannins in 'Tannat' red wine (González-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 positively 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: gelatin; 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 men 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 Δ*E** parameter was calculated for all treatments compared to CONTR (Fig. 1). GEL gave the highest Δ*E** value (8.01), followed by BEN (2.98).

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

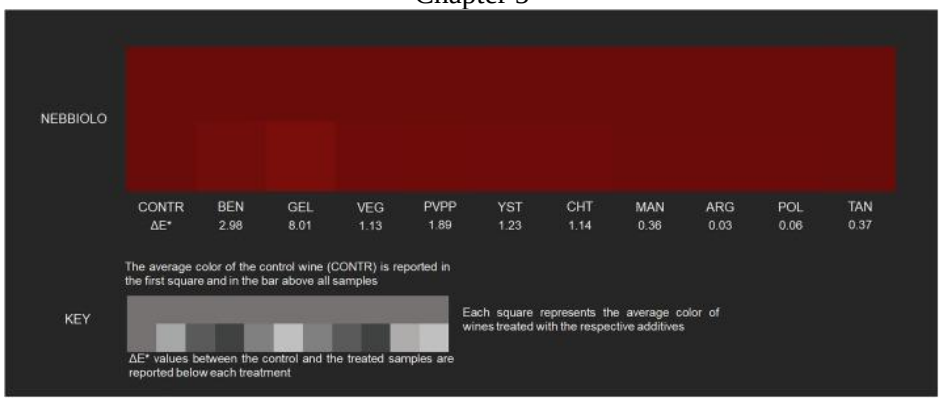

Figure 1. 'Nebbiolo' wine colour detected after the treatment with different additives and processing aids. BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: grape 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.

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 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	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	0.64 ± 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	1.90 ± 0.44 a	0.69 ± 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.

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).

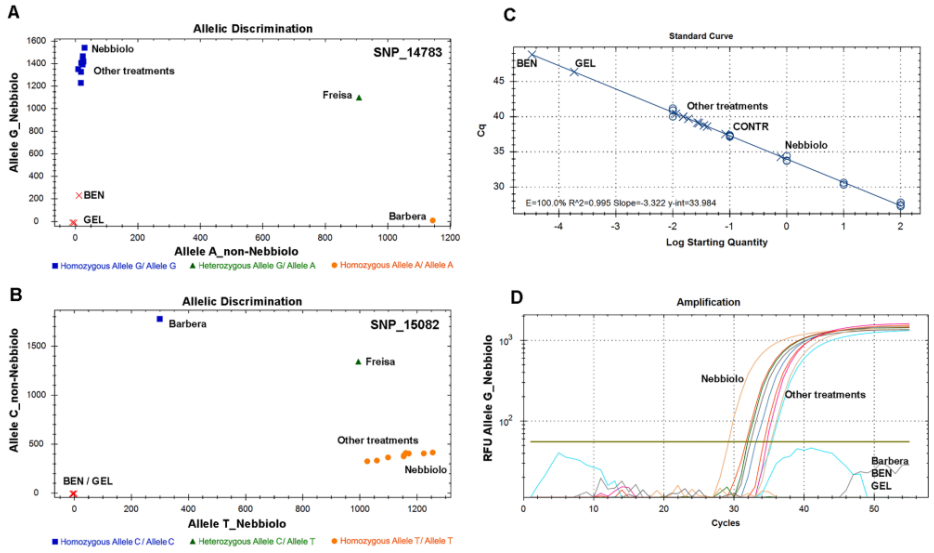

Figure 2. SNP genotyping in 'Nebbiolo' wines extracted with SirM method and previously treated with different oenological additives and processing aids. (A) Scatterplot of TaqMan® SNP_14783 genotyping assay with 'Nebbiolo' wines. (B) Scatterplot of TaqMan® SNP_15082 genotyping assay with 'Nebbiolo' wines. (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 ampli fication 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.

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 TaqMan® 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 acquisition, 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

Table S1. Primers and probles 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-TAAAAAAAGTGTTAAGGTGATAAT-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. Characterization of 'Nebbiolo' wine before treatments. Data are mean values ± SDs of two replicates.

Wine parameter	
Ethanol (% v/v)	13.61±0.00
Glycerol (g/L)	11.08±0.02
pH	3.46±0.00
Total acidity (g/L tartaric acid)	6.32±0.08
Malic acid (g/L)	0.04±0.02
Lactic acid (g/L)	1.95±0.00
Tartaric acid (g/L)	1.60±0.06
Citric acid (g/L)	0.13±0.01
Acetic acid (g/L)	0.30±0.00
Free SO_2 (mg/L)	10.50±0.71
Total SO_2 (mg/L)	76.48±0.91
Turbidity (NTU)	15.05±0.01
Color intensity (AU)	8.29±0.01
Hue	0.75±0.00
L*	16.2±0.0
a*	46.99±0.04
b*	27.12±0.02
Total phenolics (mg/L (-)–epicatechin)	3163±70
Total anthocyanins (mg/L malvidin-3-O-glucoside chloride)	148.4±1
Total flavonoids (mg/L (+)–catechin)	1063±6

Table S3. DNA quantity and quality extracted from 'Nebbiolo' wines treated with different additives and processing aids using Plant/Fungi DNA 2 Isolation Kit (Norgen). For each treatment repetition, one sample was extracted (R1, R2, and R3). Purity and yield measured using NanoDrop. 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). 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	Treatment		NanoDrop Quantification			SNP_14783			SNP_15082		
			DNA yield [ng/mL of wine]	A_{260}/A_{280}	A_{260}/A_{230}	Alleles			Alleles		
						R1	R2	R3	R1	R2	R3
CONTR20	-		33.7±6.5 a	1.35±0.15 a	0.51±0.15 a	+	+	+	+	+	+
CONTR	-		5.2±1.1 b	1.13±0.11 a	0.26±0.04 a	nd	-	nd	-	-	-
BEN		Bentonite	3.4±1.8 b	1.13±0.08 a	0.23±0.14 a	-	-	-	-	-	-
GEL		Gelatine	6.8±3.1 b	1.19±0.07 a	0.31±0.01 a	-	-	-	-	-	-
VEG		Vegetables protein	7.6±1.8 b	1.27±0.05 a	0.31±0.03 a	-	-	-	-	-	-
PVPP		Polyvinylpolypyrrolidone	5.8±0.9 b	1.05±0.19 a	0.24±0.09 a	-	-	-	-	-	-
YST		Yeasts hulls	7.5±3.1 b	1.18±0.06 a	0.27±0.02 a	nd	nd	nd	nd	nd	nd
CHT		Chitosan	7.4±2.4 b	0.98±0.06 a	0.21±0.04 a	-	nd	nd	nd	nd	-
MAN		Yeast mannoprotein	11.9±5.1 b	1.17±0.09 a	0.25±0.03 a	-	-	-	-	-	-
ARG		Arabic gum	16.6±19.9 b	1.11±0.06 a	0.29±0.06 a	nd	nd	nd	nd	nd	nd
POL		Potassium polyaspartate	6.7±0.8 b	1.08±0.09 a	0.26±0.06 a	+	nd	nd	nd	+	nd
TAN		Grape skin tannin	5.5±1.2 b	1.03±0.14 a	0.31±0.12 a	-	nd	-	nd	nd	-

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Chapter 6

Influence of filtration treatments on grapevine DNA traceability in wine

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ABSTRACT

Wine authentication through grapevine DNA traceability could be affected by wine technological processing treatments. In this study, filtration treatments including depth filter treatment using kieselguhr, perlite and membrane filtration using different types and pore sizes (0.22 and 0.45 μm) were applied on 'Nebbiolo' wine at an experimental scale. We used 'Nebbiolo' because it is an important Italian wine grape variety used to produce high-quality wines. Phenolic composition and color properties of the treated wines were examined using spectrophotometric and HPLC methods, while grapevine DNA traceability was evaluated using PCR based assays. The filtration treatments, as expected, significantly decreased turbidity compared to the unfiltered control, although total phenolics, total flavonoids and total non-flavonoids were not significantly affected. A significant reduction of acetylated anthocyanins by 6.1%–43.3% was observed in filtered wines, which could account for the reduced in total anthocyanins and color intensity of these wines. Grapevine DNA was significantly reduced in filtered wine by 37.2%–99.7%, with the reduction rate depending mainly on the properties of filter material. Polyvinylidene difluoride (PVDF) membranes with a pore size of 0.22 μm showed highest reduction of grapevine DNA in wine, resulting in the failure of TaqMan® single nucleotide polymorphisms (SNPs)-based assays used to detect grape DNA in wines.

Keywords: Wine, Clarification, Membrane filtration, Genetic traceability, Nebbiolo, SNPs

1. Introduction

Wine industry is one of the most profitable sectors, consequentially wine fraud including adulteration and counterfeiting can occur, damaging the image and market of premium wines (Ranaweera et al., 2021). In the past decades, wine fraud and mislabeling were preferentially detected using chemical-based techniques including mass spectrometry, spectroscopic methods and chromatography techniques combined with chemometrics (Sun et al., 2022; Villano et al., 2017). However, these methods are affected by various factors that impact on wine composition such as climate seasonal conditions, soil characteristics, vineyard management and enological practices (Boccacci et al., 2020; Sun et al., 2022).

DNA based methods are reported to be accurate and proficient for variety identification of wine because grapevine DNA is more resistant to the winemaking process than other wine components. DNA markers, including nuclear and chloroplast simple sequence repeats (SSRs) (Agrimonti & Marmiroli, 2018; Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; García-Beneytez et al., 2002) and single nucleotide polymorphisms (SNPs) (Catalano et al., 2016), are tested for DNA authentication of wine (Boccacci et al., 2020; Galstyan et al., 2021). Failure of amplification and inconsistent results were observed for wine varietal authentication using SSR genotyping due to DNA degradation (Savazzini & Martinelli, 2006; Vignani, Lio, ` & Scali, 2019). Whereas the high sensitivity of SNP-based assay can be observed in low-quality fragmented DNA, resulting in potential application in quantitative varietal authentication of blended wine and against fraud (Boccacci et al., 2020; Zambianchi et al., 2021).

Among wine production chain, residual DNA in wine is influenced by many factors such as wine making practice, clarification agents, aging,

and yeast/bacterial activity (Catalano et al., 2016; García-Beneytez et al., 2002; Villano et al., 2017; Zambianchi et al., 2021, 2022). Regarding wine clarification, filtration treatments are usually applied to eliminate suspended and colloidal particles in wine. These treatments, including depth filtration and membrane filtrations, can reduce in wine the content of aroma compounds, phenolics, pesticide residues, sulfide-bound copper, and other wine constituents due to the adsorption properties of filter aids and membranes (Arriagada-Carrazana et al., 2005; Doulia et al., 2016; Prodanov et al., 2019; Zhang et al., 2022). The adsorption capacity is reported to be dependent on the formation of hydrogen bonds and electrostatic interaction (Cai, Xie, Zhong, Tian, & Yang, 2021; Cassano et al., 2017). Despite research on the effects of filtration on wines, to our knowledge the detailed response of filtration treatments on DNA traceability in wine has not been studied.

Vitis vinifera L. cv. Nebbiolo is widely planted in northwestern Italy, where high-quality 'Nebbiolo' wines are produced under Protected Denomination of Origin (DOC and DOCG) (Raimondi et al., 2020). The misrepresentation of the origin and variety on 'Nebbiolo' wine labels can occur due to its high quality and economic value (Miglietta & Morrone, 2018). Cultivar-specific SNP technique was optimized for varietal authentication of 'Nebbiolo' wines (Boccacci et al., 2020; Gambino et al., 2022). However, there is limited research literature on the influence of wine processing treatments, especially stabilization treatments, on varietal identification efficiency of this cultivar wine. Gambino et al. (2022) revealed that DNA concentration was reduced by using different fining agents, especially bentonite and gelatine, which drastically reduced grapevine DNA below identification threshold. Given that filtration treatments can modify wine chemical-physical parameters depending on

the filtration method and the characteristic of the filter material, it is hypothesized that DNA traceability in wine could be influenced by filtration treatments. Therefore, the impact of commonly used filtration treatments at an experimental scale on DNA traceability of 'Nebbiolo' wine were determined in this study using TaqMan® genotyping assay. The result will provide useful information on varietal identification of wines.

2. Material and Methods

2.1 Chemicals, reagents, and wine sample

Chemical reagents including ethylene diamine tetraacetic acid (EDTA), sodium chloride (NaCl), Tris-HCl, cetyltrimethylammonium bromide (CTAB), β-mercaptoethanol and ethanol were purchased from Merck KGaA (Darmstadt, Germany). Malvidin-3-O-glucoside chloride was purchased from Extrasynthese (Genay Cedex, France). Ultrapure water was produced by a Milli-Q system (Merck Millipore, Darmstadt, Germany). TEX buffer containing 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1M Tris–HCl (pH 8.0), 3% CTAB and 1% β-mercaptoethanol and TE buffer containing 1 mM EDTA (pH 8.0) and 10 mM Tris-HCl (pH 8.0) were prepared according to Gambino et al. (2022). Proteinase K was sourced from Thermo Fisher Scientific (Waltham, MA, USA). NucleoSpin® Plant II were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). TaqMan® Environmental Master Mix 2.0, TaqMan® Exogenous Internal Positive Control (EIPC) reagents (containing primers, VIC probes specific for EIPC), TaqMan® 9-cis-epoxycarotenoid-dioxygenase gene (*VvNCED2*) assay and TaqMan® SNP Assay (containing primers, FAM and VIC probes) were from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals and reagents

were supplied by Merck KGaA (Darmstadt, Germany). Kieselguhr (KI) and Perlite (PE) were provided by IMERYS Filtration EMEA (Milan, Italy). Membrane sheets (47 mm of diameter) of different material and pore size were purchased as follows: cellulose nitrate (CE, 0.45 μm pore size) membranes were from Sartorius Stedim Biotech (Goettingen, Germany), polyethersulfone (PES, 0.22 and 0.45 μm) membranes were from Pall Corporation (Port Washington, NY, USA), polyvinylidene difluoride (PVDF, 0.22 and 0.45 μm) membranes were from Merck Millipore (Darmstadt, Germany), and polytetrafluoroethylene (PTFE, 0.45 μm) membranes were from Hermann Bohlender (Gruensfeld, Germany).

The wine used for the experiment was produced in 2022 from about 200 kg of 'Nebbiolo' grapes from Roero wine area, Cuneo, Italy. The must was inoculated with *Saccharomyces cerevisiae* active dry yeast (FERMOL Premier Cru, AEB, Brescia, Italy; 20 g/hL). Maceration lasted for 10 days, the cap was punched down once the first day, and two punches down were carried out daily until the 4th day. 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* (Malotabs™, Lallemand Inc., Montreal, Quebec, Canada). After malolactic fermentation, 50 mg/L SO_2 were added, and the wine was subsequently racked to remove the lees. The wine was stored in a steel tank in the winery for nine months until the beginning of the filtration treatments. The wine sample contained 10 mg/L of free SO_2. Wine basic physical-chemical parameters are available in Table S1.

2.2 Filtration treatments

Eleven filtration treatments using different depth filter aids and filter membranes were conducted on the same wine sample at a laboratory scale. Four different types of membranes filters including CE, PES, PVDF and PTFE with 0.45 μm pore size were applied (CE45, PES45, PVDF45, and PTFE45, respectively). PES and PVDF membranes with 0.22 μm pore size were also used for filtration treatments (PES22 and PVDF22, respectively). In addition, cellulose membrane was used in combination with two filtration aids: Kieselguhr (KI) and Perlite (PE). In both case KI and PE were used to create an alluviation panel above the cellulose membrane, to depth filtrate the wine samples. Moreover, KI and PE were used without other membranes (CF-KI and CF-PE): 1.4 g of KI or PE per liter of wine were added to the wine samples, stirred for 1.5 min and successively centrifuged at 3000×g for 5 min at 20 °C (Hettich 32R, Tuttlingen, Germany). The control (CO) was unfiltered wine. All the treatments were carried out in triplicate using a vacuum filtration system (VWR International, Milan, Italy), and collecting 500 mL of wine for each replication: 50 mL of treated wine were used for physical-chemical determination, while the remaining wine (450 mL) was bottled and stored at − 20 °C until DNA extraction.

2.3 Wine chemical-physical analysis

2.3.1 Basic chemical-physical parameters

Total acidity was analyzed by titration using OIV-MA-AS313-01 method (OIV, 2020). pH and turbidity were determined using an ino Lab® pH 730 pH meter (WTW, Weihheiim, Germany) and a TB1 portable turbidimeter

(Velp Scientifica, Usmate, Italy), respectively. Individual acids (malic acid, lactic acid, tartaric acid, citric acid, and acetic acid), alcohol, glycerol, fructose and glucose were evaluated by high performance liquid chromatography (HPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector and a refractive index detector (Giordano et al., 2009).

2.3.2 Phenolic composition and color characteristics

For each treatment replicate, 50 mL of wine samples were collected for color and phenolic analysis. All spectrophotometric measurements were performed using a UV-1800 spectrophotometer (Shimazdu, Kyoto, Japan). Wine color intensity (CI) and hue were determined by measuring the absorbance at 420, 520, 620 nm, and expressed as $A_{420}+A_{520}+A_{620}$ on 10 mm path length and the ratio between A_{420} and A_{520}, respectively, according to OIV-MA-AS2-07B method (OIV, 2020). CIELab values including L^*, a^* and b^* color components, were determined, and Chroma (C^*) and hue angle (h) and the total color difference (ΔE^*) between CO and filtration treated wines was calculated according to OIV-MA-AS2-11 method (OIV, 2020).

The total polyphenol index (TP) was determined by measuring A280 and reported as mg/L of (−)-epicatechin (Scalzini et al., 2020). Total flavonoids (TF) and total non-anthocyanins flavonoids (TNA) were analyzed based on the method reported by Petrozziello et al. (2018) and expressed as mg/L of (+)-catechin.

2.3.3 Individual anthocyanins

Wine samples were diluted one time with a HCl solution (pH 0.5), then filtered using PTFE 0.45 μm syringe filters (Lab Logistics Group GmbH, Meckenheim, Germany) before HPLC injection. Fifty μL of the samples were injected in a HPLC system (Agilent 1260, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Compound separation was performed using a LiChroCART analytical column (25 cm × 0.4 cm) obtained from Merck (Darmstadt, Germany). Mobile phase A and B were 10% formic acid aqueous solution and 10% formic acid aqueous solution containing 50% methanol, respectively. The following gradient was adopted: 0–15 min, 28%–45% B; 15–35 min, 45%–70% B; 35–45 min, 70%–90% B; 45–48 min, 99% B; 48–58 min, 28% B, with a flow rate of 1.0 mL/min. The individual anthocyanins were identified and quantified as described by Río Segade et al. (2014). Total monomeric anthocyanin content (TA) was calculated as the sum of individual anthocyanins (mg/L of malvidin-3-O-glucoside chloride).

2.4 DNA traceability analysis

2.4.1 DNA extraction from wines

CTAB based method was conducted as described by Siret et al. (2002) with some modifications (Agrimonti & Marmiroli, 2018) as reported by Gambino et al. (2022). Wines were frozen at − 20 °C for 15 days before analysis. One hundred mL of wine was centrifuged for 1 h (4000×g, 4 °C) using a Sigma 3-16 KL refrigerated centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany), then the wine pellet was dissolved in 5 mL of TEX buffer, which was incubated for 1 h at 65 °C with mixing at the

interval of 10–15 min. Five mL of chloroform: isoamyl alcohol (24:1) was added into the sample, then sequentially homogenized and centrifuged (Sigma 3-16 KL centrifuge) for 10 min (8000×g, 4 °C). The supernatant added with 0.1 volume of pre-warmed 10% CTAB (65 °C) was extracted again with 1 volume of chloroform: isoamyl alcohol. The aqueous phase added with 2 volumes of cold ethanol was store in freezer (− 25 °C) overnight. The precipitated DNA was obtained after centrifugation (10,000×g, 30 min, 4 °C) using a Hermle Z216-MK Refrigerated Microcentrifuge (Wehingen, Germany), suspended in TE buffer (250 μL) and incubated (30 min, 48 °C) with the addition of proteinase K (20 μL, 20 mg/mL). Then, the sample was added with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), homogenized and centrifuged (Z216-MK Microcentrifuge) (11,000×g, 15 min, 4 °C). The aqueous phase was added with 2 volumes of cold ethanol and 2.5 mol/L of ammonium acetate, and store in freezer (− 25 °C) for 2 h. The pellets were obtained after centrifugation (Z216-MK Micro centrifuge) for 30 min (20,000×g, 4 °C) and washed with cold ethanol (500 μL, 70%, v/v). The extracted DNA was dissolved in 100 μL of ultrapure sterile water, then purified with the NucleoSpin® Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA quantity and quality were evaluated by measuring the absorbance at 230, 260, 280 nm, using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was stored at − 20 °C until analysis.

2.4.2 Grapevine DNA quantification and PCR inhibitors determination

Quantitative PCR (qPCR) amplification of *VvNCED2* (VIT_10s0003g03750) was used for grapevine DNA quantification following the method reported by Savazzini and Martinelli (2006). TaqMan® EIPC reagents were used for the evaluation of the presence of PCR inhibitors in the extracted DNA (Boccacci et al., 2020). The qPCR mixture was composed of the extracted DNA (2.5 μL), TaqMan® Environmental Master Mix 2.0 (5 μL), 0.4 μL of EIPC DNA, 2 μL of EIPC mix (containing premixed forward, reverse primers, and VIC probe specific for EIPC) and sterile water (0.1 μL). Amplification cycles were as follows: the initial denaturation step set at 95 °C for 10 min, then 55 cycles of 95 °C for 15 s and 60 °C for 1 min. DNA standard was extracted from 'Nebbiolo', 'Barbera' and 'Freisa' young leaf using NucleoSpin® Plant Kit. Grapevine DNA and the percentage of qPCR inhibition were quantified from the calibration curves of the *VvNCED2* TaqMan® assay and EIPC, respectively, using the CFX96 Detection System from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All extracted DNA samples were analyzed in triplicate.

2.4.3 SNP genotyping

Two markers, SNP_15082 and SNP_14783, were analyzed in extracted DNA as reported by Boccacci et al. (2020). FAM and VIC probes were designed using Primer Express version 3.0 (Thermo Fisher Scientific) to genotype non-'Nebbiolo' alleles ('Barbera' and 'Freisa') and 'Nebbiolo' alleles (Table S2), The qPCR mixture consisted of the extracted DNA (2.5

μL), TaqMan® Environmental Master Mix 2.0 (5 μL), 40X TaqMan® SNP Genotyping Assay mix (0.25 μL), and sterile water (2.25 μL). The amplification cycles were the same reported in Section 2.4.2. Allelic discrimination was performed using the CFX Maestro Software version 2.0 (Hercules, CA, USA). All the extracted DNA samples were determined in triplicate.

2.5 Statistical analysis

For the wine variables, one-way analysis of variance was conducted using SPSS Version 22 (IBM, Armonk, New York, USA). The differences among the filtration treatments were separated with different letters using Tukey post-hoc test at p-value ≤ 0.05.

3. Result and discussion

3.1 Filtration treatment on turbidity, phenolic composition and color characteristics

In this study, a 'Nebbiolo' wine aged for 9 months was used for filtration treatments, and the influence of these treatments on wine turbidity, phenolic composition and color parameters were assessed. The chemical results are shown in Table 1. CF-KI and CF-PE significantly decreased wine turbidity by 62.5%, 28.6%, respectively, whereas CE filter membrane pre-coated with KI and PE treatments and other membrane filtration treatments showed a significant decrease of between 88.5% and 99.3%. Low turbidity values of wines submitted to different filtration treatments (ranged from 0.09 to 1.47 NTU) indicate a wine clarification (Ribéreau-Gayon et al., 2006).

CF-KI, CF-PE, CE-KI and CE-PE had no significant influence on phenolic compounds including TP, TF and TNA, indicating the minor adsorbent capacity of depth filter agents on wine phenolic compounds (Ribéreau-Gayon et al., 2006). Similarly, compared to the unfiltered control, membrane filtration treatments showed no significant influence on the phenolic compounds including TP, TF and TNA, in accordance with Buffon et al. (2014) who found that cross-flow microfiltration had no significant influence on wine phenolic profile. However, reductions of polyphenols in membrane filtered wine were observed in previous studies because of strong adsorption of membranes on these compounds (Arriagada-Carrazana et al., 2005; Prodanov et al., 2019). Rosária et al. (2022) revealed that the influence of filtration on wine phenolic composition was dependent on filtration type and initial wine composition.

In most cases, the hue of the filtered wines was not influenced except for CE-KI, PVDF22 and PTFE45 treatments (only decreased by 1%), indicating the unchanged proportion between yellow and red in these wines. Arriagada-Carrazana et al. (2005) reported a slight decrease of hue in membrane filtered wines. A slight reduction in CI between1.2%– 3.0% was observed in filtered wines, but not after PTFE45 treatments (Table 1). Similarly, Oberholster et al. (2013) demonstrated a CI reduction in membrane filtered wines, although Buffon et al. (2014) reported that there was no significant influence on wine color between the control and cross-flow microfiltration treated wines.

A minor difference in the CIELab coordinates (L^*, a^* and b^*) for different wines after filtration was obtained. All the filtration treatments showed significant increase in L^*, but minor decrease in a^* and b^* with respect to the control. Rosária et al. (2022) found a decrease in the a^* coordinate, but a slight increase in the L^* coordinate of filtration treated

wines. The color differences were visually confirmed in Fig. 1. Wines treated with the two depth filtration treatments (CE-KI and CE-PE) and membrane filters (PVDF45, PVDF22, PES22 and PTFE45) showed higher ΔE^* values than the generally accepted visual recognition threshold (3.0) of wine color differences (Pérez-Magariño & González-Sanjosé, 2003).

Table 1. Turbidity, phenolic composition, color parameters of 'Nebbiolo' wines subjected to filtration treatments.

Treatment	Turbidity	TF	TNA	TP	L^*	a^*	b^*	C^*	h	CI	Hue
CO	12.28 ± 0.23a	2608 ± 34a	2447 ± 34a	4836 ± 75a	34.7 ± 0.1d	53.71 ± 0.22e	42.88 ± 0.20d	68.72 ± 0.30e	38.60 ± 0.02cde	4.51 ± 0.01 ab	1.03±0a
CF-KI	4.60 ± 0.32c	2637 ± 72a	2484 ± 64a	4919 ± 102a	35.6 ± 0.1c	54.83 ± 0.12d	43.74 ± 0.07c	70.14 ± 0.13d	38.58 ± 0.03de	4.45 ± 0.01bc	1.03±0 ab
CF-PE	8.77 ± 0.69b	2638 ± 27a	2479 ± 25a	4809 ± 59a	35.7 ± 0.3bc	54.84 ± 0.19d	43.77 ± 0.22c	70.16 ± 0.28cd	38.60 ± 0.07cde	4.44 ± 0.04c	1.03±0 ab
CE-KI	0.19 ± 0.21e	2618 ± 34a	2450 ± 31a	4732 ± 85a	36.3 ± 0.1 ab	55.76 ± 0.11abc	44.59 ± 0.08b	71.40 ± 0.13 ab	38.65 ± 0.05cd	4.42 ± 0.01cd	1.02±0bc
CE-PE	0.24 ± 0.13e	2604 ± 29a	2434 ± 27a	4866 ± 75a	36.1 ± 0.1abc	55.72 ± 0.10abc	44.64 ± 0.06 ab	71.4 ± 0.11 ab	38.70 ± 0.01bc	4.44 ± 0.01bc	1.02 ±0abc
CE45	0.23 ± 0.08e	2596±7a	2434 ± 10a	4825 ± 33a	36.1 ± 0.4abc	55.28 ± 0.30cd	43.97 ± 0.21c	70.63 ± 0.37cd	38.50 ± 0.02ef	4.40 ± 0.05cd	1.03±0 ab
PES45	0.81 ± 0.13de	2598 ± 20a	2446 ± 13a	4877 ± 53a	36.4 ± 0.1a	55.46 ± 0.14bc	44.04 ± 0.13c	70.81 ± 0.19bc	38.45 ± 0.01f	4.37 ± 0.02e	1.03±0 ab
PES22	0.23 ± 0.21e	2586 ± 28a	2417 ± 21a	4754 ± 21a	36.4 ± 0.0a	55.88 ± 0.23 ab	44.77 ± 0.30 ab	71.61 ± 0.37a	38.70 ± 0.08bc	4.41 ± 0.01cd	1.02 ±0acbc
PVDF45	0.30 ± 0.11e	2635 ± 29a	2456 ± 27a	4727 ± 70a	36.4 ± 0.1a	55.72 ± 0.05abc	44.58 ± 0.06b	71.36 ± 0.08 ab	38.66 ± 0.01cd	4.40 ± 0.01cd	1.03±0 ab
PVDF22	0.09 ± 0.03e	2635 ± 13a	2464 ± 11a	4888 ± 152a	36.1 ± 0.0abc	55.92 ± 0.02 ab	44.95 ± 0.03 ab	71.74 ± 0.04a	38.79 ± 0.01 ab	4.45±0bc	1.02±0bc
PTFE45	1.47 ± 0.15d	2637 ± 15a	2473 ± 19a	4765 ± 87a	36.7 ± 0.2bc	56.01 ± 0.15a	45.10 ± 0.16a	71.91 ± 0.22a	38.84 ± 0.03a	4.52 ± 0.03a	1.02±0c

Note: Same letter in the same columns indicate no significant difference between different filtration treatments by Tukey test ($p < 0.05$). Turbidity is expressed as nephelometric turbidity units (NTU). TP, Total phenolic index, expressed as (−)-epicatechin/L. TF, total flavonoids, expressed as (+)-catechin/L. TNA, total non-anthocyanins flavonoids, expressed as (+)-catechin/L. CI, color intensity, expressed as absorbance units on 10 mm path length. CO, control. CF-KI, kieselguhr accompanied by

centrifugation. CF-PE, perlite accompanied by centrifugation. CE-KI, Cellulose membrane pre-coated with kieselguhr. CE-KI, cellulose membrane precoated with perlite. CE45, 0.45 μm pore size cellulose. PES45, 0.45 μm pore size polyethersulfone. PES22, 0.22 μm pore size polyethersulfone. PVDF45, 0.45 μm pore size polyvinylidene difluoride. PVDF22, 0.22 μm pore size polyvinylidene difluoride. PTFE45, 0.45 μm pore size polytetrafluoroethylene.

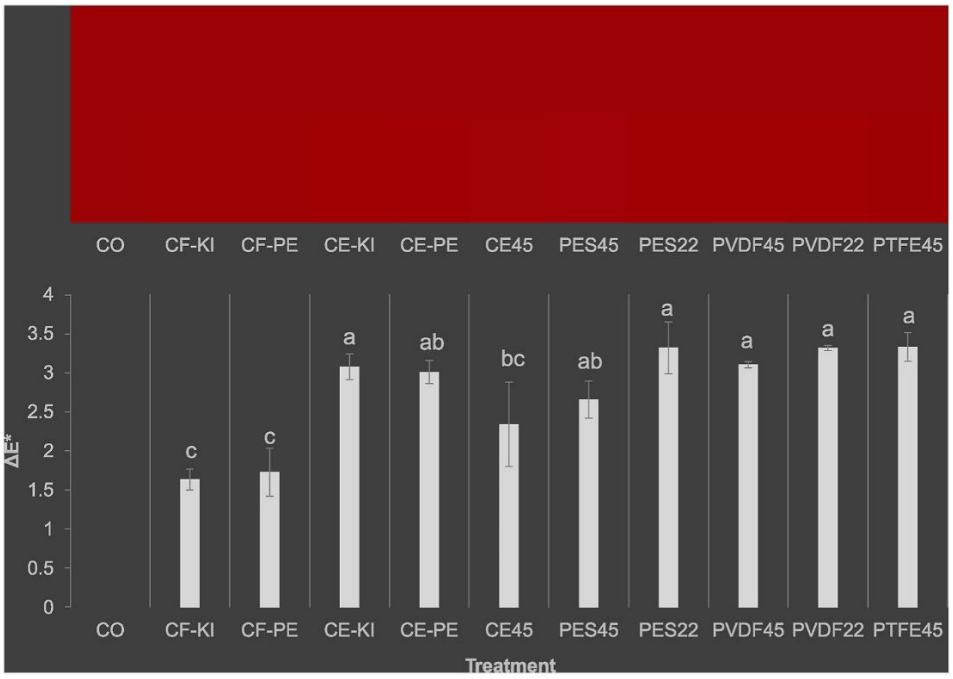

Figure 1. RGB color of 'Nebbiolo' wines subjected to filtration treatments. Same letter in the same columns indicate no significant difference between different filtration treatments by Tukey test ($p < 0.05$). CO, control. CF-KI, kieselguhr accompanied by centrifugation. CF-PE, perlite accompanied by centrifugation. CE-KI, Cellulose membrane pre-coated with kieselguhr. CE-KI, cellulose mem brane pre-coated with perlite. CE45, 0.45 μm pore size cellulose. PES45, 0.45 μm pore size polyethersulfone. PES22, 0.22 μm pore size polyethersulfone. PVDF45, 0.45 μm pore size polyvinylidene difluoride. PVDF22, 0.22 μm pore size polyvinylidene difluoride. PTFE45, 0.45 μm pore size polytetrafluoro ethylene. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

For the individual anthocyanins quantified in filtration treated wines (Table 2), malvidin-3-O-glucoside represents the majority of anthocyanins (51.8%–54.6%) in 'Nebbiolo' wines, followed by peonidin-3-O-glucoside (17.7%–19.0%). The sum of the concentrations of simple glycoside anthocyanins were found higher than those of acetyl- and cinnamoyl-glucoside anthocyanins, in agreement with previous studies (Paissoni et al., 2020; Río Segade et al., 2014).

Although a high decrease of malvidin-3-O-glucoside in wines submitted to cross-flow microfiltration was previously observed (Cameira-dos-Santos et al., 1994), CF-KI and CF-PE had no significant influence on total simple glycoside anthocyanins, and some other membrane filtration treatments including CE-KI, CE-PE, CE45, and PVDF45 slightly increased malvidin-3-O-glucoside and peonidin-3-O-glucoside up to 4.2%. The increased glucoside anthocyanins in filtered wines could be regenerated from decolorized anthocyanins by bisulfite addition since that vacuum filtration treatments caused the release of free SO_2 from anthocyanins (Berké et al., 1998; Ribéreau-Gayon et al, 2006).

Compared to the control, CF-KI and CF-PE treatments showed a reduction of acetylated glucosides by 6.1% and 13.1%, respectively, and CE-KI and CE-PE treatments led to significant decreases of these compounds by 47.6% and 50.3%, indicating the co-adsorption of CE and depth filtration aids on these compounds. Moreover, acetylated glucosides in wines subjected to membrane filtration treatments were also significantly reduced by 21.1%–43.3%, but these treatments had no significant influence on cinnamoyl-glucosides. However, Gonçalves et al. (2012) reported significant adsorption of membrane filter on coumaroylated anthocyanins compared to other forms of anthocyanins.

Vieira et al. (2018) demonstrated that anthocyanins content variations in membrane filtered wines were impacted by membrane texture surface properties with different adsorption capacity.

Compared to the control, CF-KI and PES45 treatments had a minor effect on TA with a reduction by 1.1%, whereas CF-PE treatment caused significant reduction of TA by 1.7%. Moreover, in most cases, membrane filters resulted in significant reduction of TA reaching 3.3%. The lower TA in filtration treated wines could be partially responsible for the lower CI of these wines compared to the control, given that a correlation on these two parameters was found on a large set of red wines (Giacosa et al., 2021). The decreases of TA and CI in treated wines could be partially due to the adsorption of the depth filter aids and membrane filters on acetyl anthocyanins (Arriagada-Carrazona et al., 2005; Ober holster et al., 2013; Vieira et al., 2018). In general, compared to the untreated sample, the filtration treatments caused also some modifications in the anthocyanin profile of ‘Nebbiolo’ wines, with the highest impact provided by CE-KI and CE-PE treatments (Table 2).

Table 2. Anthocyanin profile of 'Nebbiolo' wines subjected to filtration treatments

Treatment	TA	Delphinidin-3-*O*-glucoside	Cyanidin-3-*O*-glucoside	Petunidin-3-*O*-glucoside	Peonidin-3-*O*-glucoside	Malvidin-3-*O*-glucoside	∑ Delphinidin derivatives	∑ Cyanidin derivatives	∑ Petunidin derivatives	∑ Peonidin derivatives	∑ Malvidin derivatives	∑ simple glycosides	∑ acetyl glucosides	∑ cinnamoyl glucosides
CO	44.01 ± 0.10a	1.93 ± 0.03	1.34 ± 0.11a	3.05 ± 0.02	7.79 ± 0.03c	22.80 ± 0.12cde	1.93 ± 0.03	2.29 ± 0.11a	3.54 ± 0.03a	9.64 ± 0.02a	26.35 ± 0.12a	36.9 ± 0.1cd	3.74 ± 0.06a	3.37 ± 0.05
CF-KI	43.54 ± 0.21 ab	1.9 ± 0.02	1.22 ± 0.13 ab	3.04 ± 0.01	7.77 ± 0.06c	22.72 ± 0.11e	1.9 ± 0.02	2.13 ± 0.13 ab	3.51 ± 0.01a	9.58 ± 0.07 ab	26.16 ± 0.12 ab	36.65 ± 0.16d	3.51 ± 0.08b	3.38 ± 0.06
CF-PE	43.24 ± 0.17bc	1.92 ± 0.04	1.17 ± 0.01b	3.06 ± 0.02	7.75 ± 0.09c	22.72 ± 0.09de	1.92 ± 0.04	2.07 ± 0.01b	3.51 ± 0.02a	9.48 ± 0.09abc	26.00 ± 0.08abc	36.62 ± 0.16d	3.25 ± 0.09c	3.38 ± 0.04
CE-KI	42.75 ± 0.2cd	1.88 ± 0.03	1.26 ± 0.03 ab	3.05 ± 0.14	8.12 ± 0.06a	23.17 ± 0.17a	1.88 ± 0.03	2.02 ± 0.04b	3.39 ± 0.13 ab	9.49 ± 0.06abc	25.72 ± 0.17c	37.47 ± 0.21a	1.96 ± 0.04hi	3.32 ± 0.03
CE-PE	42.57 ± 0.02d	1.89 ± 0.01	1.28 ± 0.03 ab	2.97 ± 0.06	8.03 ± 0.12 ab	23.24 ± 0.08a	1.89 ± 0.01	2.03 ± 0.03b	3.25 ± 0.11b	9.40 ± 0.11c	25.74 ± 0.07c	37.41 ± 0.08 ab	1.86 ± 0.10i	3.31 ± 0.03
CE45	43.05 ± 0.19bcd	1.91 ± 0.05	1.27 ± 0.03 ab	3.05 ± 0.07	8.09 ± 0.06a	23.27 ± 0.1a	1.91 ± 0.05	2.04 ± 0.01b	3.41 ± 0.07 ab	9.51 ± 0.07abc	25.92 ± 0.12bc	37.6 ± 0.16a	2.12 ± 0.04gh	3.34 ± 0.05
PES45	43.53 ± 0.21 ab	1.93 ± 0.01	1.23 ± 0.02 ab	3.09 ± 0.05	7.89 ± 0.06bc	23.15 ± 0.07 ab	1.93 ± 0.01	2.09 ± 0.03b	3.51 ± 0.05a	9.52 ± 0.06abc	26.21 ± 0.10 ab	37.29 ± 0.14abc	2.83 ± 0.08f	3.4 ± 0.03
PES22	42.91 ± 0.15cd	1.85 ± 0.02	1.17 ± 0.01b	3 ± 0.01	7.77 ± 0.05c	22.81 ± 0.11bcde	1.85 ± 0.02	2.03 ± 0.02b	3.42±0 ab	9.42 ± 0.04bc	25.93 ± 0.11bc	36.60 ± 0.15d	2.95 ± 0.05d	3.35 ± 0.01
PVDF45	42.99 ± 0.05cd	1.89 ± 0.03	1.23 ± 0.01 ab	3.06 ± 0.01	8.06 ± 0.01 ab	23.12 ± 0.03abc	1.89 ± 0.03	2.04±0b	3.43 ± 0.01 ab	9.52±0abc	25.86 ± 0.02bc	37.36 ± 0.04 ab	2.26 ± 0.02 fg	3.36 ± 0.02
PVDF22	42.79 ± 0.01cd	1.87 ± 0.02	1.20 ± 0.02 ab	2.97 ± 0.07	7.91 ± 0.03bc	23.07 ± 0.07abcd	1.87 ± 0.02	2.01 ± 0.02b	3.35 ± 0.06 ab	9.42 ± 0.03bc	25.89 ± 0.09bc	37.02 ± 0.05bcd	2.43 ± 0.04f	3.35 ± 0.05
PTFE45	43.19 ± 0.31bc	1.86 ± 0.02	1.22 ± 0.01 ab	3.05 ± 0.1	8.01 ± 0.02 ab	23.05 ± 0.22bcde	1.86 ± 0.02	2.05 ± 0.01b	3.45 ± 0.1 ab	9.57 ± 0.02 ab	25.99 ± 0.25abc	37.19 ± 0.27abc	2.63 ± 0.07e	3.37 ± 0.03

Note: Same letter in the same columns indicate no significant difference between different filtration treatments by Tukey test ($P < 0.05$). All data is expressed as mg malvidin-3-O-glucoside chloride/L. TA, total monomeric anthocyanins. CO, control. CF-KI, kieselguhr accompanied by centrifugation. CF-PE, perlite accompanied by

centrifugation. CE-KI, Cellulose membrane pre-coated with kieselguhr. CE-KI, cellulose membrane pre-coated with perlite. CE45, 0.45 μm pore size cellulose. PES45, 0.45 μm pore size polyethersulfone. PES22, 0.22 μm pore size polyethersulfone. PVDF45, 0.45 μm pore size polyvinylidene difluoride. PVDF22, 0.22 μm pore size polyvinylidene difluoride. PTFE45, 0.45 μm pore size polytetrafluoroethylene.

3.3 Filtration treatment on grapevine DNA traceability

DNA in wine was extracted using a CTAB based method (Gambino et al., 2022) and the results were reported in Table 3. The DNA yield and two absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}), generally used to estimate the quality of extracted DNA, were determined through a spectrophotometric analysis. High quality DNA was obtained in CO and after some filtration approaches (CF-KI and PTFE45). However, after many filtration treatments the absorbance ratios, in particular A_{260}/A_{230}, were low indicating a high content of polyphenols and carbohydrates in the extracted DNA (Table 3), in accordance with previous results (Agrimonti & Marmiroli, 2018; Gambino et al., 2022; Zambianchi et al., 2021, 2022).

Further, the presence of yeast DNA and phenolic substances in the extracted DNA from wine resulted in overestimation of spectrophotometric quantified grapevine DNA yield (Boccacci et al., 2020; Gambino et al., 2022). The more reliable grapevine DNA content could be quantified by qPCR using specific DNA makers as the *VvNCED2* amplified by TaqMan® probe (Fig. S2), as previously suggested (Savazzini & Martinelli, 2006). The grapevine DNA, expressed as *VvNCED2*, accounts for up to 0.30% of the extracted DNA samples in CO and filtration treated wines, in agreement with Boccacci et al. (2020) and Gambino et al. (2022) who also confirmed the overestimation by spectrophotometric analysis of DNA extracted from ‘Nebbiolo’ wines.

All the filtration treatments reduced the DNA recovery from ‘Nebbiolo’ wine from 37.2% to 99.7% depending on the filter characteristics (Table 3). For the two depth filtration aids, CF-KI and CF-PE, treatments showed significant reductions by 60.6% and 40.8% of the concentrations of grapevine DNA with respect to the control, confirmed the different absorption capacities of KI and PE on grapevine DNA. For membranes with pore size of 0.45 μm, PTFE provided the highest grapevine DNA removal (87.0%), followed by PVDF (80.4%) and PES (72.2%). Moreover, CE45 treatment showed the lowest reduction (37.2%) of grapevine DNA. The losses of grapevine DNA in wines subjected to 0.22 μm pore size membrane filters (PVDF and PES) were higher compared to the loss due to 0.45-μm corresponding membrane filters, but significant differences were not found. It should be mentioned that the 0.22-μm PVDF membrane filter significantly removed most of grapevine DNA (99.7%). Our results confirmed preliminary observation of Catalano et al. (2016), who reported that filtration with perlites caused significant reduction of DNA yield in wine. Although DNA molecules can pass through filter used in this work, the membrane filter treatments showed a reduction of DNA yield compared to unfiltered control (CO), suggesting that DNA molecules could be adsorbed to the colloids in wine, and thus eliminated by filtration treatment. In general, all the filtration treatments lead to significant reduction of residual grapevine DNA in ‘Nebbiolo’ wine compared to the unfiltered control, and the reduction percentage depended on the treatment method and on the characteristic of material used, which have different adsorption characteristics on wine colloids and on DNA (Liang & Keeley, 2013).

Table 3. Quantity and quality of extracted DNA from 'Nebbiolo' wines subjected to filtration treatments

Sample	DNA yield ng/mL wine	A_{260}/A_{280}	A_{260}/A_{230}	VvNCED2 quantification DNA yield, pg/mL of wine	% Grapevine DNA	Loss of DNA after treatment, %	SNP_14783			SNP_15082		
							R1	R2	R3	R1	R2	R3
CO	21.56 ± 1.98a	1.91 ± 0.14a	1.53 ± 0.71bc	9.73 ± 0.80a	0.05 + 0.0006	–	+	+	+	+	+	+
CF-KI	18.53 ± 2.92a	2.02 ± 0.02a	1.88 ± 0.06 ab	3.79 ± 0.21cde	0.02 + 0.004	−60.6 ± 5.3	+	+	+	+	+	+
CF-PE	2.54 ± 0.16c	2.02 ± 0.36a	2.55 ± 0.70a	5.68 ± 0.85bc	0.23 + 0.04	−40.8 ± 13.0	+	+	+	+	+	+
CE-KI	1.76 ± 0.09c	1.70 ± 0.29 ab	0.67 ± 0.03cd	4.80 ± 1.42bcd	0.27 + 0.07	−50.5 ± 13.8	+	+	+	+	+	+
CE-PE	2.03 ± 0.25c	1.19 ± 0.47b	0.61 ± 0.05cd	4.03 ± 0.76bcd	0.20 + 0.03	−58.5 ± 7.4	+	+	+	+	+	+
CE45	1.98 ± 0.04c	1.78 ± 0.10 ab	0.61 ± 0.0cd	6.03 ± 0.99b	0.30 + 0.05	−37.2 ± 14.8	+	+	+	+	+	+
PES45	2.06 ± 0.86c	1.78 ± 0.28 ab	1.13 ± 0.324bcd	2.65 ± 0.47def	0.13 + 0.04	−72.2 ± 6.9	+	+	+	+	+	+
PES22	2.09 ± 0.07c	1.39 ± 0.16 ab	0.52 ± 0.18d	1.58 ± 0.44 fg	0.08 + 0.02	−83.7 ± 3.4	+	+	+	+	+	+
PVDF45	1.58 ± 0.36c	1.50 ± 0.27 ab	0.55 ± 0.14d	1.86 ± 0.72efg	0.12 + 0.04	−80.4 ± 8.3	+	+	+	+	+	+
PVDF22	2.04 ± 0.25c	1.33 ± 0.16 ab	0.54 ± 0.07d	0.01 ± 0.02g	0 + 0	−99.7 ± 0.3	nd	nd	+	+	nd	nd
PTFE45	8.48 ± 0.70b	1.91 ± 0.11 ab	1.55 ± 0.17bc	1.23 ± 0.36 fg	0.02 + 0.004	−87.0 ± 4.2	+	+	+	+	+	+

Note: 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. Allelic profile of genotyping assay SNP_14783, SNP_15082 for each treatment replicate (R1, R2, and R3). '+' indicates samples that correctly amplified, and 'nd' stands for 'not detected'. Same letter in the same columns indicate no significant difference between different filtration treatments by Tukey test ($p < 0.05$). CO, control. CF-KI, kieselguhr accompanied by centrifugation. CF-PE, perlite accompanied by centrifugation. CE-KI, Cellulose membrane pre-coated with kieselguhr. CE-KI, cellulose membrane pre-coated with perlite. CE45, 0.45 μm pore size cellulose. PES45, 0.45 μm pore size polyethersulfone. PES22, 0.22 μm pore size polyethersulfone. PVDF45, 0.45 μm pore size polyvinylidene difluoride. PVDF22, 0.22 μm pore size polyvinylidene difluoride. PTFE45, 0.45 μm pore size polytetrafluoroethylene.

'Nebbiolo' grape and wines can be distinguished from other grape cultivars using two previously validated 'Nebbiolo'-specific SNPs, SNP_15082 and SNP_14783 (Boccacci et al., 2020). The correct ampli fication of both SNPs by TaqMan® assays were observed in all wines except for the samples subjected to PVDF22 treatment (Fig. 2, Table 3). PCR inhibitors in extracted DNA can affect the efficiency of the genotyping assays for wine authenticity. Their presence in the samples was verified by adding an EIPC in all DNA extracts, the amplification efficiency of all wine samples ranged between 93% and 110%, without statistical differences when compared with the control containing DNA of optimal quality extracted from leaves and assuming an amplification efficiency of 100% (Fig. S1B). Therefore, the failure in varietal identification in wines subjected to PVDF22 treatment was not linked to the presence of PCR inhibitors, but was due to the low DNA yield in the extracted samples. Gambino et al. (2022) previously reported that loss of identification efficiency of grape DNA in 'Nebbiolo' wine treated with oenological processing aids and additives was observed in DNA samples with the concentration lower than 0.5 pg/mL of starting wine. This data was confirmed also in this work: after filtration of the 'Nebbiolo' wine, the PVDF22 was the only treatment with problems in SNP genotyping and

with a DNA yield quantified by qPCR of *VvNCED* lower than 0.5 pg/mL of starting wine. Thus, the failure in recognizing DNA traces by TaqMan® assay in commercial 'Nebbiolo' wines after filtration depends on the combination of the pore size of the membranes (0.22 μm) and the characteristic of material, as polyvinylidene difluoride (PVDF22) was more effective at removing DNA than polyethersulfone (PES22).

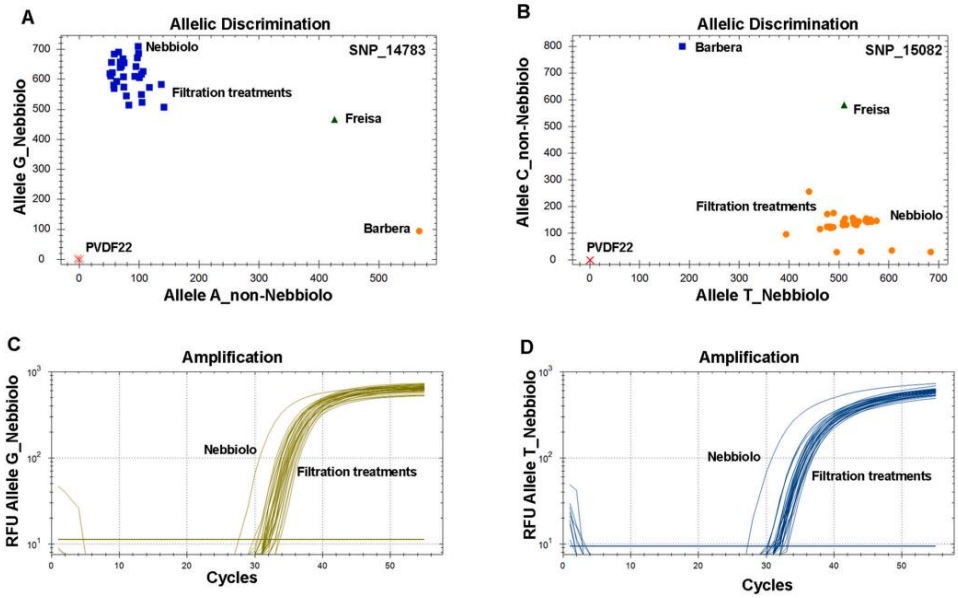

Figure 2. SNP genotyping in 'Nebbiolo' wines subjected to filtration treatments. (A, B) Scatterplots of TaqMan® SNP_14783 and TaqMan® SNP_15082 genotyping assays with 'Nebbiolo' wines. (C) Relative fluorescence unit (RFU) of the TaqMan® SNP_14783 probe tagged with VIC dye (allele G 'Nebbiolo'). (D) Relative fluorescence unit (RFU) of the TaqMan® SNP_15082 probe tagged with FAM dye (allele T 'Nebbiolo'). The control DNA from 'Nebbiolo', 'Barbera' and 'Freisa' were extracted from leaves. PVDF22: 0.22 μm pore size polyvinylidene difluoride.

4. Conclusions

In this study, the effects of filtration treatments using depth filtration aids and membrane filters on wine phenolic compounds and DNA traceability were evaluated. Although filtration treatments had no significant influence on TP, TF and TNA, those treatments decreased wine turbidity and CI. Filtration treatments showed significant reductions in the content of acetylated anthocyanins, leading to significantly decreased TA (with few exceptions), which could be responsible for the reduced CI in filtered wines. Filtration treatments can hinder genetic traceability of wine depending on the filtration method and the characteristic of material. 'Nebbiolo' was correctly identified by SNP based assay in wines subjected to depth filtration and membrane (CE, PES, PVDF, PTFE) filters with high pore size (0.45 μm) and low pore size (PES 0.22 μm). However, the membrane material PVDF with low pore size (0.22 μm) hindered TaqMan@ assay, indicating that the uncertainty of authenticity of membrane-filtered wines by SNP-based assay was affected by the porosity and membrane material. This work, together with the previous study (Gambino et al., 2022), revealed that the com bination of additives and filtration can make DNA recovery from wines very difficult, and future improvements of DNA extraction techniques from wine are needed.

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CRediT authorship contribution statement

Jianqiang Song: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Camilla De Paolis:** Validation, Investigation, Formal analysis, Data curation. **Paolo Boccacci:** Validation, Investigation, Formal analysis, Data curation. **Lorenzo Ferrero:** Validation, Resources, Investigation, Formal analysis, Data curation. **Amedeo Moine:** Investigation, Formal analysis, Data curation. **Susana Río Segade:** Writing – review & editing, Validation, Methodology, Investigation. **Simone Giacosa:** Writing – review & editing, Validation, Resources, Methodology. **Giorgio Gambino:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Conceptualization. **Luca Rolle:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Maria Alessandra Paissoni:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Table S1. Physical-chemical characteristics of 'Nebbiolo' wine before treatments

No.	Wine parameters (mean ± standard deviation, *n*=3)	
1	Ethanol (%, v/v)	14.42±0.12
2	Total acidity (g/L as tartaric acid)	6.4±0.1
3	pH	3.56±0.01
4	Glycerol (g/L)	10.63±0.08
5	Fructose (g/L)	0.33±0.01
6	Glucose (g/L)	0.20±0.27
7	Malic acid (g/L)	nd
8	Lactic acid (g/L)	1.37±0.01
9	Tartaric acid (g/L)	1.48±0.01
10	Acetic acid (g/L)	0.23±0.01
11	Succinic acid (g/L)	1.32±0.01

Note: "nd", not detected.

Table S2. 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 Rev Probe allele A Probe allele G	GAGCACAATCAACAATTATCCATTTTGGTTGTGTTA ATAGCAGGCAA FAM-TAAAAAAGTGTTAAGGTGATAAT-NFQ VIC-TAAAAAAGTGTTAAGGTGATGAT-NFQ	83
SNP_15082	chr8_19402046	T	C	For Rev Probe allele A Probe allele G	TCTCTTCTGGCATGGAAATCAATTAGATTACGGGC CAAGCTGA FAM-TCTCATTTTCCTCATTAT-NFQ VIC-TCTCATTTTCCTCATCATG-NFQ	89

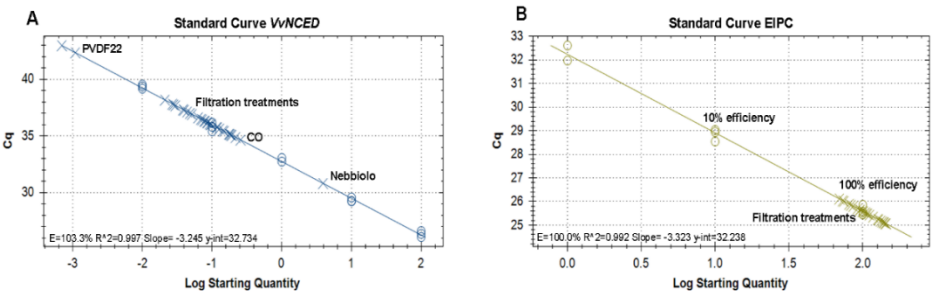

Figure S1. (A) Standard curve of *VvNCED2* TaqMan® probe used to quantify grapevine DNA in 'Nebbiolo' wines. DNA from 'Nebbiolo' leaves was used as a calibrator for the standard curve. (B) The percentage of PCR inhibition was calculated from a calibration curve with serial dilution of TaqMan® Exogenous Internal Positive Control (EIPC), assuming 100% amplification efficiency of EIPC in samples containing DNA of optimal quality extracted from leaves of 'Nebbiolo'. The amplification efficiency of all wines samples ranged between 93% and 110%, without significant differences when compared with the DNA sample from leaves. CO: untreated control; PVDF22: 0.22 µm pore size polyvinylidene difluoride.

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General conclusion

During my three-year PhD program in collaboration with Araldica Castelvero company, different research lines have been followed with the main aim to contribute to deeper explore some technical and commercials issues in the winemaking process.

In the first session (Experimental session A) the impact of climate change on the wine production has been explored, to better understand the technical fallout on the wine industry. Nowadays, climate change represents one of the most important and hard challenges that the wine producers have to face with, forcing to improve strategies, study new oenological technique and enhance methodologies to reduce alcohol content in wines. The continius collaboration between wine producers and research community is essential to improve wine quality, guarantee the developing of sustainable solution and to overcome this situation.

During harvest 2022, an innovative oenological technique, for the production of high-quality wines have been evaluated. With the cold liquid stabulation we studied the impact of a pre-fermentative maceration on lees, *maceration sur bourbes*, on the phenolic, antioxidant and aroma characteristics on aroma-neutral white grape varieties. A major role of the variety was showed for the secondary metabolites, meanwhile the treatments impacted some chemical-physical parameters. Regarding the aroma characteristics different oucomes were highlighted for the two varieties, moreover this practice did not affect the sensory features of mouthfeel and color. In the future a deeper research may interest the linkage between these first results and the grape composition. Also, in a view of sustainable production can be of interest the use of by-products,

commonly considered as waste, that can improve the neutral-varieties white winemaking.

Finally, for three consecutive vintages, 2019, 2020 and 2021, the combined effect of ripeness degree and withering process length on the physical-chemical composition of partially withered Nebbiolo grapes were carried out. The results of the study underlined that those two variables can be managed according to the desired oenological aim, showing a possible valorization of withered Nebbiolo grapes. The importance of the choice of the harvest date were highlighted and the results obtained by this work showed that an earliest harvest time, contrary to tradition, can be useful in a view of long ageing of '*Sforzato di Valtellina DOCG'* wines.

In the second session (Experimental session B), different studies on the genetic traceability of monovarietal wines were carried out.

The increasing impact of wine on the world's economy and its growing economic value is leading to a constant improvement of technique to fight against frauds and product manipulations. Nowadays, DNA-based methods are the most precise and affordable. In the first work, of this second part, a series of a new specific SNP markers were indentify for the most common Piedmont grape varieties: 'Barbera', 'Dolcetto' and 'Arneis'. Subsequentially, those markers were validated with two real time-PCR techniques, with Taq-Man® genotyping assay resulting more reliable and repetable.

With the objective of try to explain the causes of the lower efficiency in varietal identification in commercial wines two works were carried out. In the first study the impact of most common commercial oenological aids and adjuvants on the DNA-traceability of 'Nebbiolo' wine was evaluated.

The main results showed that Bentonite and Gelatine had the strongest impact, not only in turbidity, phenolic composition and color, but also on DNA reduction. Finally, with the aim to deepening, the question was about other common winemaking practices, such as filtration treatments. So, in the last study, the effects of different filtration treatments and materials on ‘Nebbiolo’ wine DNA, thought SNP-methods were assessed. The results highlited that grapevine DNA was reduced depending mainly on filter pore size and materials. Indeed, ‘Nebbiolo’ wines treated with PVDF membranes with 0.22 µm showed the highest DNA reduction, resulting in failure of SNP assay used.

Annex I – Research products

ISI index journals articles

Gerbi V. & De Paolis C. (2025) The effects of climate change on wine composition and winemaking processes. *Italian Journal of Food Science*, 37(1), 246-260. https://doi.org/10.15586/ijfs.v37i1.2775

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Other contributions

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Stabulazione liquida a freddo come tecnica enologica nella vinificazione in bianco: implicazioni tecnologiche, aromatiche e sensoriali. Candidato: Del Conte M., Relatore: Paissoni M.A., Secondo relatore: Río Segade S., Correlatore: De Paolis C.

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