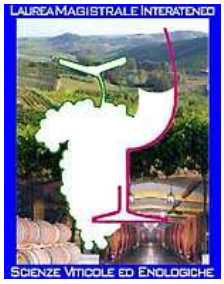

UNIVERSITA' DEGLI STUDI DI
TORINO MILANO PALERMO SASSARI FOGGIA

CORSO DI LAUREA MAGISTRALE INTERATENEO

IN

SCIENZE VITICOLE ENOLOGICHE

TESI DI LAUREA

Impact of the use of oenological processing aids and additives on the genetic traceability of 'Nebbiolo' wines

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Anno Accademico 2020/2021

SUMMARY

ABSTRACT

‘Nebbiolo’ is a well-known grapevine variety used to produce prestigious monovarietal wines, such as Barolo and Barbaresco. Genetic traceability is an important tool used to fight frauds and to protect the authenticity of high-quality wines. SNP-based assays are reported to be an effective method to reach this aim in ‘Nebbiolo’ wines, but several issues are reported for the authentication of commercial wines. We analysed the impact of the most common commercial additives and processing aids used in winemaking on the SNP-based traceability in ‘Nebbiolo’ wine. Fining agents (gelatine and bentonite) resulted to have the strongest impact on wine parameters and on grapevine DNA residual. The DNA reduction associated with the use of bentonite, gelatine, and filtration, 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.

1. INTRODUCTION

'Nebbiolo' (*Vitis vinifera* L.) is an important Italian winegrape variety from Piedmont used to produce high-quality wines. It is well diffused in north-western Italy where it is used to produce well-known DOCG (*Denominazione di Origine Controllata e Garantita*) wines, such as Barolo, Barbaresco, Roero, Gattinara and Sforzato di Valtellina. '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 take many forms: adulteration is defined as the fraudulent alteration of the wine composition; however, other types of fraud are increasingly spreading in recent years. Among them, the misrepresentation on the label of information regarding the origin and variety of wine is very common (Holmberg, 2010). Hence, there is the need to protect 'Nebbiolo' wines from fraud that could damage the image and the market of these premium wines. To protect the authenticity and verifies 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 studied the authenticity of the wine, trying to elaborate traceability methods to associate the chemical composition of wine with its varietal, geographical and productive origin. They tried to build classification and tracing models using a fingerprinting approach based on mineral content, metabolomic profile, isotopes content, infrared spectrum, phenolic and aromatic profile (Versari et al., 2014; Villano et., 2017). Nevertheless, chemometric approaches often turn out to be expensive in terms of time and resources, and they present some inaccuracies caused by the great influences that viticultural and winemaking techniques have on the wine (Versari et al., 2014). Therefore, 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 the DNA from wine and using variety-specific markers, it is possible to discriminate musts and wines (Siret et al., 2000; Pereira et al., 2012). However, the result can be very different depending on the wines, DNA extraction technique, the type of marker, and the technique used for the amplification of the genetic material. 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 molecular traceability and varietal recognition starting from residual DNA inside the 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 wine, the results of the amplification are often not reliable (Catalano et al., 2016). Indeed, several studies reported issues to use these methods for the traceability of commercial wine (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 have been performed, including 'Nebbiolo' (Gambino et al., 2017). The comparison between the different genomes available allowed the identification of several mutations and polymorphisms between the different genotypes, such as small insertions and deletions, inter- and intra-chromosomal translocations and inversions, and single nucleotide polymorphisms (SNPs). SNPs are particularly interesting because they are spread across the whole grapevine genome and have the potential to become a valid alternative to SSRs for cultivar identification (Cabezas et al., 2011). Therefore, SNPs were used also for genetic traceability of varieties in the wine (Barrias et al., 2019; Pereira et al., 2017) since they can be detected in low-quality fragmented DNA (Catalano et al., 2016). Recently, we identified SNPs for the

authentication of ‘Nebbiolo’ and developed a way for the molecular traceability of this cultivar in experimental wines based on SNPs TaqMan® assay (Boccacci et al., 2020). We successfully identified two markers, SNP_15082 and SNP_14783, to distinguish ‘Nebbiolo’ from more than 1100 genotypes, and we demonstrated the possibility to identify 1% of non-‘Nebbiolo’ cultivar in ‘Nebbiolo’ wine at the end of maceration. Nevertheless, the efficiency of the assay drops at the end of malolactic fermentation and in commercial wines due to the reduction of the amplification efficiency and to the enhancing presence of PCR inhibitors.

After malolactic fermentation, wine can undergo several winemaking practices before bottling, which may modify its composition. In winemaking, the usage of additives and processing aids to enhance wine stability is well diffused and several products are allowed for this purpose (OIV, 2016a). To produce high-quality wine stability and clarity of the product are essential. Different products can be employed as fining agents; among them, the most used are bentonite, chitosan, vegetable proteins, animal proteins, and polyvinylpolypyrrolidone (PVPP) (Castro Marin et al., 2020; Ficagna et al., 2020; Rio-Segade et al., 2020). Whereas the main products used as stabilizers are potassium polyaspartate, yeast mannoproteins, and Arabic gum (Bosso et al., 2020; Rinaldi, Coppola, & Mojo, 2019).

In literature, several problems on the amplification efficiency of DNA in commercial wines was reported in many cases (Zambianchi et al., 2021; Boccacci et al., 2020; Boccacci et al., 2012; Recupero et al., 2013). These problems are probably associated with filtration, clarification, fining agents, and/or DNAses yeast activity (Catalano et al., 2016). However, to date, no work has analyzed in detail these procedures and agents that potentially may drastically reduce the quality and the quantity of DNA in the wine after alcoholic fermentation (Faria et al., 2008; Siret et al., 2002; Siret et al., 2000). The effect of the most

common additives on the wine matrix chemical composition has been widely studied, but to the best of our knowledge, there are no studies about their effect on wine DNA traceability. This study aims to understand 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

'Nebbiolo', 'Barbera' and 'Freisa' leaves were collected and DNA was extracted from young leaves using a Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) by following the manufacturer's instructions. Accessions were genotyped at six SSR markers (This et al., 2004) by following the procedure reported by Ruffa et al., 2016 to confirm their cultivar identity, together with ampelographic observations.

2.2 Experimental vinification

Partially dehydrated 'Nebbiolo' grapes from Chiuso (Sondrio, Italy) were crushed in TEMA de-stemmer–crusher (Enoveneta, Piazzola Sul Brenta, Italy) in December 2019, and 10 mg/L of SO_2 were added to the must. After 24 h *Saccharomyces cerevisiae* (ACTIFLORE® BO213, Laffort, Bordeaux, France) was inoculated to the must at the dose suggested by the producer (30 g/hL). One punch-down was carried out the first day, then two punches down per day were carried out until the 6^{th} day. During the second week of fermentation, two pumpings over per day were performed in the first two days, while only one per day was carried out in the following days until the end of maceration, which lasted 14 days in total. The end of maceration was followed by the gentle pressing of the pomace cap using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy). Malolactic fermentation was induced by the addition of *Oenococcu oeni* (Malotabs^{TM}, Lallemand Inc., Montreal, Canada). After the malolactic fermentation, 50 mg/L of SO_2 were added and the wine was subsequently racked to remove the less. The first control (CONTR20) was collected and

Table 1. Characterization of 'Nebbiolo' wines before the treatments. Data are means ± SDs of two replicates.

Wine Parameter	
Etanol (%)	13.61±0.00
Glicerol (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)	0.3±0.00
Free SO_2 (mg/L)	10.5±0.71
Total SO_2 (mg/L)	76.48±0.91
Turbidity (NTU)	15.05±0.01
Color Intensity (PO 10mm)	8.29±0.01
Hue	0.75±0.00
L*	16.23±0.01
a*	46.99±0.04
b*	27.12±0.02
Total Phenol (mg/L Epicatechin)	3163.08±69.71
Total Anthocyanins (mg/L Malvidine-3-O-glucoside)	148.36±0.57
Total Flavonoid (mg/L Catechin)	1062.96±5.83

subsequently bottled in 0.5 L bottles and frozen for two weeks at -20°C before DNA extraction as described below. After the sulphitation, the wine was racked two times, approximately every 6 months, every time with the addition of 10 mg/L of SO_2. Table 1 shows the chemical characterization of the 'Nebbiolo' wine used in this experiment.

2.3 Sample treatment with enological additives

The most common additives and processing aids used in winemaking were selected for this experiment. In March 2021, ten additives were tested in three replicates and added to the 'Nebbiolo' wine. For each additive, the preparation has been carried out according to the instructions on the product technical sheet (Table 2). The used dose was calculated as 85% of the maximum dose suggested by the processing aids 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 filtration (FLT) was performed following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The temperature was kept constant at 16 ° C for all treatments. According to what is already present in literature (Table 2), after 7 days each trial was racked with a small laboratory peristaltic pump. For each additive and each replicate, the clear wine was collected for chemical analysis, and a 0.5 L bottle was frozen for two weeks to enhance the nucleic acid precipitation.

Table 2. Additives and processing aids used on 'Nebbiolo' wine. CONTR20: untreated wine sampled in 2020, one year before application of enological additives; CONTR: untreated wine sampled in 2021 at the time of application of additives.

Sample	Treatment	Product	Used Dose	Reference
CONTR20	-	-	-	-
CONTR	-	-	-	-
BEN	Bentonite	Gelbentonite, Dal Cin	25.5 g/hL	Ficagna et al., 2020
GEL	Animal Gelatine	Premium Gel Grado 1, Vason	25.5 g/hL	Cosme et al., 2007
VEG	Vegetable Protein	Vegecoll. Laffort	4 g/hL	Río-Segade et al., 2020 Ficagna et al., 2020
PVPP	Polyvinylpolypyrrolidone	PVPP, Alea Evolution	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	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	85 mL/hL	-
TAN	Skin Tannin	Protan Raisin, AEB	25 g/hL.	-
FLT	0,2 µm Filtration	Sterile Single Use Vacuum Filter Units, Nalgene™ Rapid-Flow™	-	-

2.4 Chemical analysis on 'Nebbiolo' wines

After the treatment, 25 mL of wine was collected to carry out the chemical-physical analysis. Titratable acidity was determined by titrimetry according to OIV-MA-AS313-01, 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 acid (malic, lactic, and citric acid) were estimated by HPLC (Agilent Technologies, Santa Clara, USA), following the method proposed by Schneider et al. (1987). Turbidity was analysed using a turbidimeter (Model TB1, Velp Scientifica, Usmate, Italy) and expressed in NTU (Nephelometric Turbidity Units), in accordance with the OIV method OIV-MA-AS2-08 (OIV, 2016b). Total and free-SO_2 were quantified by Solfotech extractor (Exacta + Optech Labcenter Spa, San Prospero, Italy) according to the OIV method OIV-MA-AS323-04A (OIV, 2016b).

Wine phenolic composition and colour parameters were evaluated following the methods reported by Petrozzielo et al. (2018), using a UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). Total anthocyanins (TA) and total flavonoid (TF) were quantified by diluting the sample with ethanol:water: 37% hydrochloric acid (70:30:1, v/v) and subsequently measuring the absorbance at 536-540 nm and 280 nm, respectively. TA were quantified as mg/L of malvidin-3-glucoside chloride and TF were expressed as mg/L of catechin. Total phenolic index (IPT) was evaluated by measuring absorbance at 280 nm of the sample diluted in water as reported by Scalzini et al., (2020). The results were expressed in mg of (−)-epicatechin/L of wine using a calibration curve obtained with standard solutions (y=82.158x). The wine color parameters were evaluated by the acquisition of the visible spectra (380-780 nm) of the undiluted samples using 1 mm optical

path cuvettes and the subsequent determination of the Colour Intensity (CI) ($A_{420}+A_{520}+A_{620}$) and Hue (A_{420}/A_{520}) on an optical path of 10 mm, following the OIV-MA-AS2-07B method(OIV, 2016b). Wine colour was also evaluated by CIEL*a*b* parameters, according to the OIV-MA-AS2-11 method (OIV,2016b). The total color difference (ΔE*) between control and treated samples was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Then, the CIEL*a*b* coordinates were converted to RGB values for journal compatibility purposes.

2.5 Grapevine DNA extraction from wines

Total DNA from wine was extracted with two different methods: i) Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) (Norgen protocol) and ii) the cetyltrimethylammonium bromide (CTAB)-based method by Siret et al. (2002) and Agrimonti & Marmiroli (2018) (SirM protocol) with some modifications.

Before the aliquots collection for DNA extraction, each wine conserved st -20°C was homogenised by inverting the bottle several times. Every replicate was extracted from 50 mL (Norgen) and 100 mL (SirM) of wine pellets obtained after centrifugation at 4,000 *g* at 4 °C for 1 h. In 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, whereas 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 of TEX buffer [20 mM EDTA pH

8.0, 1.4 M NaCl, 1M Tris–HCl pH 8.0, 3% cetyltrimethylammonium bromide (CTAB), and 1% β-mercaptoethanol] and incubated at 65 °C for 1 h, with mixing (every 10–15 min). Then, 1 volume of chloroform:isoamyl alcohol (24:1) was added and homogenized. After centrifugation at 8,000 *g* for 10 min at 4 °C, the supernatant was mixed with 0.1 volume of 10% CTAB and extracted again with 1 volume of chloroform:isoamyl alcohol. The DNA-containing upper phase was precipitated overnight at -25 °C with 2 volumes of ethanol. Then, DNA was collected by centrifugation at 10,000 *g* for 30 min at 4 °C, resuspended in 250 µL of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0), and treated with 20 µL of Proteinase K (20 mg/mL) at 48 °C for 30 min. Then, 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and samples were centrifuged at 11,000 *g* for 15 min at 4 °C. DNA was precipitated with 2 volumes of ethanol and 2.5 M of ammonium acetate at – 25 °C for at least 2 h. After centrifugation at 22,000 *g* for 30 min at 4 °C, pellets were washed twice with 500 µL of 70 % ethanol and resuspended in 45 µL of TE buffer. The final purification was performed with the kit 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 A_{260}/A_{280} and A_{260}/A_{230}. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used. DNA was stored at −20 °C.

2.6 Grapevine DNA quantification by qPCR and determination of PCR inhibitors

To quantify the grapevine DNA, all DNA samples were analysed by 9-cis-epoxycarotenoiddioxygenase (*VvNCED2*); whereas 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) to the qPCR mixture. For the grapevine DNA quantification, the primers and the TaqMan® FAM-labelled probe for *VvNCED2* reported by Savazzini & Martinelli (2006) were used. qPCR reaction was performed in a final volume of 10 µL, consisting of 2,5 µL of DNA, 5 µL of 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 of EIPC DNA, 1 µL of EIPC mix (containing premixed forward, reverse primers, and VIC probe specific for EIPC) and 0.1 µL of sterile water. Amplification cycles were characterized 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, obtained by serial dilution. The 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 were analysed by SNP_15082 and SNP_14783. According to what is reported by Boccacci et al. (2020), 'Nebbiolo' alleles and non- 'Nebbiolo' alleles were marked with different dyes (FAM and VIC dye) as reported in Table S1. 'Barbera' and 'Freisa' were selected as homozygous and heterozygous non-'Nebbiolo' cultivar, respectively. qPCR reaction for TaqMan® SNP assays was performed in a final volume of 10 µL, consisting of 2.5 µL of DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.25 µL of 40X TaqMan® SNP Genotyping Assay (containing pre-mixed forward and reverse primers, VIC probe, and FAM probe) and 2.25 µL of sterile water. The amplification profile was the same reported in 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) using the Tukey HSD post-hoc 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, 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 Enological impact of additives

The impact of additives and processing aids on 'Nebbiolo' wine phenolic composition and turbidity is reported in Table 3. Bentonite (BEN), gelatine (GEL), PVPP, and yeast hulls (YST) strongly decrease the wine turbidity, while mannoprotein (MAN), chitosan (CHT), and Arabic gum (ARG) slightly increase the NTU level compared to the untreated control (CONTR). Bentonite (a commercial product mainly composed of a natural clay known as montmorillonite) is widely used as fining agent, which can adsorb and precipitate proteins. In our study, BEN had the greatest impact on wine turbidity. These results are in agreement with those of Ficagna et al. (2020), in which clarification with BEN showed the most intensive reduction in turbidity, followed by PVPP, while vegetable proteins (VEG) treatment led to a minor reduction of NTU level. GEL also had a great impact in terms of turbidity reduction, according to what is reported by González-Neves et al. (2014).

The wine phenolic composition changed after the treatment with different processing aids. 'Nebbiolo' wines treated with grape skin tannin (TAN) showed a higher IPT and TF content compared to the CONTR; whereas, PVPP, CHT, BEN, and GEL showed significant lower IPT values. TA content was slightly affected by the usage of TAN, ARG, MAN, and CHT; whereas, BEN, VEG, PVPP, and potassium polyaspartate (POL) caused a decrease in anthocyanins content. 'Nebbiolo' wines treated with GEL showed the lowest TA values, which is 15% lower compared to the CONTR. GEL and PVPP treatments significantly affected the TF content with respect to the CONTR, leading to a reduction of 12.3 % and 5

%, respectively. Instead, other treatments did not significantly change the TF content. Among all the treatments tested in our study, GEL had the strongest impact on wine phenolic composition, showing the lowest IPT, TF, and TA values (Table 3). Our results are in agreement with the literature, BEN and GEL strongly affect the wine phenolic composition leading to a reduction of anthocyanins and tannins, respectively (González-Neves et al., 2014). 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 only marginally decrease the phenolic composition as a side effect (Castro Marin & Chinnici, 2020).

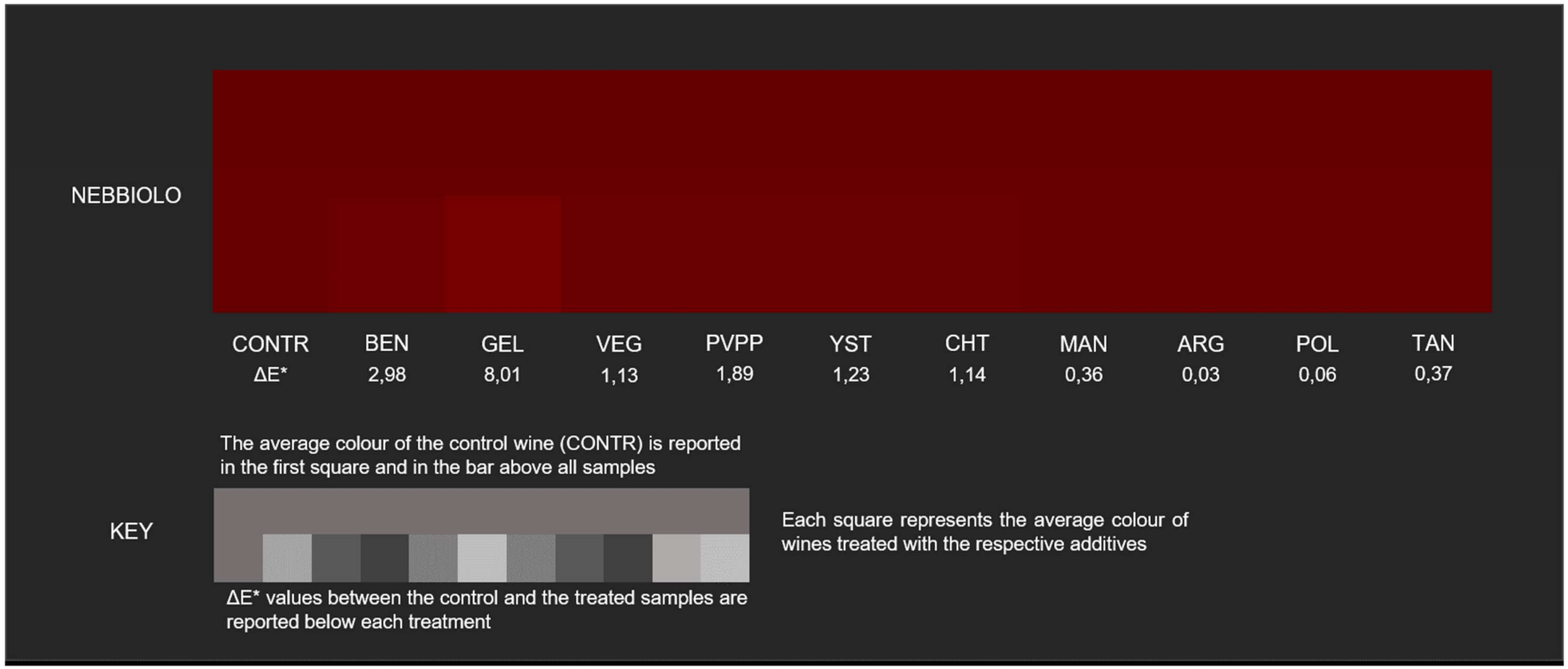
As previously demonstrated, wine colour can decrease as a secondary effect of the treatment with fining agents (Río-Segade et al., 2020). In particular, TAN increased the CI, while the lowest values were shown in wines treated with PVPP, BEN, and GEL. Regarding Hue values, VEG, YST, and CHT did not show any significant differences compared to the CONTR. BEN, ARG, MAN, POL, and TAN showed higher values, while GEL and PVPP the lowest. The reduction of CI and the increase of the Hue values is likely due to the lower TA values reported after the treatment with fining agents. The increased Hue can be justified by the loss of anthocyanins that led to a reduction of the red colour component (A_{540}). The CIEL*a*b* characterization of 'Nebbiolo' wines was performed; L* (lightness), a*, and b* (red/green color and yellow/blue color, respectively) values are reported in Table 3. According to Río-Segade et al. (2020), the usage of fining agents (BEN and GEL) can increase the Hue values, leading to a shift towards yellow and red hue, respectively. A slight increase in L*, a*, and b* values are reported also after the treatment with CHT, YST, PVPP, and VEG; whereas, all the other treatments did not change significantly the colour properties

Table 3. Phenolic composition and colour characteristic of 'Nebbiolo' wines treated with different additives and processing aids. CONTR: untreated control; BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: skin tannin. Data are means ± SDs of three replicates. Values followed by different letters within a column are significantly different ($p < 0.05$, one-way ANOVA with HDS post hoc). IPT: Total Phenolic Index; TA: Total Anthocyanins; TF: Total Flavonoid.

	Turbidity	IPT	TA	TF					
Sample	**NTU**	**mg (-)-Epicatechin/L**	**mg Malvidin-3-glucoside chloride/L**	**mg Catechin/L**	**Colour Intensity**	**Hue**	**L***	**a***	**b***
CONTR	11.59±0.04 cd	3069.97±17.10 bcd	145.80±1.24 a	1050.60±8.24 b	8.25±0.01 bc	0.75±0.00 c	16.63±0.06 ef	47.49±0.09 ef	2781±0.10 ef
BEN	2.30±0.61 g	2974.12±21.74 efg	140.14±1.68 bcd	1042.36±8.24 bc	7.82±0.01 f	0.76±0.05 a	18.07±0.06 b	49.01±0.08 b	29.93±0.09 b
GEL	6.58±1.31 e	2738.60±37.05 h	123.97±0.47 e	921.51±6.29 e	6.97±0.03 g	0.74±0.01 d	20.92±0.10 a	51.43±0.07 a	33.31±0.12 a
VEG	9.93±1.16 d	3009.72±42.16 cdef	138.79±2.60 d	1019.01±16.65 bcd	8.08±0.01 d	0.75±0.00 c	17.18±0.05 d	48.05±0.09 d	28.61±0.08 d
PVPP	4.15±0.27 f	2916.61±16.43 g	137.44±2.14 d	1005.28±10.90 d	7.89±0.02 e	0.74±0.01 e	17.60±0.05 c	48.39±0.07 c	29.16±0.08 c
YST	7.21±0.49 e	2996.03±9.49 def	139.33±2.03 bcd	1028.63±8.58 bcd	8.06±0.00 d	0.75±0.00 c	17.23±0.02 d	48.09±0.02 d	28.69±0.03 d
CHT	14.55±0.24 a	2963.17±25.10 fg	144.72±3.71 ab	1035.49±22.69 bcd	8.06±0.00 d	0.75±0.01 c	17.20±0.03 d	48.04±0.04 d	28.62±0.05 d
MAN	14.56±0.61 a	3080.93±32.86 bc	142.84±1.24 abcd	1017.64±4.12 bcd	8.30±0.03 ab	0.75±0.02 b	16.47±0.11 fg	47.27±0.14 f	27.57±0.17 f
ARG	13.61±0.37 ab	3097.36±21.74 ab	144.45±1.68 abc	1013.52±18.88 bcd	8.24±0.01 c	0.75±0.03 b	16.64±0.03 ef	47.47±0.03 ef	27.82±0.03 ef
POL	12.58±0.04 bc	3048.06±21.74 bcde	139.06±0.81 cd	1023.13±8.58 bcd	8.23±0.03 c	0.75±0.04 b	16.67±0.07 e	47.50±0.10 e	27.86±0.11 e
TAN	12.35±0.01 bc	3163.08±24.65 a	142.03±0.47 abcd	1097.29±6.29 a	8.35±0.00 a	0.76±0.05 a	16.44±0.04 g	47.28±0.06 ef	27.56±0.06 f

with respect to the CONTR. The CIEL*a*b* coordinates were converted in RGB values and the wine colour representation is reported in Figure 1. GEL gave the highest ΔE* values, followed by BEN. Wines treated with BEN and GEL reached a visually colour reduction; whereas, all the other treatments did not exceed the three ΔE* units, which is the threshold that allowed the recognition by the human eye (Pérez-Magariño & González-Sanjosé, 2003). These results confirm what was reported by Río-Segade et al.,(2020): BEN and GEL had the strongest impact on the turbidity, colour, and phenolic composition of 'Nebbiolo' wines.

Figure 1. 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: skin tannin. Each colour was acquired by spectrophotometry, expressed in CIE L*a* b* 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 enological additives

We used two DNA extraction protocols that had proven effective in 'Nebbiolo' wines, the very fast Norgen protocol with good results in experimental musts and wines, and the SirM protocol more performing in commercial wines (Boccacci et al., 2020). The DNA yield and the quality ratios of DNA extracts were initially estimated through a spectrophotometric analysis using NanoDrop 1000. The spectrophotometric quantification of wine extracted using Norgen protocol is reported in Table S2, while the quantification results of wines extracted with SirM method are reported in Table 4. 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 the CONTR and the treatments (Table S2). On the other hand, significant differences were found in wine samples extracted with the SirM method: CONTR20 ('Nebbiolo' wine sampled in 2020, one year before application of enological additives) showed the highest DNA yield while the CONTR has the lowest concentration. In contrast with the result obtained after the Norgen protocol, all the wines treated with different additives and processing aids showed a significantly higher DNA yield compared to the CONTR (Table 4).

The quality of the extracted DNA was estimated from traditional absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}). 'Nebbiolo' wines extracted using both protocols did not show any significant differences in terms of A_{260}/A_{280} and A_{260}/A_{230} ratios. However, DNA extracted with the SirM protocol showed higher quality than DNA extracted with Norgen protocol, likely due to the application of more intense DNA cleaning operation using phenol and chloroform (Table 4, Table S2). The presence of polysaccharides and phenolic substances

(including tannins), which are extremely common in grapes, negatively affects the quality of DNA extracted from wines.

Several previous works (Vignani et al., 2019; Savazzini & Martinelli, 2006) 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, the spectrophotometric quantification is often not reliable for the quantification of grapevine DNA in wines. Consequently, we adopted a more specific quantification of grapevine DNA based on *VvNCED2* amplification using TaqMan® probes, as previously suggested in different works (Savazzini & Martinelli, 2006; Vignani et al., 2019; Boccacci et al., 2020). No amplification of the *VvNCED2* was observed in any DNA samples extracted with Norgen protocol, probably the grapevine DNA present in the samples was too limited and/or too impure to allow amplification in qPCR of *VvNCED2*. Instead, the DNA extracted with SirM protocol was successfully amplified using *VvNCED2* TaqMan® probes. The values of the grapevine DNA and its percentage ratio with respect to the total DNA yield measured by NanoDrop are reported in Table 4. In general, the data obtained with Nanodrop quantification were overestimated. The CONTR showed the highest concentration of grapevine DNA with 6.73±1.13 ng per mL of wine: this amount corresponds only to the 0.29% of the DNA yield quantified by NanoDrop. For the other treatment, the percentage of grapevine DNA is lower. Likely, most of the DNA yield quantified by the Nanodrop is not grapevine DNA. Spectrophotometric quantification is a non-reliable method to quantify DNA in wine extract, regardless of the extraction method. These data confirmed the overestimation previously reported in 'Nebbiolo' wines by Boccacci et al. (2020), whose results showed that grapevine DNA can be up to 25 times less than the DNA estimated with spectrophotometer in the must and 20,000 times less in the wine after 1 year.

All treatments showed a lower grapevine DNA concentration with respect to the CONTR; therefore, all the cleaning treatments played a role in removing DNA from the wine, but the intensity of the reduction differs depending on the treatment. Interestingly, the products with the highest enological impact (Table 3) caused the highest loss of DNA compared to the CONTR. Samples treated with BEN and GEL showed the strongest reduction, with a DNA loss of 99.56% and 99.67%, respectively. The filtered samples (FLT) also showed a strong loss of DNA, with a reduction of 96.60%. Whereas, POL, ARG, and YST seem to have the least impact on grapevine DNA extraction. Moreover, CONTR20 has 41.92% more grapevine DNA compared to CONTR. Nevertheless, the reduction that occurred during one year of stocking in stainless steel with 3 rakings after the malolactic fermentation is not significant. Interestingly, the loss of DNA caused by aging is lower compared to the loss due to the treatment with fining agents (i.e. BEN and GEL) and FLT. Thus, cleaning operations play the most important role in decreasing DNA residual in wine. The literature reports the greater efficiency in reducing the turbidity of wine after the use of blends of different fining agents (González-Neves et al., 2014).

Table 4. DNA purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*. Percentage ratio between DNA quantification by *VvNCED2* and the yield measured by NanoDrop. Loss of DNA after treatment expressed as percentage ratio between the DNA (quantified by *VvNCED2*) of the Control and the treated wine. The ratio was calculated as follows: (DNA of the Control - DNA of' wine after treatment)/ DNA of the Control. Allelic profile of genotyping assay SNP_14783, SNP_15082. For each treatment replicate, one sample was extracted (R1, R2, and R3). For each sample, genotyping was performed twice (1st and 2nd repetition). '-' in the allelic profile denotes an incorrect allelic call; '+' indicates samples that correctly amplified, and 'nd' stands for 'not detected'. Data are means of 3 replicates ± standard deviation. Values followed by different letters within a column are significantly different ($p < 0.05$, Kruskal-Wallis test with Conover's Comparison test). CONTR20: untreated control sampled one year before the application of additives; CONTR: untreated control BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: skin tannin; FLT: filtration.

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		
	DNA yield [ng/mL of wine]	A_{260}/A_{280}	A_{260}/A_{230}				1st repetition						2nd repetition					
							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 efg	+41.92±12.58 a	+	+	+	+	+	+	+	+	+	+	+	+
CONTR	2.42±0.50 d	1.40±0.17 a	0.72±0.02 ab	6.73±1.13 ab	0.29±0.07 a	-	+	+	+	+	+	+	+	+	+	+	+	+
BEN	4.27±0.11 abc	1.44±0.03 a	0.66±0.03 ab	0.03±0.04 g	0.00±0.00 g	-99.56±0.35 gh	-	-	-	-	+	-	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±0.00 g	-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 def	0.03±0.02 cdefg	-80.65±12.24 defg	+	+	+	+	+	+	+	+	+	+	+	+
PVP	4.41±0.45 ab	1.43±0.06 a	0.61±0.09 ab	1.90±0.47 cd	0.04±0.01 bcde	-71.67±6.67 bcde	+	+	+	+	+	+	+	+	+	+	+	+
YST	3.80±0.52 bcd	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 bc	1.88±0.26 a	043±0.08 b	0.56±0.32 efg	0.02±0.01 defg	-91.65±5.20 efgh	+	+	+	+	+	+	+	+	+	+	+	+
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 bcdef	-78.09±3.77 cdef	+	+	+	+	+	+	+	+	+	+	+	+
ARG	3.50±0.05 bcd	1.58±0.13 a	0.69±0.03 ab	2.51±1.49 bcd	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 abc	0.10±0.07 abc	-41.00±29.33 abc	+	+	+	+	+	+	+	+	+	+	+	+
TAN	2.67±0.52 c	1.99±0.44 a	0.60±0.11 ab	1.77±0.39 cdef	0.07±0.01 abc	-73.69±6.17 cde	+	+	+	+	+	+	+	+	+	+	+	+
FLT	2.27±0.43 c	1.54±0.13 a	0.61±0.15 ab	0.23±0.25 fg	0.01±0.01 eg	-96.60±3.45 fgh	-	+	nd	-	-	-	nd	-	-	nd	nd	-

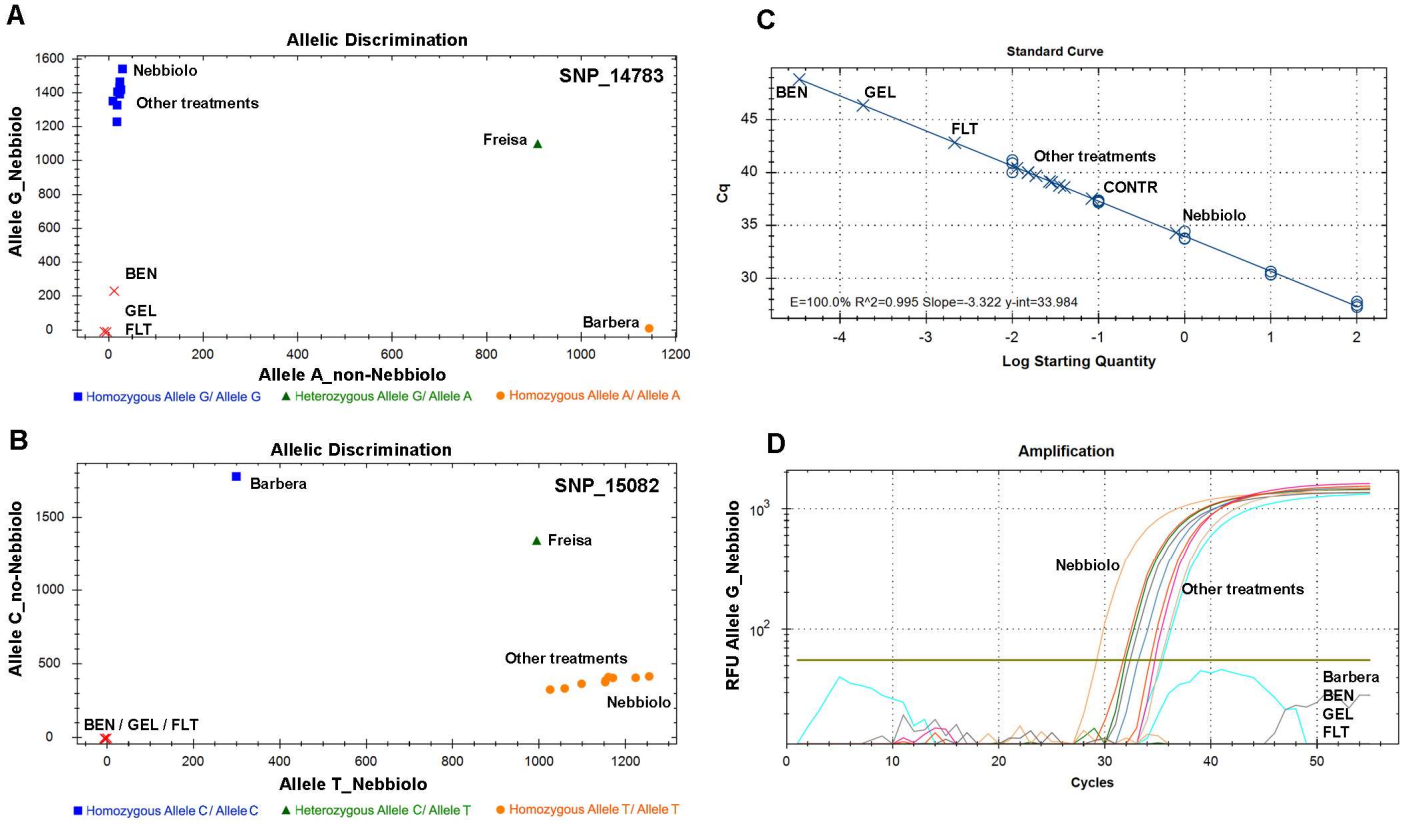
Therefore, the combined effect of these treatments can strongly reduce the DNA quality and quantity in the wine, explaining why several authors did not successfully find traces of DNA in commercial wines (Boccacci et al., 2020; Boccacci et., 2012; Catalano et al., 2016).

3.3 SNP genotyping in 'Nebbiolo' wines

The combination of the allelic calls of two specific 'Nebbiolo' SNPs (SNP_15082 and SNP_14783) is enough to recognize 'Nebbiolo' from more than 1,100 genotypes. In a precedent study, the TaqMan® assay based on these two SNP allowed the recognition of 'Nebbiolo' must and wine 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 showed a reduced identification efficiency also in 'Nebbiolo'.

The TaqMan® assays for the detection of SNP_15082 and SNP_14783 were applied to the DNA extracted with the Norgen protocol, and all samples did not amplify or incorrect calls of the genotyping assays were observed (Table S2). 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. The 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.

Figure 2. SNP genotyping in 'Nebbiolo' wines extracted with SirM methods and previously treated with different enological 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 present in 'Nebbiolo' wine extracts. DNA from 'Nebbiolo' leaves was used as a calibrator for the standard curve. (D) Relative fluorescence unit (RFU) of the TaqMan® probe tagged with VIC dye (allele G 'Nebbiolo'). The yellow line in the amplification plot indicates the RFU level of 'Barbera' (non-'Nebbiolo' control), above which, it was possible to detect 'Nebbiolo' wines. The control DNA from 'Nebbiolo', 'Barbera' and 'Freisa' were extracted from leaves. CONTR: untreated control; BEN: bentonite; GEL: gelatine; FLT: filtration.



The genotyping of DNA extracted with the SirM protocol was more successful. The CONTR and the CONTR20 correctly amplified for both the allele. Several authors (Boccacci et., 2020; Catalano et al., 2016) reported the aging time as one of the causes of the reduction of identification efficiency in commercial wines. According to our result, the reduction of the quantity of DNA that occurs over time is not responsible alone for the incorrect amplification of commercial wines reported by several studies. Despite the aging of the wine and the low-quality of the DNA, this TaqMan® assay is confirmed to be very robust and effective to identify 'Nebbiolo' wines in experimental conditions.

Interestingly, there is a clear correlation between the treatments and the success of the genotyping: ARG, TAN, CHT, MAN, VEG, POL, and PVPP treatments do not show any effect on the assay. All the repetitions correctly amplified and the allelic discrimination was always possible with a precision of 100%. Nevertheless, other treatments, such as FLT, BEN, and GEL resulted in an incorrect or the absence of SNP amplification. It is probably due to the low quantity of residual DNA in the wine. Indeed, according to the *VvNCED2* quantification result, the TaqMan® assay used in our study loses efficacy if the samples have less than 0.5 pg of DNA per mL of wine. Indeed, BEN, GEL, and FLT treatments, which are the three treatments with the lowest quantity of grapevine DNA (under 0.5 pg/mL of wine), showed amplification issues, whereas all treatments with more DNA were correctly discriminated by the assay. In addition to the low DNA concentration, the presence of PCR inhibitors in the DNA extracted 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 without significant differences. This result confirms that the amplification issues in BEN, GEL, and FLT treatments were uniquely caused by the low

quantity of DNA in wines after the treatment with fining agents and none by the presence of PCR inhibitors in the extracts.

The use of BEN and GEL, as well as FLT, represent very common practices widely used in the production of most commercial red wines, including 'Nebbiolo' wines; moreover, it is not unusual to use together these three practices. Considering our results, one of these three techniques alone can reduce the grapevine DNA by 90%; thus, their combination can have a drastic effect on the reduction of DNA residual in wines. Therefore, their combined effect on the DNA residual 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; Catalano et al., 2016; Recupero et al., 2013; Boccacci et al., 2020). To the best of our knowledge, this is the first time an experiment investigates the causes of the reduced efficiency of the genetic traceability of wines.

4. CONCLUSIONS

In our study, we have 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. A reported in literature by Boccacci et al., (2020), using two SNP markers (SNP_14783 and SNP_15082), it is possible to identify 'Nebbiolo' wines from other non- 'Nebbiolo' wines. Nevertheless, as reported by several authors, the winemaking process can affect the precision of the varietal identification. All the 'cleaning' operations aimed to reach clarity and stability of the wine after the alcoholic fermentation can reduce the quality and the amount of DNA in the wine. Our results showed an impact of fining agents on turbidity and phenolic composition in line with other studies in literature. BEN and GEL had the strongest impact on turbidity, phenolic, and colour parameters. Our study confirms the efficiency of the TaqMan® assay for the varietal identification also in aged wines; indeed, under experimental conditions, the recognition was possible in 2 years old wine with 100% of precision. The identification was also possible for the majority of the wines treated with additives or processing aids. Nevertheless, the recognition failed in wines treated with BEN, GEL, and FLT. '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 neither the DNA quantity nor the identification efficiency. Our study contributed to explaining the reasons for the decreased identification efficiency in

commercial wines and confirmed the need for future improvements of the DNA extraction techniques from wines.

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6. SUPPLEMENTARY MATERIALS

Table S1. Primers and probes used for the SNP genotyping

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

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

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