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Utilization of a freeze-thaw treatment to enhance phenolic ripening and tannin oxidation of grape seeds in red (*Vitis vinifera* L.) cultivars

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ABSTRACT

Phenolic ripening represents a major interest for quality wine producers. Nevertheless, climatic or genotypical limitations can often prevent optimal maturation process. During winemaking seeds can be easily separated and technologically processed to improve their quality. Relying on the key role of oxidation for phenolic ripening, a freeze-thaw treatment was proposed to improve the fruit quality for potential use in challenging growing conditions. The experiment was carried on in two distinctive viticultural areas, Michigan and Italy. Five cultivars (Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot noir and Chambourcin) and six cultivars (Cabernet Sauvignon, Sangiovese, Syrah, Croatina, Barbera and Nebbiolo) were used in Michigan and Italy, respectively. Samples were collected at different phenological stages, to describe the natural ripening process and grape seeds were characterized before and after a freeze-thaw treatment. Colorimetric and spectrophotometric data highlighted similarities among natural and artificial seed ripening promising future applications for the wine industries.

1. Introduction

The importance of seed color at harvest time for grape quality evaluation has been understood for millennia – the famous Roman agriculture writer Columella (4-70 A.D.) suggested the process of seed darkening as the best grape ripening index in his book: "De Re Rustica" (Rustioni & Failla, 2016). During ripening, a grape seed starts as a bright green color, and slowly changes to yellow, and eventually dark brown shades (Ferrer-Gallego, García-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2010; Ristic & Iland, 2005; Rodríguez-Pulido et al., 2012). The seed coat modifications during ripening aims to provide mechanical protection to the embryo and to maintain seed dormancy (Ristic & Iland, 2005; Rolle et al. 2013). Seed browning during berry ripening is considered to be the result of oxidation of flavan-3-ols and tannins (Adams, 2006; Kennedy, Matthews, & Waterhouse, 2000; Ristic & Iland, 2005; Rustioni & Failla, 2016), which are initiated by an oxidative burst at veraison (Pilati et al., 2007). The major role of phenolic oxidation in coat browning has been shown to occur during the development of seeds and fruits in different species including *Arabidopsis thaliana*; *Phaseolus vulgaris*; *Zea mays*; *Litchi chinensis* (Pourcel et al., 2007).

In addition to tannin color, the oxidation of phenolics is expected to affect their gustatory perception, including astringency (McRae & Kennedy, 2011). It is well known that astringency perception is a complex tactile sensation caused by a loss of lubricity in oral saliva (Cheynier et al., 2006; McRae & Kennedy, 2011). The interactions between tannins and saliva proteins that are responsible for this perception involve a number of mechanisms, including hydrophobic interactions (Van der Waals and π-π stacking), hydrogen bonding, self-association (causing cross-links between protein-tannin complexes) and finally, protein aggregation and eventual production of colloidal particles (McRae & Kennedy, 2011). Through the formation of new bonds, and the modification of the molecular structures and interactions (McRae & Kennedy, 2011; Pourcel et al., 2007), intra-molecular bonding is increased, which reduces tannin flexibility; altering linear and extended tannin forms into more condensed structures (McRae & Kennedy, 2011; Poncet-Legrand et al., 2010). Flavanol polymerization reactions, regardless of the polymers formed (proanthocyanidins, oxidation products, or ethylflavanols), generally enhance the astringency (Cheynier et al., 2006). Nevertheless, the availability of binding sites (associated to the structural flexibility) and the steric hindrance of the tannin polymer could prevent the protein access to binding sites, creating a threshold in the correlation among tannin size and protein binding efficacy (McRae & Kennedy, 2011).

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Despite the current knowledge of seed color and tannin oxidation, and their relationship to fruit quality and wine making, environmental conditions pose a challenge to their development in different viticultural areas: ideal growing conditions for *Vitis vinifera* consist of temperate climate regions with warm, dry summers seeing moderate precipitation, and mild winters. The first challenge is the increase in heat accumulation in warm viticultural grape growing regions, which is prominent in many Italian vineyards. Increased summer temperatures are capable of altering the pathways of secondary metabolites during ripening, resulting in poor polyphenolic maturation and low fruit quality at harvest (Frioni et al., 2017). The second challenge is the annual variation in climatic conditions experienced in many cool climate viticultural regions, including Michigan. Each issue can be associated with global climate change, and they are limiting the sustainable production of grapes with a consistent optimal fruit quality at harvest in several viticultural areas of the world (Schultze, Sabbatini, & Luo, 2016).

Technological procedures targeted towards improving phenolic oxidations are widespread among wine producers located in suboptimal environmental climates. The most prominent examples include: must oxygenation during maceration, use of wood barrels and, in general, wine micro-oxygenation (Gómez-Plaza & Cano-López, 2011). The main restriction of these techniques concerns the non-selective nature of tannin oxidation obtained through musts and wines, which involves interactions with other molecules that potentially affect additional organoleptic characteristics (e.g. accumulation of aldehydes). The oxidation effects on different wines has been described by Bueno, Culleré, Cacho, and Ferreira (2010).

Phenols are predominantly located in the vacuole, while oxidoreductases are found in the cytoplasm. Thus, their interaction, and subsequent induction of enzymatic browning will not occur unless the cell membranes are damaged (Li, Guo, & Wang, 2008). Freeze-thaw treatments are known to affect the ultrastructure of vacuolated cells, due to the formation of ice crystals during freezing, which affect membranous components of the protoplasm (Mohr & Stein, 1969). This process has previously been observed in studies focused on strawberry shelf-life managements (Holzwarth, Korhummel, Carle, & Kammerer, 2012; Oszmiański, Wojdyło, & Kolniak, 2009). Considering grape seeds, different techniques are already available to separate them from the must during winemaking (Canales, Llaudy, Canals, & Zamora, 2008). Nevertheless, the seed removal (recommended in case of suboptimal phenolic ripening) could also impoverish wines in terms of 'body' and 'structure', eliminating a tannin source. Thus, in our opinion, seed recycling after freeze-thaw treatment, could be recommended to improve wine quality.

Phenolic heterogeneous pigments obtained by free radical polymerization caused by oxidations (Waterhouse & Laurie, 2006) are difficult to be quantified by traditional chemical assays due to their inhomogeneity and extractability limitations. For example, the oxidation bonds are resistant to acid catalyzed thiolysis (Kennedy et al., 2000) and the tannin oxidation can lead to solubility problems (Poncet-Legrand et al., 2010; Zanchi et al., 2007). Nevertheless, the presence of conjugated double bonds in these phenolic oxidized molecules allows the absorption of light of visible wavelengths (Rustioni, 2017). In fact, the spectrum of oxidized polymeric phenolics was recently characterized in sunburn grape berries, indicating a broad absorption band in the green spectral region, with a maximum around 500 nm (Rustioni, Rocchi, Guffanti, Cola, & Failla 2014). Therefore, the optical properties of seeds measured on-solid could be impacted by the detection limitations of the oxidized polymeric phenolics. However, CIELab color parameters and image analysis have already been proposed as a technique to evaluate seed ripening (Ferrer-Gallego et al., 2010; Obreque-Slier, López-Solís, Castro-Ulloa, Romero-Díaz, & Peña-Neira, 2012; Rodríguez-Pulido et al., 2012).

The present study aims to test a new procedure based on the selective oxidation of grape seeds by a physical treatment (freezing) to improve tannin ripening. Modifications to the seed's optical properties that occur during natural ripening and freezing treatment will be investigated and described using two different analytical approaches (spectrophotometric and colorimetric), and will utilize grape cultivars grown in two viticultural areas (Italy and Michigan).

2. Materials and methods

2.1. Environmental characteristics

The experiment was carried out during the 2017 growing season in the United States (Michigan) and Italy. Benton Harbor, Michigan (Latitude 42.0841 deg, Longitude – 86.3570 deg, Elevation: 220 m) and Riccagioia, Italy (Latitude 44.98; Longitude 9.08; Elevation 144) are characterized by different climates. Following the Köppen-Geiger Classification (Köppen, 1936), Benton Harbor's climate is under the Dfa type: continental without dry season with hot summer, while Riccagioia is Cfb: temperate without dry season with warm summer. The following agro-climatological analysis were completed using data collected from weather stations located near the experimental vineyards. The Benton Harbor weather station is a part of Enviro-weather, a weather station network run by Michigan State University, and is located 14 km far from the commercial vineyard in the Lake Michigan Shore AVA (American Viticulture Area) where samples were collected. Torrazza Coste station, 1 km far from the Riccagioia experimental field, belongs to the network of Consorzio Tutela Vini Oltrepò Pavese, the local wine-growers consortium. Data were analyzed for the period 2000, January 1st – 2017, October 31st. In order to provide an evaluation of the availability of thermal resources during the different phases of the growing season two methods of analysis were used:

1. GDD – Winkler Growing Degree Days (Amerine & Winkler, 1944)
2. NHH – Normal Heat Hours (Cola et al., 2014; Parisi et al., 2014)

The advantage of NHH over GDD is the use of a response curve considering optimal temperature for grape growth, taking into account the negative effects of under and over-optimal temperatures on plant growth (Mariani, Parisi, Cola, & Failla, 2012).

Fig. 1 displays these patterns from 2000 to 2017; blue and red lines represent the average value for Benton Harbor (Michigan) and Riccagioia (Italy), respectively, in terms of the seven Winkler classes. The average value for Benton Harbor was 1576 GDD, which follows the Winkler class II: temperate cool, suitable for early ripening grapes for wines to be aged, and medium ripening grapes for white or red wines ready to drink. Riccagoia, with an average value of 2119 GDD, falls into class IV: temperate warm, suitable for late ripening grapes for white or red wines ready to be aged. The difference in thermal resources availability of the two areas is confirmed by the analysis of Fig. 2, where ten-day accumulations of GDD (a1 and a2) and NHH (b1 and b2) are presented. Red lines represent 2017 behavior, and thick, black lines indicate average 2000–2016 values. The dark grey area is bordered by an average ± 1 standard deviation and the light gray area by an average ± 2 standard deviation. The timespan between fruit-set and physiological maturity is represented by the purple area.

2.2. Plant material and experimental design

In Michigan, 5 cultivars were considered: Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot noir (*Vitis vinifera* L.) and the French-American hybrid Chambourcin (Seyval-Villard 12–417 × Chancellor). Samples (3–5 bunches) of each cultivar were collected at the beginning of ripening (T0 – BBCH 85) and harvest time (T2 – BBCH 88) from a commercial grower collection (Meier, 2001). In Italy, vines were used from the ampelographic collection located in Oltrepò Pavese (south Lombardy), described in Rustioni et al. (2013). Six (*Vitis vinifera* L.) cultivars (Cabernet Sauvignon, Sangiovese; Syrah; Croatina; Barbera

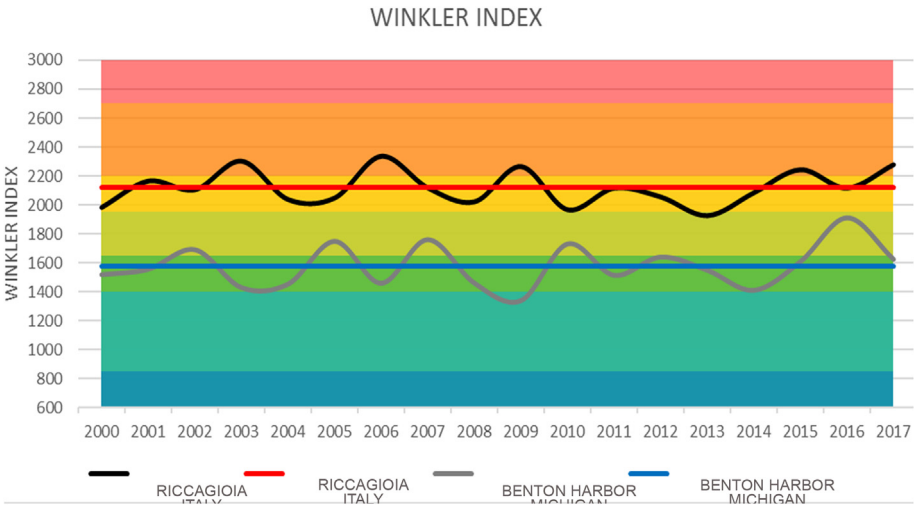

Fig. 1. Yearly Winkler Index from 2000 to 2017 in Riccagioia (Italy) and Benton Harbor (USA-Michigan). Average values are also presented. The chart is divided into the seven Winkler classes (I−, I, II, III, IV, V and V+).

and Nebbiolo) were considered. Samples (3–5 bunches) of each cultivar were collected at veraison (T0 – BBCH 83); middle ripening (T1 – BBCH 86); and harvest time (T2 – BBCH 89) (Meier, 2001).

At both research institutes, clusters were analyzed within 24 h of the sampling time. 50 seeds were randomly selected from each cultivar, and linearly arranged on a polystyrene board to maintain the seeds in position. Each seed was analyzed immediately after placement on the board, and after the freezing treatment. The procedure consisted of freezing (−20 °C) the seeds overnight, followed by a 3-h defrosting period at room temperature. A total of 1400 seeds were analyzed at two points during the experiment.

2.3. Data records, data elaboration and statistical analysis

Analyses were carried out using a Konica Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan) in Michigan, and a Jaz System spectrometer (Ocean Optics, B.V., Dunedin, USA) in Italy. Seeds were described by 3 colors parameters: lightness (L^*), chroma (C^*) and hue (h^*). The L^* parameter ranges from 100 (perfect white) and zero (black) (Obreque-Slier et al., 2012). The C^* parameter increases with chromatic saturation. The h^* parameter, indicates the tone or hue and is expressed in angle degrees (0° = red; 90° = yellow; 180° = green; 270° = blue) (http://sensing.konicaminolta.us/2015/03/understanding-the-cie-lch-color-space/). The changes to these parameters in each seed before and after treatment are representative of the artificial ripening effect. Variation in the color of each ripened seed (T2) with respect to the

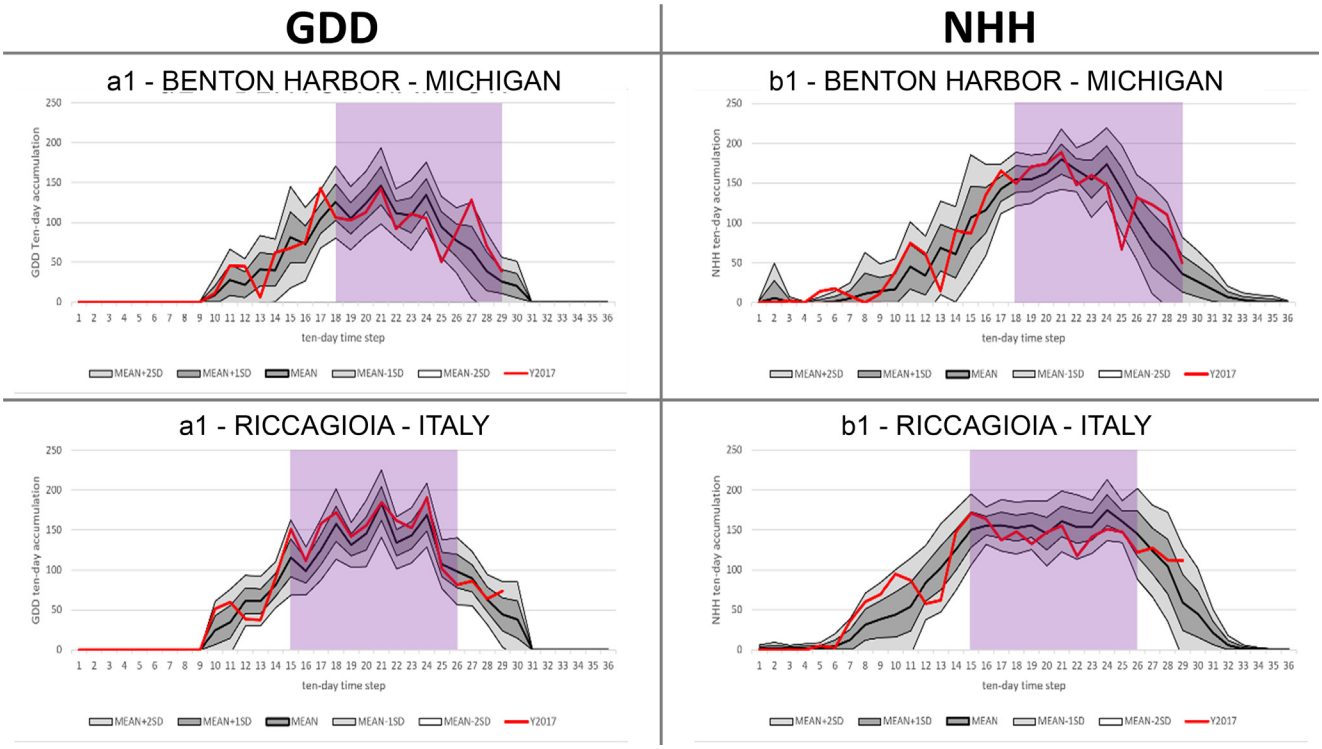

Fig. 2. GDD (Winkler Growing Degree Days) and NHH (Normal Heat Hours) behavior during 2017 in Benton Harbor (USA-Michigan) and Riccagioia (Italy). Purple areas represent the timespan between fruit set and maturity.

cultivar's average seed color at T0 are indicative of natural ripening effects. The Jaz System spectrometer (Ocean Optics, B.V., Dunedin, USA) was fitted with a DPU module, an ILX511b detector, an OFLV-3 filter, an L2 lens, and a 50 μm slit as installed options. A reflection probe QR600-7-VIS125 consisting of a tight bundle of seven optical fibers (600 μm in diameter) in a stainless-steel ferrule (six illumination fibers around one read fiber), was coupled to the spectrophotometer. A probe holder was included to ensure the analytical reproducibility – the distance between the sample surface and the probe was fixed at 12 mm. The instrument was set up with a near infrared − visible (NIR − vis) light source (Ocean Optics) 4095 power setting, and an integration time automatically corrected by the instrument. Collected spectra ranged between 341 and 1025 nm and had a spectral resolution of approximately 0.3 nm. Each spectrum was set up to be the average of 15 spectra, which were directly calculated by the instrument. A calibration with a blank was obtained using a polytetrafluoroethylene (PTFE) diffuse reflectance standard (Ocean Optics B.V.). Reflectance spectra were first elaborated using the script reported in Rustioni, Ciacciulli, Grossi, Brancadoro and Failla (2016) by R software (R Core and Team, 2015). This initial data processing allowed the spectra (400–800 nm) to be transformed to a percentage with respect to the blank; and the normalization at 800 nm (N800). Next, the N800 reciprocal was calculated, obtaining the $1/N_{800}$ spectra to approximate the graphs at the absorption spectra. Then, to highlight pigment variation as a consequence of the treatment, the initial spectrum was subtracted from the post-treatment spectrum for each seed. The compositional modifications during ripening were described by subtracting the average T0 seed spectrum of each cultivar to the spectra recorded at T1 and T2 sampling times. Statistical variability of the spectra was evaluated by considering the error bars (95% CI). Statistical analyses were obtained by using Microsoft Office Excel and SPSS statistical software (version PASW Statistics 24, SPSS, Inc. Chicago, IL).

3. Results and discussion

3.1. Natural maturity of seeds in Michigan

Regarding the colorimetric analysis of seeds collected in Michigan, the analysis of variance highlighted significant effects (Significance = 0.000) of the cultivar, sampling time and cultivar * sampling time interaction for all the variables considered (L^*; C^* and h^*). Only the L^* value showed a lower significance (0.004) concerning the cultivar * sampling time interaction. Table 1a reports the seed color variations in terms of average values and individual cultivars. The variation in lightness (L^*) values could be attributed to physical modifications of the seed surface, probably related to mechanical protection and seed dormancy maintenance. Considering the chroma and hue, we observed an increase in the C^* and h^* values in all cultivars (with the exception of h^* in Cabernet Sauvignon). This trend indicates a shift towards intense yellow colors ($h^* \approx 90$ and higher color saturation defined by higher C^* values), and could be ascribed to the oxidized phenolics responsible for the main absorption band (490–510 nm) shown in Fig. 3a and described in the following paragraph (3.3). Nevertheless, natural ripening of grapes of these cultivars in Michigan did not appear to produce highly polymerized reddish-brown pigments in seeds. The lower seasonal temperature accumulation (GDD and NHH) (paragraph 2.1) observed in this growing region compared with Italy is likely to be the cause.

Michigan, a cool climate viticulture region, is characterized by short and variable summers, and rain is often heavy during the final months of maturation. In Michigan, environmental conditions that influence ripening are highly variable from year to year. According to the Winkler GDD scale, Michigan's two primary growing regions, Northwest and Southwest, fall into regions 1a-1b and regions 1a-III. This variability indicates that the capability of grapes to reach full maturity on a consistent basis is restricted in most seasons due to lack of adequate temperature accumulation. For this reason, fruit technological maturity at harvest can be reduced by slow maturation dynamics. A favorable shift in climatic conditions has occurred over the past decades that has led to an increase in vinifera cultivated area, especially in cultivars internationally renowned (e.g. Italy, France, USA) for high quality wine production such as Merlot, Pinot noir and Cabernet Sauvignon. Despite continuous extension of grape vegetative season length due to climate change, seasonal variability is expected to be more stable in the future. This suggests the need for a solution that moderates the impact of low seasonal temperature accumulation, especially for red Vitis vinifera cultivars, that heavily rely on consistent heat units to reach full technological maturity t harvest. Even if several viticultural techniques can be adopted to mitigate the effects of climatic constraints on fruit quality, they have a limited impact on increasing polyphenols concentration of the fruit at harvest (Frioni et al., 2017).

3.2. Natural maturity of seeds in Italy

Variations of seed reflectance spectra in time, were used in the Italian samples to characterize the phenolic oxidations that occurred during fruit ripening (Fig. 3). In Fig. 3a, a spectrum representing the average values of all the considered cultivars at each sampling time can be observed. The band peaking at 678 nm is ascribed to chlorophylls (Merzlyak, Solovchenko, & Gitelson, 2003; Rocchi, Rustioni, & Failla, 2016), which significantly decreased between the first and second sampling, and remained constant until harvest. Fig. 3b indicates that chlorophyll degradation in seeds is not ubiquitous among all the studied cultivars. In Barbera and Sangiovese, no significant differences at 678 nm were recorded. This is most likely due to the low initial concentrations (Fig. SI1 a.4 and a.5). During ripening, the seed color changes, starting with a bright green, through green-yellow to yellow and then through yellow-brown to dark brown colors (Ristic & Iland, 2005). These same pigmentation changes were observed in all our samples. Thus, the degradation of chlorophyll pigments does not appear to play a crucial role in seed coat ripening; despite the quantity of chlorophyll, a brown color was obtained.

In Fig. 3a, the main absorption band (490–510 nm) is responsible for the yellowish-brownish color of the seed coat (absorbance in the green spectral region). This band is similar to the ones in grapevine woody tissues (Grossi, Rustioni, Simone Di Lorenzo, Failla, & Brancadoro, 2016) and sunburned grape berry skins (Rustioni et al., 2014) and it is characteristic of oxidized polymeric phenolics, consistent with the expected physiological oxidations of grape seed coat (Adams, 2006; Kennedy et al., 2000; Pourcel et al., 2007; Ristic & Iland, 2005; Rustioni and Failla, 2016).

This main band underwent both hyperchromic and bathochromic shifts during ripening (Fig. 3a). Furthermore, an increased asymmetry of the main band was observed due to an increased absorbance in the right shoulder ($\approx$570 nm). The hyperchromic shift can easily be explained by the increased concentration of brown pigments due to phenolic oxidations during ripening (Adams, 2006; Kennedy et al., 2000; Pourcel et al., 2007; Ristic & Iland, 2005; Rustioni and Failla, 2016). The bathochromic shift is accentuated in the Fig. 3b representation, in which the differential spectra between T2 and T0 samples in each cultivar is shown. The data indicates the prevalence of accumulation of reddish-brown pigments in all the cultivars during ripening. A broad absorption band in the green region ($\approx$430–660 nm) and a maximum absorption around 570 nm characterizes these colored compounds. Depending on the cultivar considered, this band could appear thinned, however this should be ascribed to the negative contribution of chlorophyll degradation, indicated by the spectral negative bands in the red (peak at 678 and shoulder at 650 nm) and in the blue (around 430 nm) regions. In general, the bathochromic shift observed during ripening (490–510 nm) and the observed intensification of the shoulders at higher wavelengths ($\approx$570 nm) indicate a decrease in the radiative energy required for the electron excitation of brown pigments.

Table 1
Michigan seed color variations (Lightness: L^*; Chroma: C^*; hue: h^*) in different sampling times during natural ripening (1a) and as a consequence of freeze-thaw treatment with respect to the pre-treatment values – control (1b) in terms of average values and of each cultivar.

a

		L^*	Trend	Sig.	C^*	Trend	Sig.	h^*	Trend	Sig.
Average of all the cultivars	1st sample	73.66 ± 4.35	↗	0.000	6.08 ± 2.29	↗	0.000	71.53 ± 12.33	↗	0.000
	2nd sample	77.54 ± 5.21			7.66 ± 1.58			79.21 ± 5.03		
Cabernet Franc	1st sample	75.73 ± 4.09	=	0.139	7.14 ± 2.11	↗	0.093	71.32 ± 5.86	↗	0
	2nd sample	77.09 ± 5.00			7.75 ± 1.41			76.66 ± 4.99		
Cabernet Sauvignon	1st sample	74.44 ± 3.68	↗	0	6.64 ± 1.85	↗	0.017	85.25 ± 8.31	↘	0.005
	2nd sample	79.06 ± 4.94			7.42 ± 1.30			81.44 ± 4.54		
Chambourcin	1st sample	71.02 ± 5.55	↗	0.007	3.33 ± 1.05	↗	0.000	54.35 ± 7.89	↗	0.000
	2nd sample	74.12 ± 5.70			6.22 ± 1.30			80.18 ± 5.29		
Merlot	1st sample	74.81 ± 2.75	↗	0.000	6.13 ± 1.47	↗	0.000	73.61 ± 7.27	↗	0.000
	2nd sample	78.95 ± 3.74			8.43 ± 1.11			80.32 ± 4.43		
Pinot noir	1st sample	72.31 ± 3.49	↗	0	7.17 ± 2.26	↗	0.001	73.10 ± 7.33	↗	0
	2nd sample	78.47 ± 4.97			8.47 ± 1.61			77.43 ± 4.29		

b

			L^*	Trend	Sig.	C^*	Trend	Sig.	h^*	Trend	Sig.
Average of all the cultivars	1st sample	control	73.66 ± 4.35	↘	0.013	6.08 ± 2.29	↘	0.000	71.53 ± 12.33	↘	0.000
		treated	72.67 ± 4.56			5.26 ± 1.74			60.97 ± 8.10		
	2nd sample	control	77.54 ± 5.21	↘	0.000	7.66 ± 1.58	↘	0.000	79.21 ± 5.03	↘	0.000
		treated	74.86 ± 4.59			6.34 ± 1.59			73.67 ± 6.13		
Cabernet Franc	1st sample	control	75.73 ± 4.09	↘	0.030	7.14 ± 2.11	↘	0.013	71.32 ± 5.86	↘	0.000
		treated	73.94 ± 4.02			6.17 ± 1.68			59.44 ± 4.38		
	2nd sample	control	77.09 ± 5.00	↘	0.000	7.75 ± 1.41	↘	0.000	76.66 ± 4.99	↘	0.000
		treated	73.50 ± 3.80			6.62 ± 1.43			69.98 ± 5.68		
Cabernet Sauvignon	1st sample	control	74.44 ± 3.68	↘	0.000	6.64 ± 1.85	↘	0.001	85.25 ± 8.31	↘	0.000
		treated	71.14 ± 3.61			5.52 ± 1.47			70.93 ± 7.57		
	2nd sample	control	79.06 ± 4.94	↘	0.010	7.42 ± 1.30	↘	0.000	81.44 ± 4.54	↘	0.000
		treated	76.51 ± 4.67			6.07 ± 1.40			77.51 ± 4.53		
Chambourcin	1st sample	control	71.02 ± 5.55	=	0.946	3.33 ± 1.05	↗	0.014	54.35 ± 7.89	↗	0.000
		treated	70.96 ± 4.60			3.83 ± 0.93			59.90 ± 7.18		
	2nd sample	control	74.12 ± 5.70	=	0.408	6.22 ± 1.29	↘	0.000	80.18 ± 5.29	↘	0.084
		treated	75.12 ± 6.33			4.93 ± 1.46			78.07 ± 6.76		
Merlot	1st sample	control	74.81 ± 2.75	=	0.974	6.13 ± 1.47	↘	0.005	73.16 ± 7.27	↘	0.000
		treated	74.78 ± 4.36			5.23 ± 1.68			59.80 ± 5.69		
	2nd sample	control	78.95 ± 3.74	↘	0.000	8.43 ± 1.11	↘	0.000	80.32 ± 4.43	↘	0.000
		treated	76.11 ± 3.26			6.54 ± 1.11			73.34 ± 3.71		
Pinot noir	1st sample	control	72.13 ± 3.49	=	0.801	7.17 ± 2.26	↘	0.000	73.10 ± 7.33	↘	0.000
		treated	72.53 ± 4.99			5.54 ± 1.92			54.76 ± 5.25		
	2nd sample	control	78.47 ± 4.97	↘	0.000	8.47 ± 1.61	↘	0.002	77.43 ± 4.29	↘	0.000
		treated	73.03 ± 3.22			7.55 ± 1.35			69.46 ± 3.42		

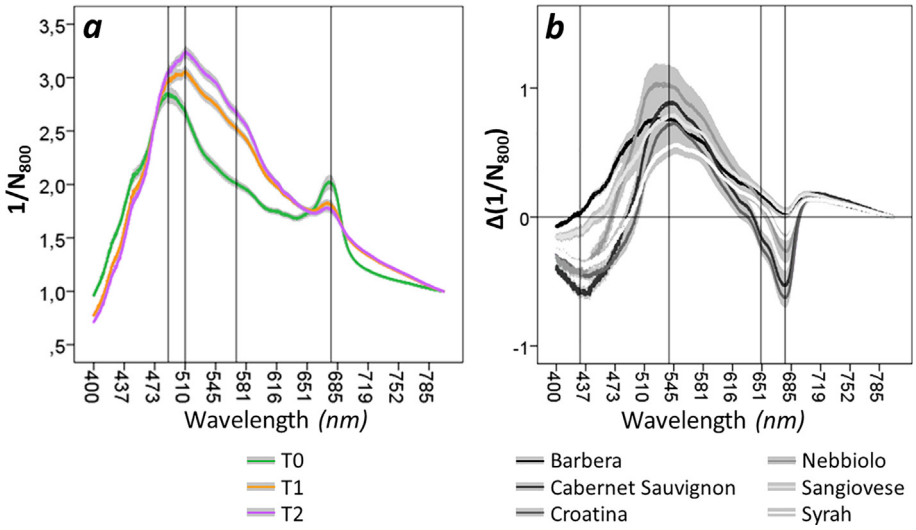

Fig. 3. Variations of reflectance spectra in the Italian seed samples during natural ripening. In Fig. 1a, the spectra are presented as average values of all the cultivars at veraison (T0 – BBCH 83); middle ripening (T1 – BBCH 86); and harvest time (T2 – BBCH 89). Vertical bars are in correspondence of 490 nm; 510 nm; 570 nm and 678 nm. In Fig. 1b, the differential spectra ($\Delta(1/N_{800})$) between T2 and T0 samples of each cultivar are shown. Positive values indicate pigment accumulation, while negative values reveal pigment loss. Vertical bars are in correspondence of 430 nm; 540 nm; 650 nm and 678 nm. The line thickness is representative of the error bars (95% CI).

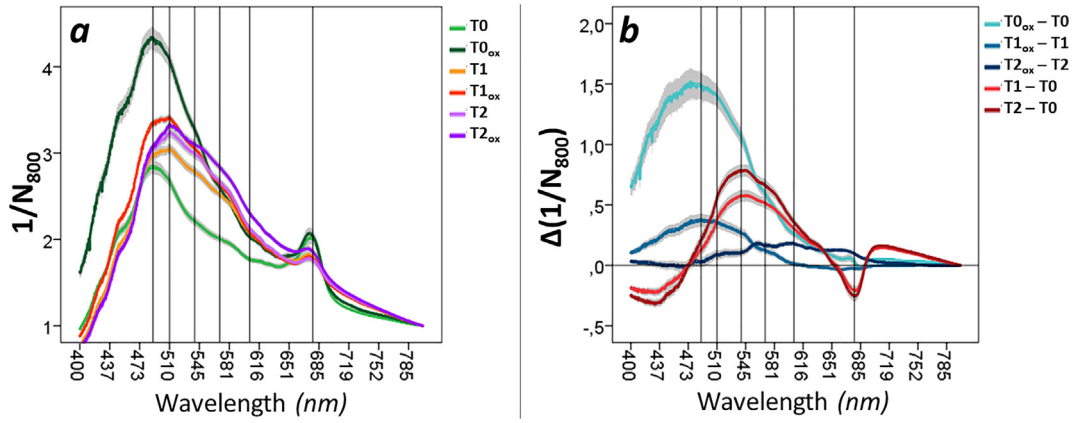

Fig. 4. Variations of reflectance spectra in the Italian seed samples as a result of a freeze-thaw treatment. Data are reported as average values of all the cultivars. Fig. 2a shows the average seed spectra before and after treatment in the three sampling times. In Fig. 2b, the spectral variations are presented as differential spectra ($\Delta(1/N_{800})$), to facilitate the comparison among natural and artificial ripening effects. Vertical bars are in correspondence of 490 nm; 510 nm; 540 nm; 570 nm; 605 nm; 678 nm. The line thickness is representative of the error bars (95% CI).

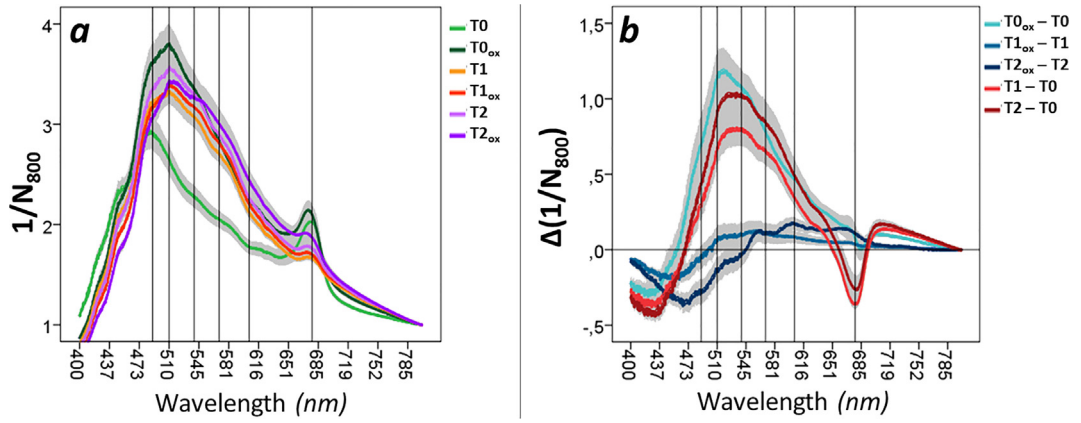

Fig. 5. Variations of reflectance spectra in Nebbiolo seed samples as a result of a freeze-thaw treatment. Fig. 2a shows the average seed spectra before and after treatment in the three sampling times. In Fig. 2b, the spectral variations are presented as differential spectra ($\Delta(1/N_{800})$), to facilitate the comparison among natural and artificial ripening effects. Vertical bars are in correspondence of 490 nm; 510 nm; 540 nm; 570 nm; 605 nm; 678 nm. The line thickness is representative of the error bars (95% CI).

Generally, bathochromic shifts of the absorption bands results from increases in the molecular complexity (e.g.: number of conjugated bonds and substituents) (Cockell & Knowland, 1999; Rustioni, Di Meo, Guillaume, Failla, & Trouillas, 2013). Continuous oxidations take place during the ripening season, and they are consistent with an increase of the number of conjugated rings in brown pigments. In fact, after oxidations, quinones undergo phenolic regenerations and could be easily involved in further polymerizations, as they phenolic oxidation products are more readily oxidized than their precursors (Adams, 2006; Li et al., 2008; Pourcel et al., 2007; Waterhouse & Laurie, 2006).

3.3. Freeze-thaw treatment and artificial seed ripening – Colorimetric characterization

With the aim of improving seed phenolic ripening in suboptimal climatic conditions, the effect of a freeze-thaw treatment on seed color was evaluated in Michigan. The analysis of variance highlighted significant effects (Significance = 0.000) of the treatment, cultivar, sampling time and their interactions for all the variables considered (L^*; C^* and h^*). The L^* value showed a slightly lower significance in the sampling time x treatment and sampling time x treatment x cultivar interactions (significance 0.003 and 0.001 respectively). The C^* value showed a slightly lower significance in the sampling time x treatment; treatment x cultivar and sampling time x treatment x cultivar interactions (significance 0.011; 0.009 and 0.001 respectively).

The treatment effect on the seed color is summarized in Table 1b. The artificial ripening process produced a decrease in lightness (L^*), but not significantly in all the cultivars and sampling times considered. The physical damages of the seed coat caused by the defrosting procedures (Mohr & Stein, 1969) could be responsible of the lightness variations. Considering the chroma and hue (C^* and h^*), we consistently observed a significant decrease in those values due to the treatment, with the exception of the first sampling time of Chambourcin grapes. The values clearly indicate a browning effect of the freeze-thaw treatment, and, thus, the production of reddish-brown pigments. The visual inspection of the seeds was also coherent with the traditionally recognized darkening characteristic of the ripening process (Ferrer-Gallego et al., 2010; Ristic & Iland, 2005; Rodríguez-Pulido et al., 2012; Rustioni & Failla, 2016). Nevertheless, seed color variations obtained with the artificial treatment appeared to be slightly different in relation to the color modifications observed during the natural fruit ripening.

3.4. Freeze-thaw treatment and artificial seed ripening – Spectrophotometric characterization

To understand the freeze-thaw treatment effects on oxidized phenolics, allowing a comparison with the naturally ripen pigmentation, the seed reflectance spectra were studied on the Italian samples. Fig. 4 details the modifications in seed pigmentation obtained by reflectance spectroscopy in the Italian samples. Fig. 4a shows the average seed

spectra before and after treatment in the three sampling times. It is interesting to note that the freeze-thaw procedure generally intensified the absorption bands (hyperchromic shifts), without affecting the spectral profile of each phenological phase. This means that the treatment enhanced the production of the pigments characteristic of each phenological phase, but did not strongly affect their molecular conformation. Observing the differential spectra ($\Delta(1/N_{800})$ – Fig. 4b), it is possible to compare the variations due to ripening (red lines) and treatment (blue lines). As previously described in the 3.2 paragraph, ripening produced a broad absorption band, spread in the entire visible spectrum, with a maximum around 540 nm. The bands obtained by the freeze-thaw treatments did not perfectly overlap with the ones produced by natural ripening. Furthermore, the bathochromic shift of the absorption band ascribable to the treatment can be observed, moving from T0, through T1 to T2 (having peak maximum around 475 nm; 500 nm and 605 nm respectively). These data suggest a major role of phenology-specific enzymatic oxidations. In fact, the cell decompartmentalization caused by the treatment (Mohr and Stein, 1969) would induce phenolic oxidations; nevertheless, the compounds obtained are similar to the ones naturally formed in each phenological stage supporting the hypothesis of enzymatic reactions. In spite of this, the treatment set up in terms of temperatures and timing for each cultivar could address the biochemical oxidation toward the target results. For example, considering Nebbiolo grapes (Fig. 5), the treatment at veraison induced the formation of the same compounds obtained during ripening, independent of the original phenological phase. Further details concerning the cultivar-specific response to the treatment are available in Supplementary information (SI1).

4. Conclusions

Phenolic ripening represents a major interest for quality wine producers. Nevertheless, climatic or genotypical restrictions can prevent the optimal maturation process. Relying on the key role of oxidation for phenolic ripening, a freeze-thaw treatment was proposed to improve the grape quality for potential use in suboptimal growing conditions. The data summarized here highlight pigment formation between natural and artificial ripening systems, however, some differences were evident, most likely due to the degree of oxidative polymerizations. Further studies should focus on the optimization of seed collection, freezing temperatures, and impact on wine sensory analysis, taking into consideration the cultivar-specific responses.

From an application perspective, this technique could be tested at the industrial scale. Currently, a number of techniques are available to separate grape seeds from the must (Canales et al., 2008). Furthermore, the freeze-thaw treatment does not rely on the use of chemical additives, which can be a concern for winemakers. For the freezing process, the use of dry ice – easily available in mostly of the winegrowing regions – could represent the best compromise in terms of costs and industrial management. In a medium sized cellar, seeds could be simply separated by precipitation in a tank or vat, during pump-overs. A freezing treatment of the seeds could be applied directly adding dry ice, allowing the thawing process at room temperature. Nevertheless, additional research could discover a technological solution more suitable for industrial winemaking. Temperature control and timing of the freeze-thaw treatments, could be also of primary importance to address the artificial ripening process to the best cultivar specific treatment conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.03.12.

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