INFLUENCE OF PRE-FERMENTATIVE STEPS ON VARIETAL THIOL PRECURSORS

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Introduction

Varietal thiols are sulfur-containing aromas conferring the typical flavour to several white wines, including Sauvignon blanc, Catarratto Bianco Comune and Grillo. Among them, 4-sulfanyl-4-methyl-2-pentanone, 3-sulfanyl-1-hexanol (3SH) and its acetate ester are considered the most important, and they have a low perception threshold in the order of magnitude of ng/L (Darriet et al., 1995; Tominaga et al., 1998; Roland et al., 2011; Coetzee and Du Toit, 2012). The varietal thiols are present in grape as non-volatile form mainly bound to glutathione (G) or cysteine (Cys), namely S-3-(hexan-1-ol)-L-glutathione (G-3SH), S-3-(hexan-1-ol)-L-cysteine (Cys-3SH), S-4-(4-methyl-pentan-2-one)-L-glutathione and S-4-(4-methyl-pentan-2-one)-L-cysteine. Recently, Thibon and co-authors (2016) identified the S-3-(hexanal)-glutathione (G-3SHal) as an additional precursor of G-3SH. The formation of volatile thiol precursors of 3SH has been associated to either (E)-2-hexenal or (E)-2-hexen-1-ol (Schneider et al. 2006, Harsch et al. 2013). Linolenic acid can release (E)-2-hexenal upon action of a lipoxygenase enzyme in the presence of oxygen; (E)-2-hexenal is then converted to G-3SH by reacting with glutathione (GSH) (Hatanaka et al. 1995, Allen et al. 2011, Harsch et al. 2013). Moreover, (E)-2-hexenal may react with hydrogen sulfide released in the early stages of the fermentation (Schneider et al. 2006, Roland et al. 2010, Pinu et al. 2012). *Saccharomyces* spp. can oxidise (E)-2-hexen-1-ol into (E)-2-hexenal under aerobic conditions, and the reverse process can occur under anaerobic conditions (Harsch et al. 2013).

Varietal thiols are released from their precursors due to the β-lyase activity of the yeasts. Both *Saccharomyces* spp. and non-*Saccharomyces* spp. have shown this ability (Fracassetti & Vigentini, 2018; Ruiz et al., 2019). Besides the yeast used for the alcoholic fermentation, other factors can affect the content of varietal thiols in wine such as the use of exogenous enzymes (Chen et al., 2018). The grape clone plays an important role the content of the thiol precursors in must (Chen et al., 2018). The grape and juice freezing was recently shown to contribute to the increased concentration of both thiol precursors and varietal thiols (Chen, Capone, Nicholson, & Jeffery, 2019). Up to now, major attention has been given to the alcoholic fermentation among the main winemaking step. Only few research studies investigated the role of pre-fermentative operations on the content of thiol precursors in must. Fracassetti, Stuknytė, La Rosa, Gabrielli, De Noni and Tirelli (2018) found the level of thiol precursors decreased as much as 95% in Catarratto Bianco Comune grape and Grillo when clear musts were compared to grape juices. The reasons why the evident loss of thiol precursors occurred in must prepared under industrial conditions are yet to be clarified. A better understanding of these aspects could have an overall impact for the flavour characteristics of the resulting wine. This study aimed to investigate the influence of Grillo grape must industrial extraction steps on the concentration of thiol precursors. Lab-made musts were also produced with the same grape variety from the same batch under different conditions in terms of presence of oxygen, addition of sulfur dioxide and depletion of copper ions. The production of musts under controlled laboratory conditions can allow to elucidate the results arising from the industrial trials and to evidence the factors affecting the concentration of volatile thiol precursors.

Materials and Methods

Grape samples
Grillo grape was collected in 5.5 ha vineyards Sant'Anna at Tenuta Regaleali planted at 520 m of average altitude and the row orientation was south-east at Sclafani Bagni (Sicily, Italy, GPS HESI coordinates: 37.71°N, 13.85°E). Grape sampling and the harvest were carried out in vintage 2018.

Preparation of must in industrial conditions
Grillo grape (6 tons) was hand harvested at ripening in small containers (20 kg) in order to prevent any berry damage. The winemaking was carried out following the procedures usually adopted by the winery. After destemming, grape berries were added with pectolytic enzyme (1 g/100 kg) and softly pressed in a closed-tank membrane press without air removal. The pressing was carried out with three sequential steps at 20, 40 and 60 kPa for 45 minutes each obtaining must yields of about 20%, 40% and 60%. An extra sampling of must was carried out after pressing at 120 kPa for 120 min in which the must yield was about 70%. The must (50 hL) was added with 30 mg/L sulfur dioxide for preventing oxidations, pumped into the clarification tank, cooled down to 7°C and kept for 12 hours settling. The must extraction process was performed under air-exposure (air-exposed musts, AEM) and air-free conditions (air-free must, AFM), the latter obtained by saturation of press, pipes and tank with carbon dioxide. Must samples for thiols precursors analysis were collected at different points of the pre-fermentative steps as schematized in Figure 1. Musts were collected after crushing, at draining, and at must extraction yield of 20%, 40%, 60% and 70% [end of pressing], during transfer in the clarification tank, in the clarification tank and after clarification. Nine must samplings were finally carried out (Figure 1). All these samples were collected in triplicate in 500 mL bottles, filled with nitrogen and readily frozen to -18 °C until analysis.

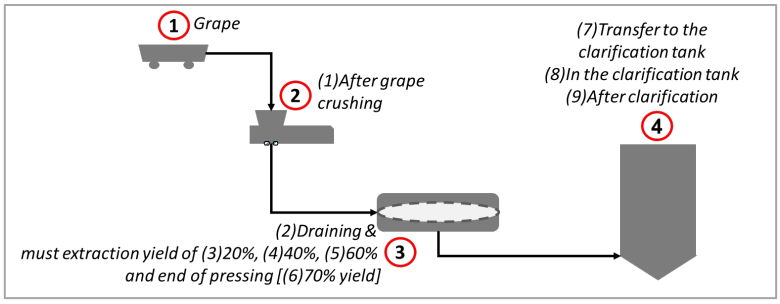

Figure 1: Sampling scheme for musts produced in the industrial plan. Sampling points are in brackets.

Preparation of laboratory-made must
The experimental design for laboratory-made must samples included 4 different conditions: i) addition of sodium fluoride and EDTA (2 mL 0.12 mol/L NaF and 0.5 mL 0.06 mol/L EDTA) (Fracassetti and Tirelli, 2015), ii) washing and gentle drying of berries skins, iii) addition of sulfur dioxide (50 mg/kg) and iv) no addition (control).
About 20 kg of Grillo grape were randomly collected from the harvested bulk and cooled to 4°C overnight prior its use. The berries were taken from the bunches by cutting the pedicle to prevent any berry damage and equally divided in four aliquots. Each aliquot was furtherly divided in 2 aliquots in order to obtain the experimental musts through (i) pressing in air-exposed condition [oxic] and (ii) pressing in air-free condition [anoxic]. The hand-pressing was performed in a beaker in case of the air-exposed condition and in gas-tight plastic bags packed under vacuum in case of air-free condition. For the air-free condition, must samples were transferred into flasks filled with nitrogen and under nitrogen flow. Each condition was carried out in triplicate for a total of 24 laboratory-made must samples produced. All the tubes were filled with nitrogen and immediately frozen at -18°C until analysis.

Determination of cysteine, glutathione and grape reaction product
The concentrations of Cys, GSH and Grape Reaction Product (GRP) in grape, must and wine samples were assessed as described by Fracassetti and Tirelli (2015). Briefly, must samples were centrifuged and 2 mL of supernatant were derivatized with *p*-benzoquinone. The concentrations of Cys, GSH and GRP were assessed by HPLC-UV at 303 nm.

Determination of varietal thiol precursors
Thiol precursors were assessed in must samples, spiked with d_1-G-3SH (500 µg/L) internal standard (IS), after SPE-purification using Strata X-polymeric cartridges (200 mg, Phenomenex, Torrance, CA, USA) (Fracassetti et al., 2018). The detection and quantification of thiol precursors was carried out by UPLC/ESI-HRMS [Acquity UPLC separation module (Waters, Milford, MA, USA) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer through an HESI-II probe for electrospray ionisation (Thermo Scientific, Waltham, MA, USA)] analysis following the separation and detection conditions reported by Fracassetti et al. (2018).

Determination of copper
The quantification of copper was carried out by an UNI CEI EN ISO/IEC 17025-accredited laboratory according to the reference method MA-F-AS322-06-CUIVRE (2010) by atomic absorption spectroscopy (detection limit: 0.05 mg/L).

Statistical analysis
The one-way ANOVA was carried out by means of Statistica 12 software (Statsoft Inc., Tulsa, 269 OK, US). Post-hoc Tukey test was performed and significant differences were judged to using a 5% significance level ($p < 0.05$).

Results and Discussion

Thiol precursors in industrial-scale musts
The content of copper was lower than 1 mg/L in all the must fractions collected during the extraction steps. While comparable levels of copper were observed in both AEM and AFM in destemming (0.87 mg/L), draining (0.57 mg/L) and 60% yield (0.81 mg/L) samples, higher concentrations were detected in 20% yield and 40% yield samples in AFMs (0.76 mg/L and 0.85 mg/L *vs.* 0.61 mg/L and 0.76 mg/L in AEM, respectively for 20% yield and 40% yield samples). This suggests the copper can be extracted faster during the pre-fermentative steps in air-free condition; nevertheless, at the end of pressing, similar concentrations of copper were found in both AEM and AFM conditions (70% yield sample, 0.50 mg/L). The pressing led to a decrease of copper in disagreement with Bekker et al. (2019).
The levels of thiol precursors were assessed in grape and must during the must extraction steps under industrial conditions. Accordingly to Fracassetti et al. (2018), 4MMP precursors, as well as GluCys-3SH and CysGly-3SH, were not detected in the grape juice and must samples. Meanwhile, Cys-3SH, GSH-3SH and G-3SHal were found. The content of G-3SHal in grape (5.21±1.28 mg/L) was higher than G-3SH (178.07±25.14 µg/L) and Cys-3SH (9.43±3.66 µg/L), but it decreased more than 90% after the grape pressing in industrial conditions (Figure 2). Lower loss rates were detected for G-3SH and Cys-3SH throughout the must extraction steps. In most of the samples, when an increase of G-3SH was found, an increase of Cys-3SH was also observed as the must extraction yield increased, especially under air-exposed conditions. Similar findings were described by Roland et al. (2010) who observed an increase in G-3SH in hand-pressed must supplemented with air during pressing. In the must fractions collected during the pre-fermentative steps, the concentration of Cys-3SH increased (Figure 2A). We can suppose the peptolysis of G-3SH occurred in the must as soon as the must extraction began since Cys-3SH was missing in the grape (Figure 1). However, the concentration gradient of thiol precursors detected in the berry cannot fully explain the higher G-3SH and Cys-3SH levels detected in the musts produced under AEM (Figure 2A and

Figure 2B). The G-3SH levels detected in in AEM samples were significantly higher than that found in AFM samples (Figure 2B). A different behaviour was shown by the G-3SHal which concentration dramatically decreased following to the grape crushing and randomly waved among the grape must fractions. Nevertheless, G-3SHal showed higher values in AEM, except in clarification tank sample (Figure 2C). The massive loss of G-3SHal after the grape crushing (-95%) could be due to the addition of metabilsufite carried out at destemming. The supplementation of sulfur dioxide led to lower concentrations of G-3SHal especially in AFM samples (Figure 2 and Table 1), and it might be due to limited oxidative synthetic pathways. These results are in agreement with those reported by Thibon et al. (2016) who highlighted the importance of sulfur dioxide addition into the press. Similarly, considering the data related to "No addition/treatment" laboratory-made must samples (Table 1) under laboratory condition, the role of air exposure resulted even clearer when the G-3SHal is considered, as it accounts up to 1780±1717 µg/L under air exposure condition but it's only 342±206 µmol/L when the air-free condition was applied. This might be related to a major lipoxygenase activity improving the availability of (E)-2-hexenal as well as the resulting formation of G-3SHal by addition of GSH, as previously reported in the literature (Roland et al., 2010; Thibon at al. 2016; Clark & Deed, 2018). This assumption could be supported by the undetectable amounts of free GSH in destemming sample (data not shown) and in "No addition/treatment" laboratory-made must samples (Table 1).

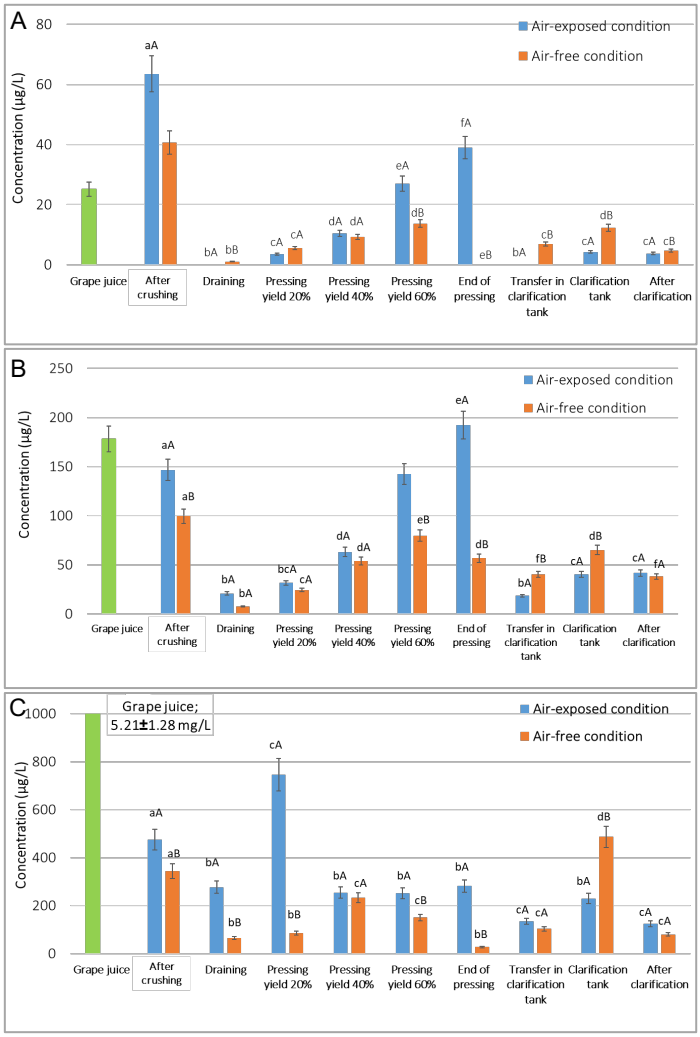

Figure 2: Concentrations (µg/L) of (A) S-3-(hexan-1-ol)-L-cysteine (Cys-3SH), (B) S-3-(hexan-1-ol)-L-glutathione (G-3SH), and (C) S-3-(hexanal)-glutathione (G-3SHal).
Legend: different letters mean significant difference (p<0,05). Capital letters refer to air-exposure vs. air-free conditions; lowercase letters refer to must samples collected in different pre-fermentative steps for the same condition of air-free/air-exposure.

Thiol precursors in lab-made musts

The addition of sulfur dioxide to grape just before the juice extraction in laboratory-made preparation allowed to attain the highest levels of GSH and the lowest levels of GRP in AEM samples. Moreover, the addition of sulfur dioxide hampered the formation of G-3SHal (Table 1) as the hydrogen sulfite addition-product is favoured over the free aldehyde (Clark and Deed, 2018). The addition of sulfur dioxide allowed to detect Cys otherwise present in trace amounts or even missing in all the other pressing conditions considered (Table 1). GRP was found in concentration range 0.38-3.83 mg/L. Since this compound has an oxidative origin, similar results were expected in AFM samples, but instead GRP levels were comparable, or even higher, to those revealed in AEM samples. The loss of GSH is far from being explained by the low GRP levels detected in AEM treatments, but it was quantitatively comparable to the formation of G-3SHal when the grape juice extraction occurred in the absence of sulfur dioxide (Table 1). Therefore, even though the addition of sulfur dioxide prevents the polyphenol-oxidase activity, it can limit the formation of G-3SHal and, consequently, of G-3SH (Thibon et al., 2016).

The presence of copper is another potential cause of G-3SH loss. Like it occurs to other thiol compounds, GSH can readily bind copper to form barely insoluble stabile copper sulfide complexes whose formation is oxygen independent (Ahmed, 2014; Ngamchuea et al., 2016). Under AEM condition the copper-GSH complex could be slowly formed and GSH could be oxidized by the copper to give oxidized glutathione (GSSG) under oxidative condition. In order to assess the role of copper ions on the content of thiol precursors, a laboratory-made must was obtained after the addition of metal ion-chelating compounds (NaF and EDTA) (Janovitz-Klapp et al. 1990; Fracassetti and Tirelli, 2015). The data highlights the remarkable effect of copper ions: the chemical removal of copper allowed the massive formation of G-3SHal, especially under air-exposed condition (14573±881 µg/L) (Table 1). This might be due to the higher amount of (E)-2-hexenal produced (Roland et al., 2010). In the samples where NaF and EDTA were added, the formation of G-3SH was prevented, with the exception of no addition/treatment samples in which G-3SH was comparable to that found in sample added with NaF and EDTA. The formation of G-3SHal in the juice also increased when the copper present in grape skins was removed by a citric acid solution but at far less extent than it occurred with chelating salts (Table 1). However, under the latter condition the highest level of G-3SH, as well as Cys-3SH, was detected meaning that the grape rinsing prevented the outer copper-dependent interferences in the synthesis pathway of thiol precursors. Interestingly, GSH depletion was strongly prevented when sulfur dioxide was added into the juice, thus suggesting the chemical and enzymatic interactions involving GSH were prevented and the formation of a sulfite adduct of GSH can avoid any nucleophilic addition (Jastrembski et al., 2017) (Table 1). In any case, the losses of thiol precursors cannot be explained by their conversion in dipeptidic precursors (Thibon et al., 2018) as GluCys-3SH and CysGly-3SH were not detected.

Juice extraction condition	*Air exposure condition*	*G-3SHAl (µg/L)*	*G-3SH (µg/L)*	*Cys-3SH (µg/L)*	*Cys (mg/L)*	*GSH (mg/L)*	*GRP (mg/L)*
No addition/ treatment	Oxic	1780±1717^{a}	95.0±20.2^{a}	126.6±64.1^{a}	trace	trace	2.23±0.53^{a}
	Anoxic	341.8±206.2^{c}	86.3±0.8^{a}	79.3±10.9^{b}	trace	nd	2.72±1.88^{a}
Addition of sodium fluoride and EDTA	Oxic	14573±881^{b}	110.5±1.4^{a}	94.3±6.8^{ac}	trace	2.56±1.31^{b}	1.86±0.16^{a}
	Anoxic	9456±575^{d}	84.6±28.2^{a}	66.4±0.7^{b}	trace	1.23±0.22^{b}	3.83±0.65^{b}
Rinsing of berry skins	Oxic	339.9±69.1^{c}	163.5±15.8^{b}	101.3±7.7^{a}	nd	nd	3.43±0.82^{a}
	Anoxic	3146±702^{e}	151.0±24.2^{b}	146.1±30.9^{c}	nd	nd	3.75±1.42^{b}
Addition of sulfur dioxide	Oxic	16.19±16.43^{f}	106.9±3.1^{a}	83.1±0.7^{a}	1.04±0.25^{a}	26.35±5.12^{a}	0.50±0.03^{c}
	Anoxic	trace	107.0±9.2^{a}	83.1±3.1^{a}	1.22±0.81^{b}	32.81±3.12^{b}	0.38±0.32^{b}

Table 1: Concentrations of S-3-(hexan-1-ol)-L-cysteine (Cys-3SH), S-3-(hexan-1-ol)-L-glutathione (G-3SH), S-3-(hexanal)-glutathione (G-3SHal), cysteine (Cys), glutathione (GSH) and Grape Reaction Product (GRP) in lab-made musts. Legend: different letters mean significant difference (p<0,05).

Conclusions and future perspectives

This study highlights the importance of the pre-fermentative steps on the content of thiol precursors. In particular, the presence of sulfur dioxide and copper ions limited the formation of G-3SHal available for its reduction to G-3SH. The air-exposed conditions can favour the formation of thiol precursors that might be due to an increased synthesis of (E)-2-hexenal. The oxidative loss can be also excluded. The clarification was responsible for higher decrease in air exposed conditions probably due to the adsorption on the solid parts favoured by the presence of oxygen. At industrial conditions particular attention should be given to the grape pressing for limiting the loss of thiol precursors. The proper management of must contact with air, addition of sulfur dioxide, extraction conditions of juice and contact time between grape juice and solids can allow to preserve major levels thiol precursors and the protect the overall aromatic characteristics of wine.

On-going researches on this topic will be related to the Trebbiano di Lugana grape and the enzymatic activities involved into the thiol precursors formation and degradation.

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