Thiol precursors in Catarratto Bianco Comune and Grillo grapes and effect of clarification conditions on the release of varietal thiols in wine

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Short title: Thiols in Catarratto Bianco Comune and Grillo wines

Abstract

Background and Aims: Varietal thiols characterise the typical aroma of several white wines, such as Sauvignon Blanc. Their presence and sensorial perception were suggested in two Sicilian grape cultivars, Catarratto Bianco Comune (CBC) and Grillo, though it has not been analytically proven.

Methods and Results: Varietal thiol precursors and free varietal thiols were assessed in CBC and Grillo grapes, musts and wines by ultra-performance liquid chromatography/high resolution MS (UPLC/ESI-HRMS). The isobaric compounds S-3-(hexanal)-glutathione (GSH-3MHAl) and S-3-(4-mercapto-4-methylpentan-2-one)-glutathione (GSH-4MMP) were discriminated by comparing their accurate masses and HR-MS/MS spectra with those of their synthetic standards. S-3-(Hexanal)-glutathione, S-3-(hexan-1-ol)-glutathione (GSH-3MH) and S-3-(hexan-1-ol)-cysteine occurred in grape, must and wine, while GSH-4MMP and its hydrolysed forms did not. Their concentration decreased during winemaking, mostly after grape pressing. We compared clarification conditions exposing must to either air or CO_2 on the concentration of thiol precursors and free thiol in wine, however, negligible differences were observed. The concentration of free thiols in the range 400–1100 ng/L were found in the wines and they were unaffected by the two clarification conditions tested.

Conclusion: The isobaric GSH-3MHAl and GSH-4MMP were clearly distinguished for the first time by UPLC-HRMS through their retention times and MS spectra. These varietal thiols were revealed in CBC and Grillo wines for the first time. The air-free and air-exposed clarification treatments had little effect on the concentration of the varietal thiols.

Significance of the Study: This research highlights the major impact of the varietal thiols (mainly 3–mercapto-hexan-1-ol and its acetate form) on the sensory properties of CBC and Grillo wines.

Keywords: Catarratto Bianco Comune cultivar, Grillo cultivar, thiol precursors, UPLC/ESI-HRMS, varietal thiols.

Introduction

Varietal thiols are grape-derived sulfur compounds contributing to the typical flavour of many white wines such as Sauvignon Blanc; and 3-mercaptohexan-1-ol (3MH), 3-mercaptohexylacetate (3MHA), and 4-mercapto-4-methylpentan-2-one (4MMP) are considered as the most important and pleasant among them (Darriet et al. 1995, Tominaga et al. 1998b, 2000). 3-Mercaptohexan-1-ol and 3MHA have an olfactory perception threshold of 60 and 4 ng/L, respectively, and they are responsible for passionfruit-like and grapefruit-like olfactory notes (Tominaga et al. 1998b). These compounds have also been found in red wines (Bouchilloux et al. 1998), 4-Mercapto-4-methylpentan-2-one has an olfactory perception threshold of 0.8 ng/L, and its aroma is described as box tree-like, black currant-like, or even cat urine-like when occurring at high concentration (Tominaga et al. 1998a).

Varietal thiols occur as non-volatile precursors in the grape berry, where they share their sulfur atom with a cysteine residue. 3-Mercaptohexan-1-ol bound as cysteinyl-conjugate (Cys-3MH), a glutathionyl-conjugate (GSH-3MH) and also a cysteinylglycin-conjugate (CysGly-3MH) has been reported (Coetzee and du Toit 2012, Peña-Gallego et al. 2012). Either (*E*)-2-hexenal or (*E*)-2-hexen-1-ol can act as a precursor of 3MH (Schneider et al. 2006, Harsch et al. 2013). (*E*)-2-Hexenal can be released from linolenic acid in the presence of oxygen by a lipoxygenase/lyase sequence, and then converted to GSH-3MH by coupling with glutathione (Hatanaka et al. 1995, Allen et al. 2011, Harsch et al. 2013). Under aerobic conditions, *Saccharomyces* can oxidise (*E*)-2-hexen-1-ol into (*E*)-2-hexenal, and the reverse process can occur under anaerobic conditions (Harsch et al. 2013). (*E*)-2-Hexenal may act as a precursor when hydrogen sulfide is released in the early part of the fermentation (Schneider et al. 2006, Roland et al. 2010, Pinu et al. 2012). 4-Mercapto-4-methylpentan-2-one occurs in grape and must as the cysteine conjugate (Cys-4MMP) and glutathione conjugate (GSH-4MMP) (Fedrizzi et al. 2009, Roland et al. 2010). The free forms of varietal thiols are released along with the alcoholic fermentation by a *S. cerevisiae* lyase

(Tominaga et al. 1998b, Murat et al. 2001). Recently, Thibon and coauthors (2016) identified *S*-3-(hexan-1-al)-glutathione (GSH-3MHAl) in Sauvignon Blanc juice. This compound can be considered as a precursor of thiol aromas.

Varietal thiols have been found in the some Italian grape cultivars, such as Verdicchio Bianco (also known as Trebbiano di Lugana) (Mattivi et al. 2012) and Arneis (Piano et al. 2014), and they were supposed to occur in Grillo wine (Calò et al. 2006) which is used to produce Marsala wine together with the Catarratto Bianco Comune (CBC) cultivar. Both cultivars are important autochthonous grape cultivars of Sicily, the largest and the southern-most Italian wine region accounting for about 17.5% of the overall Italian wine production (De Lorenzis et al. 2014). This makes Sicily the major Italian region for the grape production. Catarratto Bianco Comune is the most widespread grape cultivar in Sicily (Carimi et al. 2010), and the second most important white grape cultivar in Italy (Robinson et al. 2012). The Grillo grape was supposed to be the offspring of CBC and Muscat of Alexandria (Robinson et al. 2012). Both its high vigour and sugar concentration make the Grillo grape more suitable than CBC to produce Marsala wine (Calò et al. 2006). Even though the role of the varietal thiols in the flavour of CBC and Grillo wines has been suggested (Corona 2010), it has not been analytically assessed to date.

This research aimed to characterise Grillo and CBC grapes, musts and wines based on the concentration of the varietal thiols and their precursors. The effect of must exposure to air on the formation of the thiol precursors was also evaluated, since it could increase the level of thiol precursors and, as a consequence, the concentration of varietal thiols in wine (Mattivi et al. 2012).

Materials and methods

Chemicals and reagents

Methanol, ethanol, dichloromethane (DCM), acetonitrile, formic acid, anhydrous tetrahydrofuran, ammonium acetate, sodium fluoride, glutathione (GSH), (*E*)-2-hexenal, 2-hexyn-1-ol, thioacetic acid, manganese(IV) oxide activated, deuterium oxide, pyridine, Vitride (Red-Al® sodium bis(2-methoxyethoxy) aluminum hydride), *p*-benzoquinone (pBQ), 3-mercaptopropanoic acid (3MPA), sodium chloride, sodium borohydride ($NaBH_4$), ethanolamine (EA), *o*-phthaldialdehyde (OPA), anhydrous sodium sulfate, calcium carbonate and boric acid were purchased from Sigma-Aldrich (St Louis, MO, USA); octadecyl-functionalised silica gel 60 RP-18 (40–63 mesh) and sodium hydroxide (NaOH) from Merck Millipore (Darmstadt, Germany); sodium metabisulfite from J.T. Baker (Deventer, The Netherlands); and polyvinylpolypyrrolidone (PVPP) resin from Dal Cin Gildo (Concorezzo, Italy). All chemicals were at least of analytical grade. Water of UPLC-grade was obtained by a Milli-Q system (Merck Millipore, Darmstadt, Germany).

Grape sampling

Berry samples (500 g each) of CBC and Grillo cultivars were collected during the ripening period until the harvest in vintage of 2014. Overall, four samplings of CBC and three of Grillo grapes were made. Berry samples were taken from the 5.5 ha vineyards (GPS coordinates: 37.705927 N, 13.850155 E) by cutting the pedicel to prevent any berry damage. A maximum of three berries per sampled bunch was taken. The sampling position on the bunch (front, back, top and bottom) was varied in and with each bunch sampled in order to collect a representative sample of grape berries. The samples were stored in aluminum containers at -18°C.

Winemaking

Catarratto Bianco Comune grape bunches (81 000 kg) and 52 000 kg of Grillo grape bunches were hand harvested at ripening, and the winemaking followed the procedures usually adopted by the winery for both cultivars. In detail, during destemming and crushing,

10 g of pectolytic enzyme /1000 kg were added to the juices. Respectively,20 000 and 14 000 kg of CBC and Grillo berries, were pressed at 60 kPa in a 120 min cycle in a closed-tank membrane press without air removal. The extracted juice received 40 mg/L SO_2 in the collection vessel and was cooled to 12°C in a heat exchanger. The two juices were directly transferred to stainless steel tanks. For each cultivar, the juice (12 hL from CBC and 80 hL from Grillo grapes) was pumped into two tanks through plastic pipes. One of the tanks was full of air [samples named air-exposed must(AEM)], while the second tank was flushed and filled with carbon dioxide gas [samples named air-free must (AFM)] in order to strip oxygen. Both musts were cooled to 7°C in order to slow microbial growth and then submitted to pump-overs after 12 and 24 h (with air exposure for AEM and under CO_2 for AFM) before undergoing 12 h settling. The CBC musts were inoculated with *Saccharomyces cerevisiae* 20 CRU611 strain. The Grillo musts were inoculated with *S. cerevisiae* 25 NT116 strain. Moreover, 15 g/hL of diammonium phosphate (DAP) and thiamine were added, and the temperature was raised to 10–15 °C. When about one third of the sugar was fermented, the musts were further supplemented with 15 g/hL of DAP and thiamine. The Grillo musts were also supplemented with 20 g/hL of yeast hulls. The density of the alcoholic fermentations was monitored daily with a hydrometer and the temperature corrected. As soon as the alcoholic fermentation was complete, the wines were cooled to 10°C, racked off, supplemented with SO_2 up to 50 mg/L as the free form and bottled. The bottles were stored at 15°C until sensory evaluation. Winemaking for each cultivar was in duplicate.

Must and wine sampling

The must samples were collected from the tank sampling valve into 500 mL plastic bottles after grape pressing, must clarification, yeast inoculation and at the end of fermentation. All these samples were collected in triplicate, filled with N_2 and immediately frozen at -18°C until analysis.

Determination of glutathione and grape reaction product

149 The concentration of GSH and grape reaction product (GRP) in grape, must and wine
150 samples was assessed as described by Fracassetti and Tirelli (2015).
151 *Determination of thiol precursors*
152 **Synthesis of γ-L-glutamyl-S-[(1*R/S*)-1-(2-hydroxyethyl)butyl]-L-cysteinyl-glycine**
153 **(GSH-3MH).** The compound GSH-3MH was prepared according the procedure reported by
154 Grant-Preece et al. (2010) with a slight modification. (*E*)-2-Hexenal (0.16 g, 1.63 mmol) was
155 added to a suspension of GSH (0.5 g, 1.63 mmol) in 50% aqueous acetonitrile (10 mL),
156 following supplementation with pyridine (0.3 g, 3.8 mmol). The clear solution was stirred for
157 64 h at 25°C, diluted with water (10 mL) and washed with DCM (4x8 mL). The aqueous layer
158 was concentrated under reduced pressure to obtain a yellowish solid. A solution of this
159 product in water (10 mL) was cooled to 0°C and treated with $NaBH_4$ (0.1 g, 2.6 mmol). The
160 mixture was stirred at 0°C for 4 h before being quenched with 10% aqueous HCl to pH 3.
161 After evaporation of the solvent the resulting solid was purified by means of a C18 reversed-
162 phase low-pressure column chromatography, (LiChroprep RP18, 40-63 μm, art.13900
163 Merck, 3 g, 4x1.3 cm bed for 0.1 g of product). The column was subsequently eluted with
164 water (20 mL), 1% aqueous ethanol (20 mL), 5% aqueous ethanol (20 mL) and 15%
165 aqueous ethanol (20 mL). Fractions of the last eluent were evaporated under reduced
166 pressure to obtain GSH-3MH as a white solid (35%). It was a mixture of two epimers at $C_{1'}$
167 as evidenced from NMR spectrum; ^1H NMR spectra were recorded with a Varian-Gemini
168 200 MHz spectrometer (Agilent, Santa Clara, CA, USA). Chemical shifts (δ) are given in
169 ppm in relation to tetramethylsilane (TMS). The MS spectra were recorded with a *LCQ*
170 advantage AP electrospray/ion trap equipped instrument (Thermo Fisher Scientific, San
171 Jose, CA, USA) using a syringe pump device to directly inject sample solutions. The
172 structure of GSH-3MH was confirmed by NMR spectrum.
173

^1H *NMR* (δ, D_2O): 0.73, 0.74 (3H, 2t,*J*=7.0 Hz, $H_{4'}$); 1.20-1.80 (6H, m, $H_{2',3',1''}$); 2.00 (2H, q, *J*=7.3 Hz, H_9); 2.38 (2H, 2t, *J*=8.0 Hz, H_8); 2.65-2.89 (2H, m, $H_{1',12a}$); 2.92 (1H, dd, *J*=13.9, 5.1 Hz, H_{12b}); 3.52-3.62 (2H, m, $H_{2''}$); 3.64 (1H, t, *J*=6.2 Hz, H_{10}); 3.79 (2H, s, H_2); 4.39 (1H, m, H_5).

Synthesis of γ-L-glutamyl-S-[(1*R/S*)-1-(2-hydroxyethyl)butyl-1*d*]-L-cysteinyl-glycine (*d*1-GSH-3MH). Labeled compound *d*1-GSH-3MH (Figure 1a), was synthesised using (*E*)-2-hexenal-3-*d* prepared starting from the commercial 2-hexyn-1-ol as reported by Bennani et al. (2009). Glutathione (0.465 g, 1.515 mmol) was treated with (*E*)-2-hexenal-3-*d* (0.15 g, 1.515 mmol) and pyridine (0.285 g, 3.53 mmol) exactly as described above for the synthesis of GSH-3MH. The subsequent reduction with $NaBH_4$ (0.093 g, 2.42 mmol) and purification by means of a C18 reversed-phase low-pressure column chromatography, (LiChroprep RP18, 40-63 μm, art.13900 Merck) resulted in the compound *d*1-GSH-3MH (Figure 1a) as a white solid (32%). It was a mixture of two epimers at $C_{1'}$ as evidenced from the NMR spectrum.

^1H *NMR* (δ, D_2O): 0.73, 0.74 (3H, 2t, *J*=7.0 Hz, $H_{4'}$); 1.20-1.75 (6H, m, $H_{2',3',1''}$); 2.01 (2H, q, *J*=7.0 Hz, H_9); 2.38 (2H, 2t, *J*=8.5 Hz, H_8); 2.71 (1H, dd, *J*=13.7, 8.7 Hz, H_{12a}); 2.92 (1H, dd, *J*=13.7, 5.1 Hz, H_{12b}); 3.52-3.61 (2H, m, $H_{2''}$); 3.66 (1H, t, *J*=6.4 Hz, H_{10}); 3.81 (2H, s, H_2); 4.4 (1H, m, H_5).

Synthesis of γ-L-glutamyl-S-[(1*R/S*)-1-(2-oxoethyl)butyl]-L-cysteinyl-glycine (GSH-3MHAl). Compound GSH-3MHAl was prepared as described above for the GSH-3MH except that the intermediate aldehyde was isolated. (*E*)-2-Hexenal (0.16 g, 1.63 mmol) was added to a suspension of GSH (0.5 g, 1.63 mmol) in 50% aqueous acetonitrile (10 mL), following supplementation with pyridine (0.3 g, 3.8 mmol). The clear solution was stirred for 64 h at 25°C, then diluted with water (10 mL) and washed with DCM (4x8 mL). The aqueous

layer was concentrated under reduced pressure to obtain a yellowish solid (0.66 g) that was purified by means of a C18 reversed-phase low-pressure column chromatography, (LiChroprep RP18, 40-63 μm, art.13900 Merck, 3 g, 4x1.3 cm bed for 0.1 g of product). The column was subsequently eluted with water (20 mL), 5% aqueous ethanol (20 mL) and 15% aqueous ethanol (20 mL). Fractions of the last eluent were evaporated under reduced pressure obtaining GSH-3MHAI as a white solid (40%). The ^{1}H NMR spectrum was in agreement with what previously reported (Thibon et al. 2016).

Synthesis of γ-L-glutamyl-S-(1,1-dimethyl-3-oxobutyl)-L-cysteinyl-glycine (GSH-4MMP). The compound GSH-4MMP was prepared according to the procedure reported by Fedrizzi et al. (2009).

Synthesis of S-[(1*R,S*)-1-(2-hydroxyethyl)butyl]-L-cysteine (Cys-3MH). The compound Cys-3MH was prepared according to the procedure reported by Pardon et al. (2008) and the chloro-hydrate Cys-3MH was obtained.

Sample preparation for thiol precursor analysis

Grape juice, must and wine samples of both cultivars were analysed for thiol precursors. The grape juice was obtained from frozen grape samples, as described by Fracassetti and Tirelli (2015). The must and wine samples were collected directly from the tanks during and after fermentation.

All the samples were purified by solid phase extraction (SPE) as described by Capone et al. (2010) with slight modification. The SPE cartridges (Strata X-polymeric sorbent 200 mg, Phenomenex, Torrance, CA, USA) were activated with 5 mL of methanol and 5 mL of Milli-Q-treated water. The of sample (2 mL) was spiked with *d*1-GSH-3MH (500 μg/L; exact mass $[M + H^{+}]^{+}$: 409.159) internal standard (IS), loaded onto the SPE column and eluted with

4 mL of methanol after a washing step with 2 mL water. The solvent was evaporated under N_2 flow to 400 µL, the sample was analysed by ultra-performance liquid chromatography coupled to electrospray ionisation-high resolution MS (UPLC/ESI-HRMS).

UPLC/ESI-HRMS for the quantification of thiol precursors

The thiol precursors were analysed 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, San Jose, CA, USA)]. The HESI source parameters were optimised using the automated script in the Q Exactive acquisition software. To assess the thiol precursors, 4 µL of SPE-purified wine extracts was separated on an Acquity UPLC BEH C18 column (50×2.1 mm, 1.7 µm, 130 Å) (Waters) kept at 40°C, and using 0.1 mL/100 mL of formic acid (FA) in MilliQ-treated water (solvent A) and 0.1 mL/100 mL FA in acetonitrile (solvent B) as eluting solutions. For the UPLC separation, a linear elution gradient was applied (2 to 50% of solvent B in 5 min) at a flow rate of 0.5 mL/min. The eluate was analysed by MS using a full scan and data dependent tandem MS analysis ($ddMS^2$) of the nine most intense ions (Top9) from the inclusion list. The resolution was set at 70 000 and 17 500 for Full MS and $ddMS^2$ scan types, respectively. The automatic gain control targets were 5×10^5 and 2×10^5, and maximum ion injection times were 100 ms and 60 ms for Full MS and $ddMS^2$ scan types, respectively.

The MS data were processed with the Xcalibur 3.0 software (Thermo Scientific). Peak areas were calculated from extracted ion chromatograms of the thiol precursors with a 3-ppm mass tolerance. For quantification, a deuterated IS was used. Peak area ratios were compared with a five-point standard calibration curves obtained with synthetic thiol precursors.

Determination of varietal thiols

Synthesis of (*R/S*)-3-mercapto-1-hexan-3d-ol (*d*1-3MH). Labeled *d*1-3MH (Figure 1b) was synthesised using (*E*)-2-hexenal-3-*d* prepared starting from the commercial 2-hexyn-1-

ol as reported by Bennani et al. (2009). Thioacetic acid (0.73 mL, 10.2 mmol) was added to a solution of (*E*)-2-hexenal-3-*d* (1.0 g, 10.1 mmol) in DCM (20 mL) under N_2. The mixture was stirred at 25°C for 60 h. The solvent was removed under reduced pressure and the pale yellow liquid obtained was used for the next step without further purification. It was dissolved in methanol (45 mL) and cooled on ice. After a solution of $NaBH_4$ (0.766 g, 10.1 mmol) in water (30 mL) was added dropwise to the stirred solution, the mixture was maintained for 1 h on ice. Subsequently, a solution of sodium hydroxide (0.310 g, 10.1 mmol) in water (12 mL) was added dropwise and the mixture was stirred for 2 h on ice. The pH was adjusted to 2 by adding 2 N sulfuric acid and the solution was extracted with DCM (4x8 mL). The combined extracts were dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. Purification by column chromatography (SiO_2, CH_2Cl_2) resulted in the compound *d*1-3MH (Figure 1b) as a colorless oil (45%). The compound structure was confirmed by NMR spectrum.

^{1}H NMR (δ, *$CDCl_3$*): 0.92 (3H, t, *J*=6.7 Hz, H_6); 1.35-1.75 (5H, m, SH, H_4, H_5); 1.83 (1H, broad s, OH); 1.95 (2H, m, H_2); 3.81 (2H, m, H_1).

Sample preparation for varietal thiol analysis. The varietal thiols were assessed as described by Piano et al. (2015). Each wine sample was spiked with the *d*1-3MH (500 µg/L; exact mass OPA-derivatised *d*1-3MH $[M + H^+]^+$: 295.159) prior to the sample preparation. Thiols were extracted by liquid/liquid extraction with DCM. After the solvent evaporation under vacuum and nitrogen flow, the samples were derivatised with OPA and ethanolamine prior to the chromatographic separation.

Quantification of varietal thiols with UPLC/ESI-HRMS. The varietal thiols were determined with UPLC/ESI-HRMS as follows:, 3 µL of SPE-purified wine extracts (spiked with deuterated IS) were separated on a Kinetex phenyl-hexyl column (150×2.1 mm, 2.6

277 µm, 100 Å) (Phenomenex) kept at 28°C, and using 10 mmol ammonium acetate in MilliQ-
278 treated water (solvent A) and methanol (solvent B) as eluting solvents. For UPLC separation,
279 a linear elution gradient was applied (50 to 100% of solvent B in 14.5 min) at a flow rate of
280 0.25 mL/min. The LC eluate was analysed by MS using a full scan and data dependent
281 tandem MS analysis (ddMS^2) of six of the most intense ions (Top6) from the inclusion list.
282 The MS parameters were as for thiol precursor analysis.
283
284 The MS data were processed using the Xcalibur 3.0 software. Peak areas were
285 calculated from extracted ion chromatograms with a 3 ppm mass tolerance of OPA-
286 derivatised thiols. For quantification, deuterated IS was used. Peak area ratios were
287 compared with six-point standard calibration curves obtained using synthetic OPA-
288 derivatised 3MH and 3-MHA.
289 *Sensory analysis*
290 A panel of 11 expert judges (three females, eight males) was enrolled for the sensory
291 analysis of bottled CBC and Grillo wines. The difference between the AEM wine and AFM
292 wine for CBC and Grillo was evaluated with triangle tests. Each judge had a different
293 randomised order of samples for both tests.
294 *Statistical analysis*
295 STATISTICA software (Statsoft, Tulsa, OK, USA) was used for all statistical analysis. The
296 equations of the calibration curves were assessed by the linear regression analysis.
297 Differences were evaluated by the *t*-test, and significance was set at a value of $P < 0.05$.
298 **Results and discussion**
299 *Identification of thiol precursors*
300 The thiol precursors were identified in samples of SPE-purified CBC and Grillo grape juice,
301 must and wine by UPLC/ESI-HRMS using synthetic standards as reference compounds,
302 including GSH-3MH, Cys-3MH, GSH-4MMP and GSH-3MHAl. Such an analytical approach

for assessing these compounds was essential to properly identify them in grape juice, must and wine samples. These compounds were revealed based on their retention time in UPLC, their exact mass calculated according to the chemical formula, and their MS/MS fragmentation spectrum (Table 1). Furthermore, the MS and MS/MS data were compared to those from the literature in order to confirm the assignment. In detail, two chromatographic peaks were assigned to GSH-3MH (not shown) both of them showing the same MS/MS fragmentation pattern. They corresponded to the (*S*)- and (*R*)-diastereomers of the thiol precursor, as elsewhere reported (Capone et al. 2010, Kobayashi et al. 2010, Roland et al. 2010). Moreover, the analysis of a standard mixture of (*R*)- and (*S*)-diastereomers of GSH-3MH resulted in the same chromatographic and MS/MS behaviour. Accurate mass and MS/MS fragmentation spectrum of GSH-4MMP were the same as those reported by other authors (Fedrizzi et al. 2009, Roland et al. 2010, Larcher et al. 2013). The accurate mass of GSH-3MHAl was in accordance with Thibon et al. (2016). The MS/MS spectra of GSH-4MMP and GSH-3MHAl were similar in terms of accurate mass of the fragments, however, the relative abundance of their fragments showed different patterns (Figure 2). The UPLC/ESI-HRMS of the synthetic GSH-4MMP and GSH-3MHAl permitted discrimination of the two isobaric compounds based on their retention time (Table 1).

Determination of thiol precursors and varietal thiols in grapes, musts and wines

The thiol precursors were quantified in grape juice, must and wine of CBC and Grillo grapes, for the first time, and the varietal thiols were investigated in the corresponding wine samples. Two must clarification treatments based on either exposure of must to air or exclusion of air were evaluated, since oxygen could increase the concentration of thiol precursors and, consequently, the concentration of varietal thiols in wine (Mattivi et al. 2012). Either an air-exposed must (AEM) or a CO_2-must stored in a CO_2-filled tank (AFM) were obtained and vinified. Under such conditions, different exposure to oxygen can be obtained (Ribéreau-

329 Gayon et al. 2006, Concejero et al. 2016). The amount of oxygen is difficult to quantify under
330 commercial winemaking conditions owing to the uneven oxygen concentration inside the air-
331 exposed bulk, as well as to the oxygen consumption due to both the enzymatic and chemical
332 oxidation phenomena occurring during the lengthy clarification process. The main chemical
333 parameters of musts, obtained from both cultivars subjected to the two clarification
334 treatments, showed negligible differences (Table 2). All alcoholic fermentations ran regularly
335 to dryness though the AFM lasted 2 days longer than that of the AEM (data not shown). At
336 the end of the alcoholic fermentation, negligible difference in the composition of the AEM
337 and AFM wines of the two cultivars, except the pH and acidity of Grillo wines (Table 2).
338 Since high amounts of GSH had been reported to give rise to a significant
339 concentration of thiol precursors (Roland et al. 2010), a GSH concentration of at least few
340 tens of mg/L was expected in the grape juice. We detected, however, only trace levels of
341 GSH (<1 mg/L) in all grape juice samples. Such quantities are among the lowest values
342 reported in the literature, since several authors found GSH concentration up to 200 mg/L in
343 grape juice (Cheynier et al. 1989, Okuda and Yokotsuka 1999, Janěs et al. 2010, Fracassetti
344 and Tirelli 2015). It is difficult to ascribe the low GSH concentration to sample storage issues,
345 since GRP concentration values lower than 4 mg/L (i.e. less than 2 mg/L of GSH) were
346 found in the grape juices. A comparable concentration of GSH was detected in CBC and
347 Grillo musts during fermentation, being in the range 8–9 mg/L in the final wines (Table 2).
348 Similar data are reported in the literature (Cassol and Adams 1995, Du Toit et al. 2007,
349 Kritzinger et al. 2013).
350 The 4MMP precursors, as well as GluCys-3MH and CysGly-3MH, were not detected
351 in the grape juice, must and wine samples of either cultivar. Meanwhile, Cys-3MH, GSH-
352 3MH and GSH-3MHAl were identified in grape juice, must and wine samples of both cultivars
353 (Table 3). The concentration of Cys-3MH and GSH-3MH in Grillo grape juice changed little
354 during the monitored 3 weeks of ripening. Conversely, the concentration of the 3MH

precursors in CBC grape juice halved during the last 2 weeks of ripening, and their values were roughly half of the corresponding values found in Grillo grape juices. Ripening behaviour comparable to CBC grape was reported by Peyrot de Gachons et al. (2005) and Roland et al. (2010) for Sauvignon Blanc and Mellon B grape cultivars. The concentration of Cys-3MH and GSH-3MH matched those reported in previous research concerning Sauvignon Blanc and Pinot Gris grapes (Capone et al. 2010). The concentration of GSH-3MHAl decreased over the ripening Grillo grape by 66% , while it greatly increased (more than 12-fold) in the CBC grape. Overall, when the two cultivars were compared at harvest, Grillo grape contained a higher concentration of both GSH-3MH and Cys-3MH, whereas CBC was higher in GSH-3MHAl (Table 3).

The concentration of thiol precursors dramatically decreased in all the musts compared to that of the corresponding grape juices. About 50% of GSH-3MH and up to 98% of GSH-3MHAl were lost following the grape pressing (Figure 3a,b, Table 3). To the best of our knowledge, such a substantial loss of precursors under commercial pressing conditions is evidenced for the first time. Capone et al. (2011) proved CysGly-3MH to be an intermediate of GSH-3MH degradation to Cys-3MH, however, this intermediate was not detected in the CBC and Grillo musts. Therefore, the loss of the glutathionyl- precursors cannot be ascribed to the hydrolysis of the GSH moiety of GSH-3MH. Moreover, the Cys-3MH concentration found in both the ripe grape juices matched the concentration found in the corresponding musts (Figure 3a,b, Table 3). Therefore, the degradation of GSH-3MH leading either to the release of its amino acid units or the oxidative phenomena involving the cysteinyl-3MH moiety can hardly have a major role in the loss of the GSH-3MH, since thiol precursors are stable in oxic conditions owing to their thioether bond (Roland et al. 2010). A similar behaviour was reported for the loss of GSH following grape pressing under several commercial winemaking conditions (Fracassetti and Tirelli 2015). Therefore, factors

affecting the GSH portion of the precursors, other than oxidation or proteolysis, could induce the loss of the thiol precursors.

The substantial loss of GSH-containing precursors that occurred following extraction and clarification of the must did not change the thiol precursor pattern observed in CBC and Grillo grape juices, since the higher concentration of GSH-3MH and Cys-3MH and lower concentration of GSH-3MHAl were still detected in Grillo must compared to that of CBC must, independent of the clarification treatment applied (Figure 3). Negligible differences were also observed between differently clarified Grillo musts, whereas AFM of CBC contained higher concentration of GSH-3MH and GSH-3MHAl compared to that of the corresponding AEM, however, only the concentration of GSH-3MHAl was statistically different. It is difficult to ascribe the observed high concentration of GSH-3MHAl to the applied anoxic conditions. The reducing conditions could hinder the lipoxygenase activity needed to produce additional of 2-hexenal. Moreover, the negligible GSH concentration did not allow the formation of the precursor. More likely, the higher precursor concentration in the grape was retained in the must. The high concentration of GSH-3MHAl in AFM of CBC was expected to increase the formation of a higher concentration of GSH-3MH and then 3MH in wine due to the yeast activity. No significant increase in the concentration, however, of either GSH-3MH or 3MH in wine was detected, despite the GSH-3MHAl lost following vinification (Figures 3a, Figure 4). In all musts, the Cys-3MH concentration was lower than that of GSH-3MH and GSH-3MHAl, which is consistent with other published data (Capone et al. 2010, Thibon et al. 2016).

A high concentration of the thiol precursors still occurred in the wines even if at lower values in comparison to that of the corresponding musts (Figure 3). The alcoholic fermentation, however, poorly affected the concentration of GSH-3MH in CBC wine, likely due to the reduction of GSH-3MHAl by the yeast. An amount of GSH-3MH exceeding 50% was lost in Grillo musts following vinification. In spite of the large number of differences

406 involved in the investigated vinification processes (grape cultivar, harvest date and
407 processing conditions, fermenting yeast strains, thiol precursor concentration in the musts),
408 the CBC and Grillo wines showed a similar concentration of each precursor; in addition a
409 similar concentration of GSH-3MH and GSH-3MHAl was observed in each wine (Figure
410 3a,b). This behaviour is likely due to physical-chemical phenomena affecting solubility
411 and/or adsorption on the lees rather than to microbial or chemical modifications, since the
412 release of aromatic free thiols negligibly affected the loss of precursors. Once again, this
413 suggests that thioether-bonded GSH derivatives can be lost in oenological matrices,
414 although, explicit analytical data concerning their fate are needed to substantiate such an
415 hypothesis.

416 In agreement with the previous research, no clear correlation between precursor
417 concentration in the must and free thiol concentration in the wine was observed (Murat et
418 al. 2001, Peyrot des Gachons et al. 2002, Dubourdieu et al. 2006, Subileau et al. 2008,
419 Grant-Preece et al. 2010, Kobayashi et al. 2010, Roland et al. 2010, Winter et al. 2011,
420 Concejero et al. 2016). Moreover, no significant difference was detected between AEM and
421 AFM samples, though the AEM wines showed a slightly higher concentration of free thiols
422 independent of the grape cultivar.

423 The concentration of 3MH and 3MHA in CBC wine samples was consistently higher
424 than that in Grillo wine (Figure 4) in spite of the lower concentration of GSH-3MH and Cys-
425 3MH occurring in the former. Notably, CBC AEM and AFM contained more GSH-3MHAl (21
426 and 81%, respectively), which might have yielded the release of varietal thiols (Thibon et al.
427 2016). However, different yeast strains were employed, and their major role in yielding
428 different thiol concentration cannot be excluded (Murat et al. 2001). The concentration of
429 3MH and 3MHA in Sauvignon Blanc wines from different countries have been reported to
430 be in the range 688–18 681 ng/L and 10–2507 ng/L, respectively (Lund et al. 2009, Benkwitz
431 et al. 2012, Piano et al. 2015). The concentration of varietal thiols found in CBC and Grillo

wines was in the above-mentioned ranges and greatly exceeded their perception threshold. The calculated olfactory index of 3MH (expressed as a concentration detected to the perception threshold ratio) was 14.4 and 18.8 for AEM and AFM CBC wines, respectively, and 7.6 and 6.3 for AEM and AFM Grillo wines, respectively. The index of 3MHA was 17.2 and 15.5 for AEM and AFM CBC wines, respectively, and 3.8 and 1.6 for AEM and AFM Grillo wines, respectively. Such values highlight the major role of the varietal thiols in affecting the aromatic character of the wines made from CBC and Grillo.

Based on our data, a theoretical conversion yield of precursors into free thiols in the range 1–68% can be calculated (Table 4). However, the thiol formation arising from the Michael addition involving H_2S and 2-hexenal during the fermentation makes such yield values poorly reliable as it occurred in the AEM CBC wine where the calculated 68% yield is hardly plausible. The conversion yield of Cys-3MH into 3MH and 3MHA has been reported to be in the range 0.1–12%, while the conversion yield of GSH-3MH to 3MH and 3MHA is known to be less than 5% (Cotzee and Du Toit 2012, Concejero et al. 2016). Such a difference can be mainly ascribed to the yeast strains, as well as to their different kinetics of H_2S production (Harsch et al. 2013).

Finally, the sensory characteristics of AEM and AFM wines were compared with a triangle sensory test in order to confirm the analytical data for both CBC and Grillo wines. No significant difference was found between the CBC wines since only five out of 11 judges provided the correct response. The same result was obtained when the AEM and AFM Grillo wines were tested. These findings suggest that the winemaking conditions adopted for these two Sicilian grape cultivars did not quantitatively affect the varietal thiols, and they even underline the main role of varietal thiols in the typical flavour of CBC and Grillo wines.

Conclusions

We have for the first time clearly distinguished the isobaric GSH-4MMP and GSH-3MHAl from each other, not only by their retention time in UPLC, but even by means of their HR-

458 MS/MS spectra, and have demonstrated by analytical and sensory approaches the essential
459 role of 3MH and 3MHA in CBC and Grillo grape flavour. Since none of the 4MMP precursors
460 were detected in both must and wine, a major role of this flavor compound in CBC and Grillo
461 wines can be ruled out.
462 The applied vinification conditions (air-free and air-exposed clarification) had little effect on
463 the final aromatic thiol concentration in wine as was observed by Larcher et al. (2014).
464 The substantial loss of glutathionyl precursors that occurred during juice extraction
465 may detrimentally affect the sensory properties of Grillo and CBC wine and should be better
466 investigated.

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645

Figure captions

Figure 1. Synthesis schemes of (a) γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl-*1d*]-L-cysteinyl-glycine (*d*1-GSH-3MH) and (b) (*R*/*S*)-3-mercapto-1-hexan-*3d*-ol (*d*1-3MH).

Figure 2. High resolution-MS/MS spectra of the synthetic standards: (a) (*S*)-3-(4-mercapto-4-methylpentan-2-one)-glutathione (GSH-4MMP) and (b) (*S*)-3-(hexan-1-al)-glutathione (GSH-3MHAl).

Figure 3. Effect of must treatment on the concentration of thiol precursors in must (■,■) and wine (■,■) produced from (a) Catarratto Bianco Comune and (b) Grillo grapes. The must was either air-exposed during clarification (■,■) or air-free during clarification (■,■). Different letters mean significant difference (P<0.05) between the two clarification treatments.

Figure 4. Effect of must treatment on the concentration of volatile thiols in Catarratto Bianco Comune (■,■) and Grillo (■,■) wines produced from air-exposed must during clarification (■,■), and from air-free must during clarification (■,■). Different letters mean significant difference (P<0.05) between the two clarification treatments.

663 **Table 1.** Identification of thiol precursors and *o*-phthaldialdehyde derivative varietal thiols by
664 liquid chromatography coupled to electrospray ionisation-high resolution MS (UPLC/ESI-
665 HRMS).

Compound	Formula	Retention time (min)	Exact mass $[M + H^+]^+$	MS/MS fragments
Cys-3MH	$C_9H_{19}NO_3S$	2.15 (S diastereomer) 2.17 (R diastereomer)	222.116	83.086, 101.096, 205.089, 176.074
CysGly-3MH	$C_{11}H_{22}N_2O_4S$	n.d.	279.137	nd
GluCys-3MH	$C_{14}H_{26}N_2O_6S$	n.d.	351.158	nd.
GSH-3MH	$C_{16}H_{29}N_3O_7S$	2.58 (S diastereomer) 2.62 (R diastereomer)	408.180	83.086, 162.022, 262.111, 333.148
GSH-3MHAl	$C_{16}H_{27}N_3O_7S$	2.60	406.164	162.022, 174.095, 179.048, 259.111, 277.122, 331.132
Cys-4MMP	$C_9H_{17}NO_3S$	n.d.	220.100	nd
CysGly-4MMP	$C_{11}H_{20}N_2O_4S$	n.d.	277.122	nd.
GluCys-4MMP	$C_{14}H_{24}N_2O_6S$	n.d.	349.143	nd.
GSH-4MMP*	$C_{16}H_{27}N_3O_7S$	2.20 (n.d.)	406.164	162.022, 174.095, 179.048, 259.111, 277.122, 331.132
3MH-OPA	$C_{16}H_{23}NO_2S$	7.95	294.151	83.086, 176.052, 194.063
3MHA-OPA	$C_{18}H_{25}NO_3S$	10.15	336.162	83.086, 143.106, 194.063

666 nd, not detected.

667 **Table 2.** Composition of Catarratto Bianco Comune and Grillo musts and wines.

Must	**Clarification treatment**	**Sugars** (g/L)	**Total SO_2** (mg/L)	**pH**	**TA** (g/L tartaric acid)	**Readily assimilable nitrogen** (mg/L)
CBC	AEM	226±2	42±3	3.45±0.03	6.15±0.05	206±8
	AFM	226±2	38±2	3.45±0.03	6.10±0.05	210±8
Grillo	AEM	222±3	36±2	3.25±0.03	6.05±0.05	232±9
	AFM	222±3	36±2	3.25±0.03	6.05±0.05	232±9

Wine	**Clarification treatment**	**Sugars** (g/L)	**Free SO_2** (mg/L)	**Total SO_2** (mg/L)	**pH**	**TA** (g/L tartaric acid)	**Volatile acidity** (g/L acetic acid)	**Ethanol** (% v/v)	**Glutathione** (mg/L)
CBC	AEM	1.1±0.1	56±4	80±6	3.33±0.01	6.35±0.15	0.41±0.02	13.30±0.02	9.38±0.47
	AFM	1.2±0.3	50±2	86±4	3.51±0.01	6.20±0.05	0.42±0.04	13.66±0.06	7.85±0.39
Grillo	AEM	2.9±0.2	53±3	83±3	3.13±0.01	7.28±0.12	0.18±0.01	12.81±0.03	8.04±0.40
	AFM	2.3±0.6	35±2	74±3	3.14±0.03	6.50±0.15	0.13±0.01	12.58±0.33	8.24±0.41

668 AEM, air-exposed must during clarification; AEF, air-free must during clarification.CBC, Catarratto Bianco Comune.

669 **Table 3.** Concentration of thiol precursors in Catarratto Bianco Comune and Grillo grape
670 juices during the ripening in vintage 2014.

Grape	Sampling date	Concentration (µg/L)		
		Cys-3MH	**GSH-3MH**	**GSH-3MHAl**
CBC	12.09	16.0±4.0	445±35	635±51
	20.09	50.8±4.1	475±37	2679±214
	29.09	15.5±3.7	199±16	10672±854
	08.10†	19.0±4.8	216±17	8096±667
Grillo	21.08	25.5±6.6	436±34	7710±632
	03.09	30.8±2.4	479±37	7294±584
	10.09†	27.3±2.2	460±35	2644±217

671 †Harvest date. CBC, Catarratto Bianco Comune. Cys-3MH, S-3-(hexan-1-ol)-cysteine; GSH-3MH, S-3-(hexan-1-ol)-
672 glutathione; GSH-3MHAl, S-3-(hexanal)-glutathione.

673 **Table 4.** Theoretical conversion yield of thiol precursors into free thiols following vinification
674 of Catarratto Bianco Comune and Grillo musts.

Wine	Clarification treatment	Loss of thiol precursors† in vinification (nmol/L)	Free thiols‡ in wine (nmol/L)	Conversion yield (%)
CBC	Air	10.07	6.82	67.73
	CO_2	53.89	6.52	12.10
Grillo	Air	340.03	3.50	1.02
	CO_2	194.54	2.87	1.48

675 †Cys-3MH, S-3-(hexan-1-ol)-cysteine + GSH-3MH, S-3-(hexan-1-ol)-glutathione.‡3MH, 3-mercaptohexan-1-ol +
676 3MHA, 3-mercaptohexylacetate. CBC, Catarratto Bianco Comune.