DOI:10.1021/jf904164t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Evolution of *S*-Cysteinylated and *S*-Glutathionylated Thiol Precursors during Oxidation of Melon B. and Sauvignon blanc Musts

Aurélie Roland,*,† Jérôme Vialaret,§ Alain Razungles,# Peggy Rigou,# and Rémi Schneider⊥

†Interloire, 12 rue Etienne Pallu, B.P. 1921, 37019 Tours Cedex 01, France, §EPFL, Proteomics Core Facility, AI 0151, Station 15, CH-1015 Lausanne, Switzerland, #UMR 1083 Sciences pour l'œnologie, INRA, SupAgro, Université Montpellier I, 34060 Montpellier Cedex 01, France, and ⊥Institut Français de la Vigne et du Vin, at UMR 1083 Sciences pour l'œnologie, INRA, 34060 Montpellier Cedex 01, France

Thiol precursor content in Melon B. and Sauvignon blanc grape juices obtained under vacuum was determined by quantifying cysteinylated and glutathionylated conjugates of 3-mercaptohexan-1-ol (3MH) and 4-methyl-4-mercaptopentan-2-one (4MMP). This characterization allowed the study of thiol precursor evolution during ripening of Sauvignon blanc grapes in several viticultural situations together with grape reaction product (GRP) and the main substrate of polyphenoloxidase, that is, caftaric acid. Concentration of precursors greatly increased during ripening except for the cysteinylated conjugate of 4MMP. Precursor evolution was also monitored during the oxidation of grape juice. Addition of oxygen to a grape juice set off the enzymatic oxidation of hydroxycinnamic acids but did not negatively affect precursor concentrations. Part of the glutathionylated precursor of the 3MH was produced during prefermentative operations (up to 140% in Sauvignon blanc). Consequently, this precursor naturally occurring in grapes was also formed during prefermentative operations. The proportion of biogenetic and prefermentary formation of the glutathionylated precursor of 3MH was different under industrial conditions depending on the grape variety considered. Addition of glutathione and hexenal in grape juices of Melon B. and Sauvignon induced an increase of the production of 3MH and consequently of its acetate in the resulting wines. Residual glutathione in must has to be preserved to enhance the aromatic potential of grapes.

KEYWORDS: Oxygen; glutathione; hexenal; aroma precursors; thiols; Sauvignon blanc; Melon B.

INTRODUCTION

Young wines of Sauvignon blanc and Melon B. exhibit fruity notes when grape harvest is protected against oxidation using carbon dioxide, additions of ascorbic acid or sulfur dioxide, or the application of moderate temperatures. Some varietal thiols are responsible for these organoleptic sensations: 4-methyl-4-mercaptopentan-2-one (4MMP) (*1*), reminiscent of box tree and black currant bud; and 3-mercaptohexan-1-ol (3MH) (*2*) and 3-mercaptohexyl acetate (3MH-A) (*3*), responsible for the fruity and citrus notes of lots of wines. 3MH and 4MMP resulted from odorless precursors in grapes, identified as cysteinylated (*4*) and glutathionylated (*5*, *6*) conjugates. 3MHA came from the acetylation of 3MH by the yeast during the alcoholic fermentation. Both cysteinylated precursors (*4*) and G3MH (*7*, *8*) are cleaved during alcoholic fermentation by the yeast.

Aromatic degradation observed in wines is well documented. During aging, an important decrease of 3MH occurs in wine due to the presence of dissolved oxygen (*9*). 3MH is probably oxidized to its disulfide. Nevertheless, experiments under controlled conditions have shown that the disappearance of 3MH in oxygenated wine is not concomitant with the oxygen consumption but occurred 48 h later (*9*). Consequently, thiols could react with reactive species present in wine such as polyphenols (*10*, *11*) and more specifically with the quinones of catechin and epicatechin. Additional investigations showed that Fe(III) plays a crucial role in the oxidation of polyphenols and, so, on the trapping of thiols in a wine model solution (*12*). On the contrary, specific conditions of aging (total lees) or the presence of chemical molecules (free sulfur dioxide and anthocyanins such as malvidin-3-glucoside (*9*)) enhance the stabilization of thiols in wine.

On the contrary, there is a lack of data in the literature to support the technical observation of a better production of thiols when the must is protected from oxidation as reported above. Oxygen consumption induces a color change of the must that can contribute to intensity of darkness. Indeed, release of *trans*-caftaric acid during crushing of the berries induced the production of quinones, via the enzymatic activity of polyphenol oxidase and in the presence of oxygen. Quinones are chemically unstable and can condense with other phenolic compounds such as flavonoids to form polymerized adducts. According to their

*Address correspondence to this author at INRA-UMR Sciences Pour l'œnologie, 2 place Pierre Viala, F-34060 Montpellier, France (telephone +33 4 99 61 22 28; fax +33 4 99 61 28 57).

 Published on Web 03/03/2010

condensation degree, such polymers give yellow to brown pigments in must (*13*). Quinones can undergo Michael addition of glutathione to form the so-called grape reaction product (GRP) (*14*, *15*). This derivative is not oxidizable by the polyphenol oxidase and does not modify the color of the must. However, it can undergo an additional oxidation under the laccase action (*16*) from *Botrytis cinerea*, on botrytized grapes. The laccase oxidation of GRP gives the corresponding *o*-quinones, which, in turn can proceed to brown polymers. As long as glutathione is available, GRP formation prevents quinones from participating in coupled reactions leading to pigment formation. At this step of winemaking, thiols, present as precursors, are not sensitive to oxidation because the sulfhydryl group is involved in a C−S bond. Thus, to our knowledge, there is no evidence to support an oxidation of thiol precursors consecutive to the enzymatic browning of must.

The aim of our work was first to characterize grape juices of Melon B. and Sauvignon blanc to estimate their aromatic potential. Second, we studied the influence of oxygen consumption by grape juices on aroma precursors and oxidation markers to elucidate the mechanism that could support the improved production of thiols observed in wine when must is prevented from oxidation.

MATERIALS AND METHODS

Chemicals. All solvents were of analytical pure grade (>98%). L-Glutathione reduced was purchased from Duchefa Biochemie (Amsterdam, The Netherlands) and from Sigma-Aldrich (St Quentin en Fallavier, France). *trans*-2-Hexen-1-al, dithiothreitol, cation exchange resin Dowex 50WX4-100, sodium metabisulfite, benzene sulfinic acid, 5-sulfosalicylic acid, and hydrochloric acid were purchased from Sigma-Aldrich. Sodium sulfate was purchased from Merck (Darmstadt, Germany). All gases, nitrogen, isobutane, and helium, were purchased from Air Product (Paris, France). Ammonium dihydrogenphosphate was purchased from Acros Organics (Halluin, France). Sep-Pak C18 cartridges were purchased from Waters (Baden, Switzerland). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). Pentane and dichloromethane were from Riedel de Haen (St Quentin en Fallavier, France). Formic acid and isopropanol were purchased from Fluka (Epalinges, Switzerland, or St Quentin en Fallavier, France), and methanol was purchased from Merck.

Grape Sampling. Ten parcels were selected in three different vineyards of the Val de Loire. Two grape varieties were harvested for comparison: Melon B. and Sauvignon blanc. Harvest was carried out on three different dates, D−7, D, and D+7, where D represents the date of harvest.

Sampling was made by picking one bunch per vine along one row of the parcel. About 30 bunches were collected to constitute one sample. On the sampling dates, Melon B. grapes exhibited levels of sugars ranging from 154 to 184 g/L and total acidity ranging from 7.6 to 5.6 g H_2SO_4/L. Sauvignon blanc grapes exhibited levels of sugars ranging from 184 to 201 g/L and total acidity ranging from 6.8 to 5.7 g H_2SO_4/L.

Elaboration of Grape Juices at Laboratory Scale. To clarify the terms used in this paper, "juices" refer to berries crushed manually in our reactor without fermentation, whereas "musts" were produced from pilot-scale experiments and were fermented (see later).

Grape juices (0.5 L) were prepared by crushing fresh entire berries of Melon B. and Sauvignon blanc (1.5 kg) under vacuum. The system used for grape juice preparation was based on Rigaud et al.'s (*17*) procedure but adapted to our study. Entire berries were manually introduced into a bag-in-box connected to a vacuum pump and sampling bottle. Crushing was started as soon as the system was completely under vacuum to avoid the occurrence of oxidative mechanisms in the bag-in-box. The resulting juice was directly recovered through a perforated pipe in a bottle containing benzene sulfinic acid (1 mg/mL) and sodium metabisulfite (4.5 mg/mL). Samples were then stored at −20 °C until analysis. These triplicate samples were used for the chemical characterization of grapes and to study the influence of ripening on thiol precursors.

Addition of Oxygen, Glutathione, and Hexenal in Grape Juices. Homogeneous samples (3 kg of healthy berries) of Melon B. and Sauvignon blanc were used to produce a larger volume of juice (1.5 L). Two different experimental procedures were used for harvests 2007 and 2008.

In 2007, oxidations of grape juice were performed in a 1.5 L reactor specifically designed to allow the successive additions of oxygen through a septum located at the bottom of the glassware reactor. Dissolution and consumption of oxygen was monitored in real time using a Clark electrode introduced in the bottom of the reactor. A total of 1.7 mg of oxygen per liter of juice was added in 10 successive spikings. When all oxygen was consumed, 150 mL of juice was sampled in a flask containing benzene sulfinic acid (1 mg/mL) and sodium metabisulfite (4.5 mg/mL).

In the experiments set up in 2008, a 1.5 L reactor used to elaborate grape juice was connected to a 0.225 L reactor used to oxidize the juice. As for the 2007 setup of reactors, oxygen additions were performed at the bottom of the 0.225 L reactor via a septum. Oxygen dissolution and consumption were monitored using a chemiluminescence sensor Presens. Five different volumes of oxygen were spiked (300, 600, 1200, 2400, and 5000 μL for 0.225 L of juice), and the total oxygen added ranged from 2 to 32 mg/L of oxygen. Juice was aliquoted (125 mL) at each oxidative phase and transferred in a 150 mL flask containing benzene sulfinic acid (1 mg/mL) and sodium metabisulfite (4.5 mg/mL) when the consumption of oxygen was finished.

Using the second-generation reactor (2008 harvest), an additional experimental set was designed to study the influence of glutathione, hexenal, and both compounds during the oxidation process. Experiments were set up as follows:

(*A*) *Control Experiment*. Each juice was progressively oxidized with the addition of increasing volumes of oxygen as previously described.

(*B*) *Glutathione Addition*. Glutathione was spiked in juices at two levels of concentration: 50 mg/L for Melon B. and 100 mg/L for Sauvignon blanc.

(*C*) *Hexenal Addition*. Hexenal was spiked in juices at 1 mg/L for both grape varieties.

(*D*) *Glutathione and Hexenal Spiking*. Glutathione and hexenal were spiked into juices at levels described in experimental sets B and C.

Sampling was made when the added oxygen was completely consumed by juice (measured by the chemiluminescence sensor Presens). Each experiment was performed in duplicate.

Fermentation Experiments. Industrial grape musts of Melon B. from Nantes (50 L) and Sauvignon blanc from Tours and Sancerre (10 L) were spiked with glutathione (50 mg/L for Melon B. and 100 mg/L for Sauvignon Blanc) and/or hexenal (1 mg/L for both grape varieties). Addition of glutathione was performed at the beginning of crushing, whereas hexenal was added at the end. Sauvignon blanc from Sancerre was inoculated with the yeast strain IOC 18-2007 at 20 g/hL. Fermentation temperature was maintained at 20 °C. Sauvignon blanc from Tours and Melon B. from Nantes were inoculated with the yeast strain Vitilevure KD at 20 and 15 g/hL, respectively, and fermentations were conducted at a temperature ranging between 18 and 20 °C.

Analysis of *trans*-Caftaric Acid and Grape Reaction Product. *trans*-Caftaric acid and GRP were analyzed by HPLC-DAD (Waters Millenium) by adapting a previously published method developed by Cheynier et al. (*15*). Analytes were either isolated from grapes (caftaric acid) or produced in a model solution (GRP) as described in ref *18*.

Juice samples were centrifuged (15000 rpm, 5 min, 4 °C) and then analyzed by HPLC-DAD. Separation was performed on a reversed phase column (dC18 Atlantis, Waters, 4.6 mm × 250 mm × 5 μm) protected with a guard column (Licrospher 100-RP18, Merck, 4 mm × 4 mm × 5 μm). Oven temperature was maintained at 30 °C, and a volume of 10 μL was injected. The mobile phases were composed of water/formic acid (98:2 in %) (A) and acetonitrile/water/formic acid (80:18:2 in %) (B), and the following gradient was used for the separation: A 100 (5 min), A/B = 90:10 (20 min), A/B = 80:20 (15 min), A/B = 75:25 (5 min), A/B = 20:80 (5 min), and B 100 (5 min). The flow rate was maintained at 1 mL/min. Detection was made at 320 nm, and external quantification was performed using Empower software.

Analysis of Glutathione by Amino Acid Analyzer. Juice samples (800 μL) were mixed with an aqueous solution of 5-sulfosalicylic acid at 25% (200 μL) during 1 h at 4 °C to precipitate the proteins. After centrifugation (15 min at 4 °C, 15000 rpm), samples were filtered on a polyvinylidene fluoride membrane (porosity = 125 μm). Sample analysis

Table 1. Glutathione Analysis Conditions

time (min)	temperature (°C)	buffer[a]	pH	flow (mL/h)	ninhydrin
1	55	2	2.80 (0.2 M)	25	on
0	55	2	2.80 (0.2 M)	25	on
1	55	2	2.80 (0.2 M)	25	on
30	55	2	3.00 (0.3 M)	25	on
20	85	2	3.00 (0.3 M)	25	on
10	85	3	3.15 (0.5 M)	25	on
15	85	4	3.15 (0.5 M)	25	on
6	85	6	3.50 (0.3 M)	25	on
6	85	1	3.50 (0.9 M)	25	on
25	55	1	3.50 (0.9 M)	30	off
6	55	1	3.50 (0.9 M)	25	on

[a] Buffers 1−4, lithium citrate at various molarities and pH; buffer 6, lithium hydroxide at 0.3 M.

was performed with an amino acid analyzer (Biochrom 30). Reduced glutathione was separated on Ultrapac Resin 8 (Biochrom) according to a specific pH gradient (**Table 1**), and a postcolumn derivatization with ninhydrin was performed. Detection was made at 570 nm with a UV−visible detector.

Analysis of Aroma Precursors by Nano-LC-MS/MS. 4-*S*-Glutathionyl-4-methylpentan-2-one (G4MMP), 4-*S*-cysteinyl-4-methylpentan-2-one (Cys4MMP), 3-*S*-glutathionylhexan-1-ol (G3MH), and 3-*S*-cysteinylhexan-1-ol (Cys3MH) were quantified by stable isotope dilution assay and external calibration, using a well-optimized method previously published (*19*). Standards were synthesized according to published methods [cysteinylated precursors from Dagan et al. (*20*), G3MH from Roland et al. (*8*), and G4MMP from Fedrizzi et al. (*5*)]. Centrifuged samples (juices or musts, 1200 μL) were spiked with internal standards (labeled compounds), and analytes were selectively extracted on a cation exchange resin Dowex 50WX4-100 (50 mg). The resin was washed with water (1 mL) and elution was made with a phosphate buffer ($NH_4^+H_2PO_4^-$, 1 M, 1 mL). Extracts were then desalted on reverse phase cartridges (Sep-Pak, Waters), and analytes were finally eluted with methanol (600 μL). Final extracts were concentrated to dryness and then diluted in an accurate volume of deionized water (50 μL). Analytes were analyzed by nano liquid chromatography (Waters Acquity) hyphenated with a triple-quadrupole mass spectrometer (Thermo TSQ Vantage Extended Mass Range). Target compounds were trapped on a NanoEase Atlantis dC18 precolumn (Waters, 0.18 mm × 23.5 mm × 5 μm), and separation was performed using a Magic-C18 column (Waters, 75 μm × 100 mm × 5 μm). Flow rate was maintained at 800 nL/min, and mobile phases were composed of water with 0.1% of formic acid (A) and acetonitrile with 0.1% of formic acid (B). The gradient profile started from 0.5% B for 1.5 min, increased to 5.0% B in 0.5 min, increased to 30.0% in 16 min, increased to 40% B in 2 min, and then increased to 85.0% B in 6 min before returning to the initial conditions after 2 min of column rinse. Ionization was performed in positive mode using nano electrospray ion source maintained at a voltage equal to 1.0 kV and a temperature equal to 200 °C. Detection was performed in selected reaction monitoring (SRM) mode.

Analysis of Varietal Thiols by GC-MS/MS. 3-Mercaptohexan-1-ol (3MH) and its acetate (3MH-A) were quantified in pilot-scale wines of Melon B. and Sauvignon blanc according to a published method (*21*, *22*).

Volatile compounds were extracted from wines (250 mL) at 0 °C using pentane/dichloromethane azeotrope (2:1; 100 mL). The organic phase was separated, dried (Na_2SO_4), and concentrated under vacuum to a final volume of 2 mL.

Each extract was then purified using covalent chromatography on mercuric bound agarose gel (1 mL) previously conditioned using isopropanol (5 mL) and pentane/dichloromethane azeotrope (5 mL). After loading and washing using pentane/dichloromethane azeotrope (20 mL), elution was performed with a solution of 1,4-dithio-DL-threitol in pentane/dichloromethane azeotrope (5 mM, 5 mL). Extracts were washed with Millipore water (1 mL), dried (Na_2SO_4), and concentrated at 35 °C (final volume of 300 μL) with subsequent analysis by GC-IT MS/MS (Varian 3800 gas chromatograph coupled to a Varian Saturn 2000 ion trap mass spectrometer). Separation was performed on a DB-WAX column (30 m, 0.25 mm i.d., 0.25 μm thickness film; J&W Scientific). The carrier gas was helium 6.0 with a constant flow of 1 mL/min. Oven temperature was programmed as follows: 60 °C for 3 min, increased to 110 °C at 3 °C/min, then to 140 °C at 1 °C/min and to 245 °C at 15 °C/min for 10 min. The transfer line and the trap were set at 230 and 140 °C, respectively. Detection was performed using chemical ionization with isobutane as reactive gas and MS/MS mode.

Analysis of (*E*)-2-Hexenal and (*E*)-2-Hexenol by GC-MS. (*E*)-2-hexenal and (*E*)-2-hexenol were quantified in juice by stable isotope dilution assay according to a previously published method (*23*) and modified as follows: 2 mL of centrifuged juice (3000 rpm, 15 min, 4 °C) was diluted five times in water (Millipore), and 4 g of NaCl was added. Then, internal standards were added at 50 and 15 μg/L for (*E*)-2-hexenal-d_2 and (*Z*)-2-hexenol-d_2, respectively. Natural compounds were commercially available, whereas the deuterated ones were synthesized (*8*).

Calibrations were performed in a blank juice (a nonoxidized juice prepared under vacuum and enzymatically blocked by sodium metabisulfite and benzene sulfinic acid) by adding known amounts of analytes from stock solutions, previously quantified by GC-AED using hexan-1-ol as internal standard.

Analyses were performed on a gas chromatograph (Hewlett-Packard 6890) hyphenated with a quadrupole mass spectrometer (Hewlett-Packard 5973). Analytes were first trapped on a carboxen/PDMS fiber (Varian, 2 cm) at room temperature during 20 min, and then they were separated on a DB-WAX column (30 m × 0.25 mm × 0.25 μm) after desorption at 280 °C. The carrier gas was helium 6.0 with a constant flow equal to 1.2 mL. Injection was performed in split mode. Oven temperature was programmed as follows: 40 °C for 5 min, increased to 120 °C at 2 °C/min and then to 230 °C at 10 °C/min for 1 min. The transfer line was maintained at 250 °C. Detection was performed using electronic impact in selected ion monitoring (SIM) mode. Three ions per analyte were monitored, one for the quantification (**bold**) and two for the identification: *m*/*z* **98**, 83, 55 for (*E*)-2-hexenal; *m*/*z* **100**, 85, 57 for (*E*)-2-hexenal-d_2; *m*/*z* 100, 82, **57** for (*E*)-2-hexenol; and *m*/*z* 102, 84, **59** for (*Z*)-2-hexenol-d_2.

RESULTS AND DISCUSSION

Validation of the Crushing System under Vacuum. All precautions were taken to avoid the occurrence of oxidation during juice preparation. Grape juices were collected in sampling bottles containing benzene sulfinic acid and sodium metabisulfite at high concentrations. Benzene sulfinic acid allowed the trapping of quinones that could be formed in the system from hydroxycinnamic acids, whereas sodium metabisulfite, at such a high concentration (4.5 mg/mL), inhibited polyphenol oxidase activity.

The airtightness and the absence of oxidation were evaluated a posteriori by quantifying the production of GRP, which represents a good oxidation marker in grape juice. GRP was formed as soon as polyphenol oxidase catalyzed the formation of quinones from caftaric acid and, subsequently, can undergo a Michael addition of glutathione (*14*, *15*). For all samples, the production of GRP was below 1% (corresponding to the amount of converted caftaric acid), indicating sufficient airtightness of the crushing system.

Characterization of Melon B. and Sauvignon blanc in Composition. Grapes of Melon B. and Sauvignon blanc were crushed under vacuum to avoid oxidation prior to quantify their aromatic potential via the analysis of 3MH and 4MMP precursors: G4MMP, Cys4MMP, G3MH, and Cys3MH. Analysis of thiol precursors was performed according to a previously published method (*19*) allowing a good sensitivity (LOQ < 4.8 nmol/L), accuracy (mean value close to 95%), and precision (RSD < 10%).

In parallel, *trans*-caftaric acid, GRP, and glutathione were quantified in the same samples to correlate all data.

Grape Variety Effect. G3MH seemed to be ubiquitous because it occurred in Melon B. and Sauvignon blanc, whereas G4MMP, more specifically, was identified in only Sauvignon blanc. These data were coherent with the more specific presence of 4MMP in Sauvignon blanc and the ubiquity of 3MH in wines (*24*).

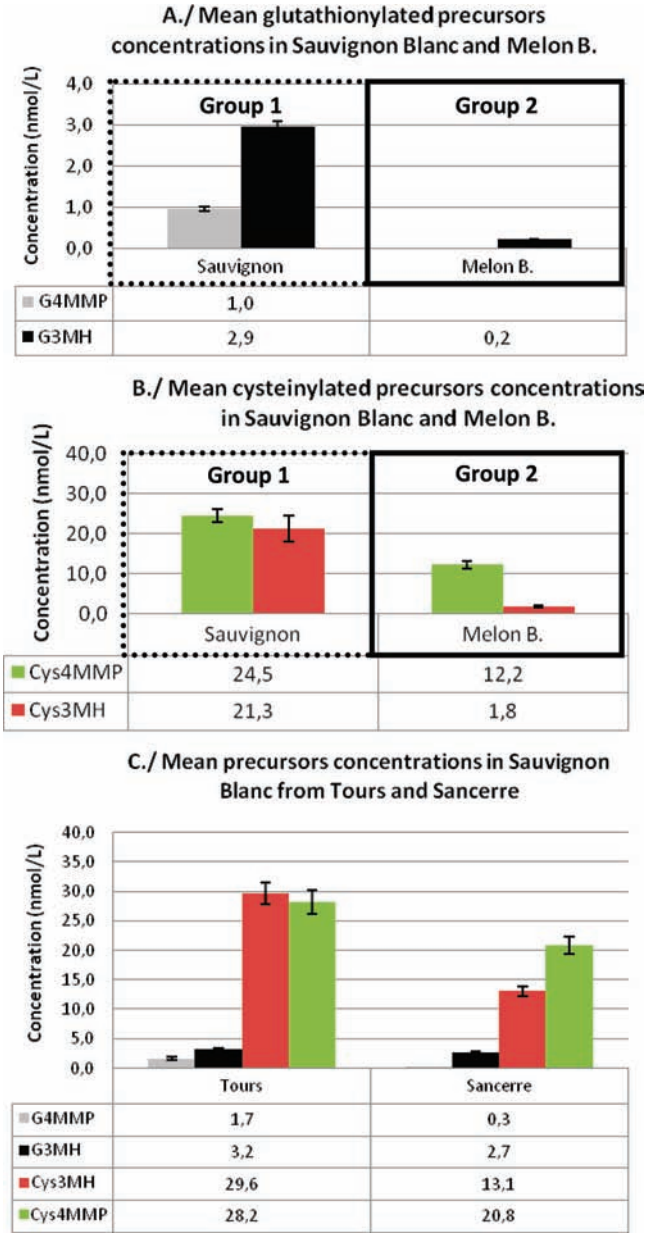

Figure 1. Precursor concentrations (**A**, **B**) and distribution (**C**) in grape juices of Melon B. and Sauvignon blanc (attribution of groups 1 and 2 was made using the Newmann−Keuls test, $\alpha = 0.05$).

Mean G3MH concentrations in grapes were 0.2 and 2.9 nmol/L for Melon B. and Sauvignon blanc, respectively. As observed for the cysteinylated precursors (*25*), G3MH in Sauvignon blanc is 3 times more abundant in grapes than G4MMP and 10 times lower than Cys3MH (**Figure 1**).

Melon B. exhibited cysteinylated and glutathionylated precursor concentrations systematically and significantly lower than Sauvignon blanc (Newmann−Keuls test with $\alpha = 0.05$) (**Figure 1**). A statistical treatment made on Sauvignon blanc samples showed a difference of concentration and distribution for cysteinylated precursors, depending on the grapes' origin (Tours and Sancerre). Indeed, concentrations of Cys3MH and Cys4MMP were similar in Sauvignon from Tours (close to 29 nmol/L), whereas the Cys4MMP amount was twice higher than Cys3MH in Sauvignon from Sancerre (close to 20 and 13 nmol/L, respectively).

Our handmade juices exhibited lower amounts of precursors than industrial ones reported by Capone et al. (*26*). For Cys3MH, this observation could be explained by its preferential location in the grape skin (*27*). Even if our handmade juices contained lower quantities of such compounds, the mechanisms involved during oxidation remained identical whatever the level of precursors occurring in juice.

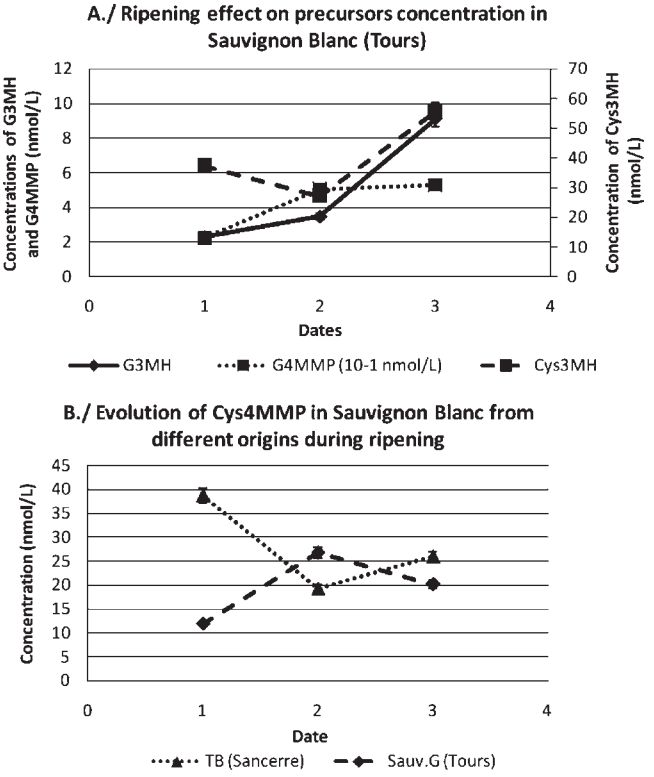

Figure 2. (**A**) Influence of ripening on precursor concentration in grapes of Sauvignon blanc (Tours) and (**B**) evolution of Cys4MMP in Sauvignon blanc from different origins during ripening.

Ripening Effect on Precursor Concentrations. Ripening effect on precursor concentrations is shown in **Figure 2**. In Sauvignon blanc, G3MH and Cys3MH concentrations increased considerably between D−7 and D+7. G4MMP concentration increased slightly between D−7 and D+7 in comparison with G3MH and Cys3MH (**Figure 2**). Cys4MMP evolution was quite different depending on the samples' origin. Indeed, Cys4MMP amount in Sauvignon blanc from Sancerre was at lowest at harvest date, whereas, at the same date, it was highest for Sauvignon blanc from Tours.

In Melon B., no statistically significant variation of Cys3MH, G3MH, and G4MMP concentrations during ripening was observed. However, Cys4MMP concentrations exhibited important variations depending on the parcel considered.

Classification of Grape Variety According to Composition and Ripeness Criteria. A principal component analysis (PCA) was run on precursors, *trans*-caftaric acid, GRP, GSH, and ripeness data (**Figure 3**) using Statgraphics Plus 5.0 software.

In that PCA, caftaric acid, GSH, and 4MMP precursors contribute mainly to the first axis, which explained 57% of the variability, whereas assimilable nitrogen and malic acid are the main contributors of the second axis (17% of variability). In that first plane (axes 1 and 2, explaining 74% of the variability), Melon B. differed from Sauvignon blanc according to the first axis. As reported by Cheynier et al. (*28*), the ratio between glutathione and *trans*-caftaric acid could represent the oxidizability of grape juice. Thus, the distinction between Melon B. and Sauvignon blanc consisted of the higher oxidizability of the Melon B. juice and its lower 4MMP precursor content. Indeed, initial concentrations of

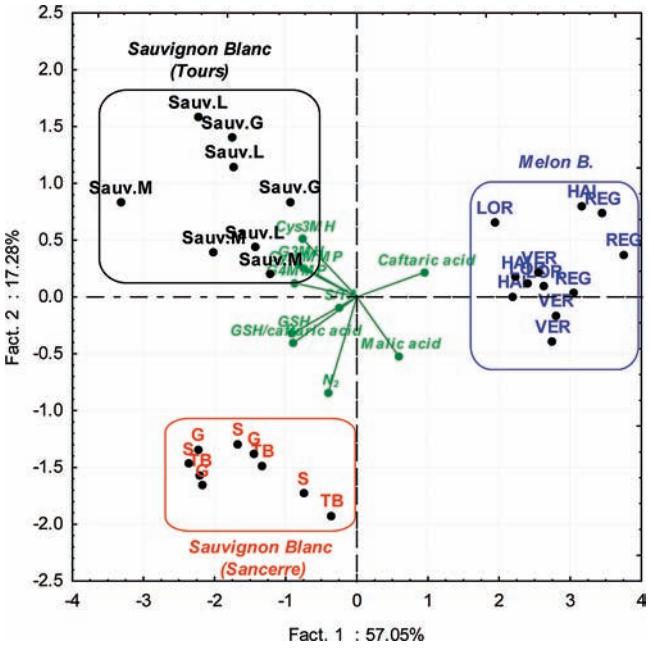

Figure 3. Principal component analysis on Melon B. and Sauvignon blanc composition. Abbreviations correspond to the names of parcels used for sampling. For Melon B. parcels: HAI, La Haie Fouassière; REG, La Regrepierre; LOR, Le Loroux; VER, Vertou. For Sauvignon blanc from Tours: Sauv.L, Seigy; Sauv.G, Meusnes; Sauv.M, Pouillé. For Sauvignon blanc from Sancerre: G, Griotte; S, Silex; TB, Terres Blanches. Each abbreviation appeared in triplicate on PCA, corresponding to the average value of the three sampling dates.

trans-caftaric acid in Melon B. and Sauvignon blanc juices were close to 1.6 and 0.6 mM, respectively, whereas the maximum amount of GRP produced was around 0.2 mM for both grape varieties.

Sauvignon blanc from Tours and Sancerre were separated in two different groups according to the second axis, that is, mainly according to the assimilable nitrogen content of the corresponding juice and, to a minor extent, their malic acid concentrations.

Finally, it could be noted that variables corresponding to thiol precursors (glutathionylated and cysteinylated) were correlated together, which could suggest a common biogenetic origin in grapes. On the contrary, precursors were not correlated with assimilable nitrogen because vector eigenvalues were orthogonal. This observation was not in coherence with the experimental data reported by Choné et al. (*29*).

Influence of Oxygen Consumption on Precursor Concentration. Mechanisms of enzymatic oxidation of polyphenols in must are well-known (*15*, *30*). Significant modification of must's composition occurs during its preparation. Indeed, enzymatic activities such as polyphenol oxidase are able to convert hydroxycinnamic acids and, particularly, *trans*-caftaric acid, the most abundant one, into their corresponding quinones in the presence of oxygen. The quinone of *trans*-caftaric acid can undergo a Michael addition of glutathione to produce the grape reaction product (*14*). No precise data are available on thiol precursor evolution during must oxidation.

The effect of oxygen consumption on thiol precursors was studied at laboratory scale, using a 1.5 L reactor designed for the 2007 harvest experiments. It was equipped at the bottom with a septum for oxygen additions and with a Clark electrode to measure the dissolution and consumption of oxygen. Using this system, the juice was completely oxidized within 3 h at room temperature.

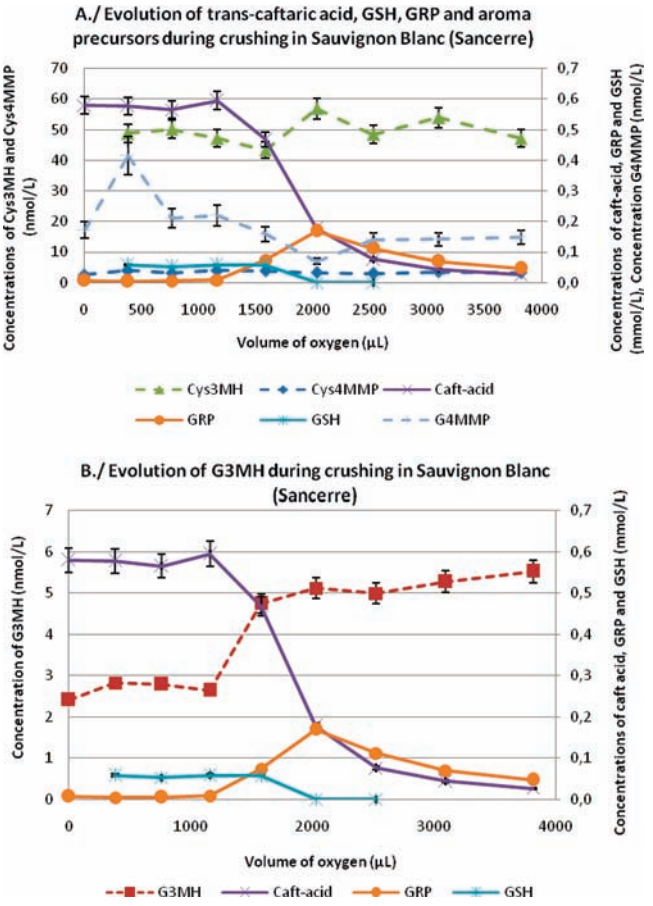

Figure 4. (**A**) Evolution of oxidation markers and aroma precursors and (**B**) production of G3MH under oxidative conditions.

As expected (*28*), these oxidized juice samples showed that reduced glutathione completely disappeared with the concomitant formation of GRP (**Figure 4**). However, cysteinylated precursors and G4MMP were not affected by oxygen consumption in juice because their concentrations did not vary along the oxidation process (**Figure 4**). Indeed, because of their chemical structure, such molecules cannot be oxidized because the thioether bond is stable under these conditions.

It must be noted that, after the addition of 1500 μL of oxygen to the juice (i.e., 2.1 mg/L), the initial concentration of G3MH (close to 2 nmol/L) increased significantly to 5 nmol/L (i.e., 2 μg/L). For example, we observed an important production of G3MH close to 140% in Sauvignon blanc from Sancerre when oxidation occurred during 3 h (5.7 mg/L of O_2 consumed) (**Figure 4**). This production could be responsible for 3MH release close to 60 ng/L if the average conversion yield of G3MH into 3MH is assumed to be equal to 4.4% (*8*).

Global production of G3MH in Melon B. and Sauvignon blanc juices ranged from 67 to 144%, respectively, under laboratory conditions. THe oxidation process lasted 3 h using the first-generation reactor (used for Sauvignon samples) against 1 h with the 2008 one (used for Melon B. sample). The difference in duration and the higher oxidizability of Melon B. could be responsible of the difference in G3MH production observed during the oxidation of Melon B. and Sauvignon blanc juices.

This production of G3MH during prefermentative operations could be due to the addition of residual glutathione on hexenal released from unsaturated fatty acids in the presence of lipoxygenase enzyme. Michael addition is a conjugate addition so that the adduct production curve is expected to be linear. The

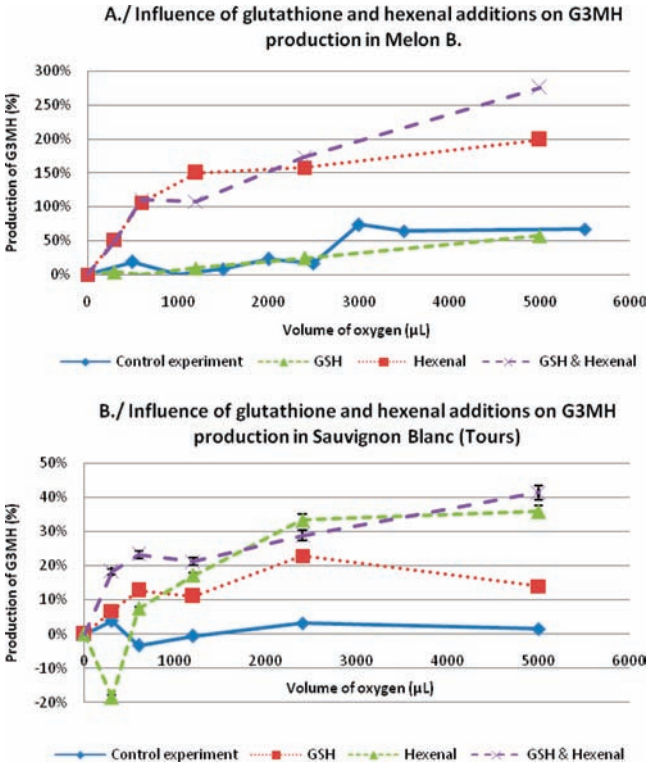

Figure 5. Influence of glutathione and hexenal additions on G3MH production in Melon B. (**A**) and Sauvignon blanc (**B**). Initial concentrations of G3MH were close to 1 and 4 nmol/L for Melon B. and Sauvignon blanc, respectively.

production shape of G3MH, similar to a Michaelis curve (**Figure 4**), could be explained by the enzymatic release of hexenal in juice.

G4MMP could be formed according to the same mechanisms in must by the addition of reduced glutathione on mesityl oxide. Nevertheless, this compound has never been identified in grape juice (*31*), which could explain the absence of G4MMP production under oxidative conditions.

Effect of Glutathione and (*E*)-2-Hexenal Addition in Grape Juices on Precursor Concentration. To investigate the influence of glutathione and hexenal on G3MH production during winemaking, a new reactor was designed for the 2008 harvest. The use of a smaller reactor for oxidation compared to the one used for 2007 harvest allowed a better oxygen dissolution in juice because headspace volume was minimized. Thus, under these conditions, complete oxidation of juice occurred in only 1 h.

The use of two different reactors did not affect the global oxidation mechanism occurring in juice because we adjusted the volume of oxygen added to the volume of juice The monitoring of oxygen consumption was performed using two different means: Clark electrode and chemiluminescence sensor Presens. Even if these two techniques could provide different values, we just measured the absence of oxygen dissolved to be sure that all added oxygen was consumed by the juice. Consequently, our two reactors were convenient to design the evolution of precursors during oxidation.

In Melon B., the initial concentration of G3MH (0.9 nmol/L) increased from 60% in the control experiment to 200% in samples supplemented with hexenal (1 mg/mL) (**Figure 5**). The addition of glutathione did not induce the production of G3MH. These observations demonstrated that hexenal was the limiting reagent to form G3MH under our conditions. To consolidate our statement, we measured the release of hexenal in our handmade juices

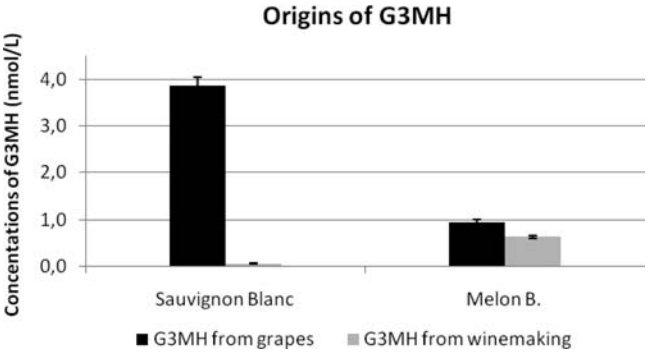

Figure 6. Origins of G3MH in Melon B. and Sauvignon blanc.

in the control experiment, and its concentration at the end of oxidation $< 200\ \mu g/L$.

In Sauvignon blanc, the initial concentration of G3MH (close to 4 nmol/L) increased for juices supplemented with either glutathione (+15%) or hexenal (+35%) in separate additions (**Figure 5**). In that case, hexenal and then glutathione were limiting reagents for G3MH production. Hexenal release was also monitored in the control experiment juice, and a final concentration close to 300 $\mu g/L$ was detected.

The amount of available hexenal seemed to be crucial for G3MH production, and a value around 200 $\mu g/L$ could be the specific threshold for its formation.

The proportions of G3MH originating from grapes and from the winemaking process for Melon B. and Sauvignon blanc were compared. In Melon B., 67% of G3MH came from prefermentative processes, whereas this was only 2% for Sauvignon blanc (**Figure 6**). Therefore, prefermentative operations that are often described as negatives can positively contribute to the production of G3MH and, consequently, to the positive improvement of the aromatic potential.

Effect of Glutathione and Hexenal Additions in Must on Varietal Thiol Production. To emphasize the influence of glutathione and hexenal on wine aroma in our context, experiments at pilot scale were performed that included fermentations and analysis of thiols released in wines, which are considered as key aroma compounds responsible for fresh and citrus fruit notes in many white wines and, especially, Sauvignon ones. Our finished wines were tasted by a trained sensorial panel, and no defect was detected due to the use of GSH or hexenal at such concentrations in must (data not shown).

For Melon B., the addition of glutathione did not change the production of 3MH and 3MHA in the resulting wines (**Figure 7**). Nevertheless, hexenal addition in must resulted in an increase of 3MH and 3MH-A equivalent to 61%. This observation is coherent with the increase of G3MH (+200%) observed under laboratory conditions when hexenal was added to the must (**Figure 5**). This suggests that hexenal is the limiting reagent for the Michael addition of GSH. However, we could not exclude, in those pilot-scale experiments, that 3MH overproduction when hexenal was added is due to the presence of other sulfhydryl donors such as H_2S in fermentation.

In Sauvignon blanc, additions of glutathione or hexenal induced significant increases in the production of 3MH and 3MH-A of 25 and 41%, respectively (**Figure 7**). This result is also in accordance with observations made at laboratory scale on the same grape variety and in the same conditions.

These experiments emphasized the key role of glutathione during prefermentative processes. Residual glutathione in juice could explain technological achievements as antioxidant at the

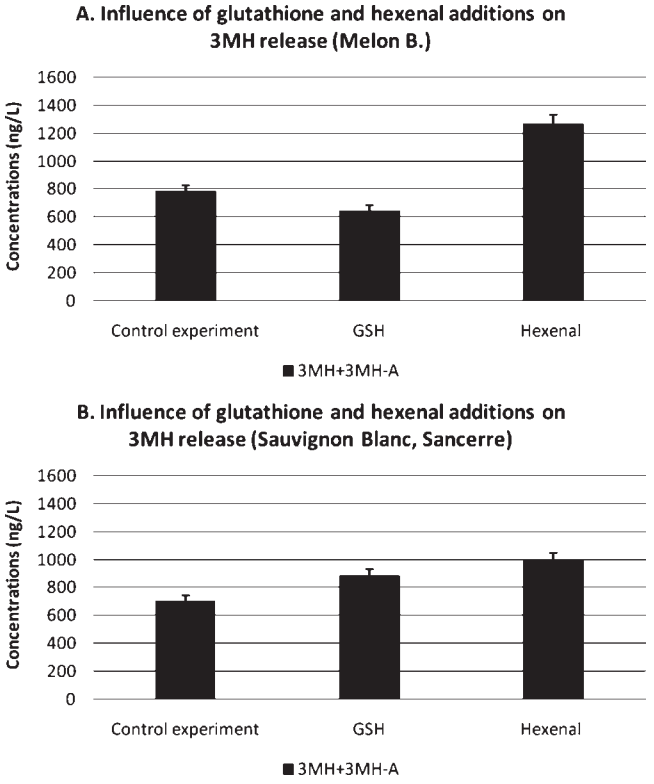

Figure 7. Influence of glutathione and hexenal additions on 3MH release in Melon B. (**A**) and Sauvignon blanc (**B**) wines during alcoholic fermentation.

beginning of alcoholic fermentation by preventing the trapping of thiols by reactive species. Glutathione was also involved in varietal thiol production through the G3MH production. Oxidation compounds such as hexenal are often considered to be off-flavors, but seem to have also a key role in G3MH formation. When present at low but sufficient concentration, they could be involved in positive reactions such as thiol production.

Production of the 3MH in wines seems to have two different origins: the first one from precursors naturally occurring in grapes and the second one linked to the winemaking technology (hexenal and G3MH pathways). The hexenal pathway described by Schneider et al. (*32*) implicated sulfur donors during winemaking, and glutathione seemed to be one of them producing the G3MH precursor.

ABBREVIATIONS USED

G4MMP, 4-*S*-glutathionyl-4-methylpentan-2-one; Cys4MMP, 4-*S*-cysteinyl-4-methylpentan-2-one; G3MH, 3-*S*-glutathionylhexan-1-ol; Cys3MH, 3-*S*-cysteinylhexan-1-ol; GRP, grape reaction product; GSH, glutathione; 3MH, 3-mercaptohexan-1-ol; 3MH-A, 3-mercaptohexyl acetate; 4MMP, 4-methyl-4-mercaptopentan-2-one; SIM, selected ion monitoring.

ACKNOWLEDGMENT

We thank Diego Chiappe and Jean-Paul Lepoutre for technical support in LC-MS/MS and GC-MS/MS devices.

LITERATURE CITED

(1) Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J. N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragrance J.* **1995**, *10*, 385–392.

(2) Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998**, *13* (3), 159–162.

(3) Tominaga, T.; Darriet, P.; Dubourdieu, D. Identification of 3-mercaptohexanol acetate, compound having a powerful odor reminiscent of box-tree, involved in the aroma of Sauvignon wines. *Vitis* **1996**, *35*, 207–210.

(4) Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursor in *Vitis vinifera* L. cv. Sauvignon Blanc: *S*-cysteine conjugates. *J. Agric. Food Chem.* **1998**, *46*, 5215–5219.

(5) Fedrizzi, B.; Pardon, K. H.; Sefton, M. A.; Elsey, G. M.; Jeffery, D. W. First identification of 4-*S*-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon Blanc juice. *J. Agric. Food Chem.* **2009**, *57* (3), 991–995.

(6) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Sulfur aroma precursor present in *S*-glutathione conjugate form: identification of *S*-3-(hexan-1-ol)-glutathione in must from *Vitis vinifera* L. cv. Sauvignon Blanc. *J. Agric. Food Chem.* **2002**, *50*, 4076–4079.

(7) Grant-Preece, P. A.; Pardon, K. H.; Capone, D. L.; Cordente, A. G.; Sefton, M. A.; Jeffery, D. W.; Elsey, G. M. Synthesis of wine thiol conjugates and labeled analogues: fermentation of the glutathione conjugate of 3-mercaptohexan-1-ol yields the corresponding cysteine conjugate and free thiol. *J. Agric. Food Chem.* **2010**, *58*, 1383–1389.

(8) Roland, A.; Schneider, R.; Guernevé, C. L.; Razungles, A.; Cavelier, F. Identification and quantification by LC-MS/MS of a new precursor of 3-mercaptohexan-1-ol (3MH) using stable isotope dilution assay: elements for understanding the 3MH production in wine. *Food Chem.* **2010**, DOI: 10.1016/j.foodchem.2009.12.095.

(9) Darriet, P. Caractérisation des composés volatils associés à la vigne et au vin. Applications technologiques; Université Victor Segalen Bordeauxm **2002**; p 97.

(10) Blanchard, L.; Darriet, P.; Dubourdieu, D. Reactivity of 3-mercaptohexanol in red wine: impact of oxygen, phenolic fractions, and sulfur dioxide. *Am. J. Enol. Vitic.* **2004**, *55* (2), 115–120.

(11) Murat, M. L.; Tominaga, T.; Saucier, C.; Glories, Y.; Dubourdieu, D. Effect of anthocyanins on stability of a key odorous compound, 3-mercaptohexan-1-ol, in Bordeaux rosé wines. *Am. J. Enol. Vitic.* **2003**, *54* (2), 135–138.

(12) Nikolantonaki, M.; Chichuc, I.; Teissedre, P.-L.; Darriet, P. Reactivity of volatile thiols with polyphenols in a wine-model medium: impact of oxygen, iron, and sulfur dioxide. *Anal. Chim. Acta* **2009**, *668* (1−2), 102–109.

(13) Singleton, V. L. Oxygen with phenols and related reactions in musts, wines and model systems: observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38* (1), 69–77.

(14) Singleton, V. L.; Salgues, M.; Zaya, J.; Trousdale, E. Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. *Am. J. Enol. Vitic.* **1985**, *36* (1), 50–56.

(15) Cheynier, V. F.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-*S*-glutathionyl caftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34* (2), 217–221.

(16) Salgues, M.; Cheynier, V.; Gunata, Z.; Wylde, R. Oxidation of grape juice 2-*S*-glutathionyl caffeoyl tartaric acid by *Botrytis cinerea* laccase and characterization of a new substance 2,5-di-*S*-glutathionyl caffeoyl tartaric acid. *J. Food Sci.* **1986**, *51*, 1191–1194.

(17) Rigaud, J.; Moutounet, M.; Cheynier, V. Relation entre la consommation d'oxygène et la composition en derivés hydroxycinnamiques de quatre moûts de raisins blanc. *Sci. Aliment.* **1988**, *8*, 467–477.

(18) Cheynier, V. F.; Van Hulst, M. W. J. Oxidation of trans-caftaric acid and 2-*S*-glutathionylcaftaric acid in model solutions. *J. Agric. Food Chem.* **1988**, *36*, 10–15.

(19) Roland, A.; Vialaret, J.; Moniatte, M.; Rigou, P.; Razungles, A.; Schneider, R. Validation of a nano liquid chromatography-tandem mass spectrometry method for the identification and the accurate quantification by isotopic dilution of glutathionylated and cysteinylated precursors of 3-mercaptohexan-1-ol and 4-mercapto-4-methylpentan-2-one in white grape juices. *J. Chromatogr., A* **2010**, *1217*, 1626–1635.

(20) Dagan, L. Potentiel aromatique des raisins de *Vitis vinifera* L. cv. Petit Manseng et Gros Manseng. Contribution à l'arôme des vins de pays Côtes de Gascogne; Ecole Nationale Supérieure Agronomique de Montpellier, **2006**; p 238.
(21) Kotseridis, Y.; Ray, J. L.; Augier, C.; Baumes, R. Quantitative determination of sulfur containing wine odorants at sub-ppb levels. 1. Synthesis of the deuterated analogues. *J. Agric. Food Chem.* **2000**, *48*, 5819–5823.
(22) Schneider, R.; Kotseridis, Y.; Ray, J. L.; Augier, C.; Baumes, R. Quantitative determination of sulfur-containing wine odorants at sub parts per billion levels. 2. Development and application of a stable isotope dilution assay. *J. Agric. Food Chem.* **2003**, *51*, 3243–3248.
(23) Schneider, R. Contribution à la connaissance de l'arôme et du potentiel aromatique du Melon B. (*Vitis vinifera* L.) et des vins de Muscadet; Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France, **2001**; p 222.
(24) Baumes, R. Wine aroma precursors. In *Wine Chemistry and Biochemistry*; Moreno-Arribas, V., Polo, C., Eds.; Springer: Berlin, Germany, 2009; p 261.
(25) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Measuring the aromatic potential of *Vitis vinifera* L. cv. Sauvignon Blanc grapes by assaying *S*-cysteine conjugates, precursors of the volatile thiols responsible for their varietal aroma. *J. Agric. Food Chem.* **2000**, *48*, 3387–3391.
(26) Capone, D. L.; Sefton, M. A.; Hayasaka, Y.; Jeffery, D. W. Analysis of precursors to wine odorant 3-mercaptohexan-1-ol using HPLC-MS/MS: resolution and quantitation of diastereomers of 3-*S*-cysteinylhexan-1-ol and 3-*S*-glutathionylhexan-1-ol. *J. Agric. Food Chem.* **2010**, *58*, 1390–1395.
(27) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Localisation of *S*-cysteine conjugates in the berry: effect of skin contact on aromatic potential of *Vitis vinifera* L. cv. Sauvignon Blanc must. *Am. J. Enol. Vitic.* **2002**, *53* (2), 144–146.
(28) Cheynier, V.; Souquet, J. M.; Moutounet, M. Glutathione content and glutathione to hydroxycinnamic acid ratio in *Vitis vinifera* grapes and musts. *Am. J. Enol. Vitic.* **1989**, *40* (4), 320–324.
(29) Choné, X.; Lavigne-Cruège, V.; Tominaga, T.; Leeuwen, C. V.; Castagnède, C.; Saucier, C.; Dubourdieu, D. Effect of vine nitrogen status on grape aromatic potential: flavor precursors (*S*-cysteine conjugates), glutathione and phenolic content in *Vitis Vinifera* L. cv. Sauvignon Blanc grape juice. *J. Int. Sci. Vigne Vin* **2006**, *40* (1), 1–6.
(30) Cheynier, V.; Rigaud, J.; Souquet, J. M.; Duprat, F.; Moutounet, M. Must browning in relation to the behavior of phenolic compounds during oxidation. *Am. J. Enol. Vitic.* **1990**, *41* (4), 346–349.
(31) *TNO, Volatile Compounds in Foods. Qualitative and Quantitative Data*; Nutrition and Food Research Institute: Zeist, The Netherlands, 1996.
(32) Schneider, R.; Charrier, F.; Razungles, A.; Baumes, R. Evidence for an alternative biogenetic pathway leading to 3-mercaptohexanol and 4-mercapto-4-methylpentan-2-one in wines. *Anal. Chim. Acta* **2006**, *563* (1−2), 58–64.

Received for review November 26, 2009. Revised manuscript received February 19, 2010. Accepted February 20, 2010.