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New Precursor of 3-Mercaptohexan-1-ol in Grape Juice: Thiol-Forming Potential and Kinetics during Early Stages of Must Fermentation

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Supporting Information

ABSTRACT: Two volatile thiols, 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), are key aroma impact compounds in many young white wines, especially of the variety Sauvignon blanc (SB). Although great effort has been invested to identify their precursors in recent years, the origin of the majority of 3MH and 3MHA generated during wine fermentation still cannot be explained. Here we demonstrate that supplying an external source of hydrogen sulfide to grape juice hugely increases its thiol-forming potential. We further describe the discovery of (*E*)-2-hexen-1-ol as an additional new thiol precursor and demonstrate that it possesses, together with (*E*)-2-hexenal, an immense thiol-forming potential during fermentation. Both C6-compounds are extremely rapidly metabolized by yeast during the first hours after inoculation, even under commercial conditions, and can be interconverted during this phase depending on their initial concentration in the grape juice. Spiking grape juice with additional acetaldehyde greatly enhanced the (*E*)-2-hexen-1-ol to (*E*)-2-hexenal conversion rate. Delaying the metabolization of the two unsaturated C6-thiol precursors by yeast, at the same time as increasing hydrogen sulfide production early in fermentation, opens up a great opportunity to tap into this enormous potential 3MH and 3MHA source in grape juice and extends the possibility of thiol production to other non-grape-based alcoholic beverages as well.

KEYWORDS: *C6, (E)-2-hexenal, (E)-2-hexen-1-ol, thiol precursor, 3-mercaptohexan-1-ol, 3MH, wine, grape, Sauvignon blanc, HS-SPME, GC-MS, kinetics*

INTRODUCTION

The volatile thiols 3-mercaptohexan-1-ol (3MH) and its acetate ester 3-mercaptohexyl acetate (3MHA) are important contributors to the aroma of young wines made from many grape varieties. Their influence is due to their associated pleasant fruity to tropical scents combined with their very low perception thresholds even in very complex wine matrixes.[1] Depending on these complexities and their individual concentrations, 3MH and 3MHA become the major flavor impact compounds in some varieties, as in wines made from Sauvignon blanc (SB) grapes especially from New Zealand.[2,3] Although 3MH has been recently detected in very low quantities in Australian SB grape juice for the first time,[4] 3MH and consequently 3MHA are normally not measurable in noninoculated commercially processed grape juice with subdued microbial activity. These two volatile thiols are synthesized by yeast during fermentation, reportedly through cleavage of mainly odorless precursors, such as conjugates with cysteine, glutathione, and intermediates thereof (see reviews in refs 5 and 6 and references therein). Subsequent yeast-driven acetylation of 3MH yields 3MHA, which normally does not exceed the concentration of its substrate in the final wine.[7] The odoriferous unsaturated carbonyl compound (*E*)-2-hexenal can contribute to the formation of 3MH during grape must fermentation by enzymatic or chemical conjugation with glutathione in the grape juice followed by a reduction and breakdown of the formed glutathionylated precursor by yeast.[8−10] More directly, it has been hypothesized that a 1,4-addition of a sulfhydryl moiety (e.g., from hydrogen sulfide or cysteine) to the β-carbon of the α,β-unsaturated (*E*)-2-hexenal and subsequent reduction or conversion of the intermediates by yeast can also give rise to 3MH.[11,12] Despite an immense influx of data in recent years leading to the identification of this multitude of thiol precursors (see reviews 5, 6, and 13), the concentration of cysteinylated and glutathionylated precursors in juice does not correlate with final thiol yields in wine,[14] and to date, only 10−15% of the total 3MH and 3MHA (3MH/A) synthesized during fermentation in wine can be attributed to originate from these precursors.[11,15,16] This knowledge gap begs to be narrowed, in order to be able to provide viticulturists and wine makers with the tools needed to predict volatile thiol potential of grapes in the vineyards and subsequently to control thiol formation at will during vinification in the winery.

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Focusing on the second objective, we took a new look at the thiol-forming potential of unsaturated C6-compounds in grape juice and investigated their kinetics during the first hours after yeast inoculation.

Six-carbon (C6) alcohols and aldehydes in plants are mainly generated through the enzymatic breakdown of C18 poly-unsaturated fatty acids contained in plant membranes.[17] The initial catabolic enzymes responsible for these reactions belong to the lipoxygenase (LOX) family, which can oxidize the carbon double bonds of linolenic and linoleic acids, the major polyunsaturated fatty acids in plant tissues, at the ninth and 13th position. The resulting oxygenated fatty acids, so-called oxylipins, are finally cleaved by different hydroperoxide lyases (HPL) into C6-compounds (i.e., (*Z*)-3-hexenal and hexanal) and various saturated and unsaturated C9- and C12-compounds.[18] The first C6-compound synthesized from linolenic acid by the LOX/HPL pathway is (*Z*)-3-hexenal, which can be either isomerized into (*E*)-2-hexenal, or, like (*E*)-2-hexenal and hexanal, further reduced into its corresponding alcohol by enzymes of the alcohol dehydrogenase family.[19] Although the LOX/HPL pathway is active in intact plant cell tissues such as grape berries,[20] the majority of these C6-compounds are rapidly formed upon damaging of plant cells, such as crushing of grapes, when oxygen is present.[17] Evolutionarily speaking this makes sense, since some C6-compounds were attributed to have bactericidal and fungicidal functions.[21,22] Additionally, some exhibit pheromone-like properties involved in plant-to-plant communication,[23] are able to repel herbivores, and can attract their carnivore antagonists (ref 24 and references therein).

In contrast to the nonvolatile thiol precursors, (*E*)-2-hexenal has a green cut-grass-like aroma[25] and is among the major contributors of the characteristic odor of freshly damaged green leaves, like that of tea leaves from which it was first isolated in 1912.[19] This green aroma is common to all C6-alcohols and -aldehydes derived from the LOX/HPL pathway and is the reason that they are sometimes referred to as green leaf volatiles (GLVs). GLVs often impair the aroma of wine, especially when under-ripe grapes are vinified.[26] However, in young white wines they can positively contribute to the perceived freshness.[27]

Although much research has focused on the synthesis and intricate kinetics of GLVs in intact and damaged plant cell tissues as well as on their development during ripening of different fruits, to the best of our knowledge only three studies have dealt with the fate of these compounds in the presence of yeast. Joslin and Ough[28] performed such experiments under aerobic conditions in buffer solution individually spiked with GLVs at −1.1 °C, whereas Herriaz et al.[29] and Dennis et al.[30] conducted experiments with more practical relevance in synthetic and natural grape must at laboratory scale. All three studies were either too far removed from conditions encountered during winemaking,[28] did not sample frequently enough, especially during the first two days of fermentation,[29,30] or failed to monitor the evolution of the important (*E*)-2-hexenal in their time course experiments completely.[29] Since 3MH is one of the most important aroma compounds in wine science of recent years and (*E*)-2-hexenal can act as its direct (reaction with H_2S) and indirect (via glutathione and cysteine) precursor, we investigated the thiol-forming potential of this carbonyl compound and its corresponding alcohol (*E*)-2-hexen-1-ol under laboratory conditions and took a new look at the kinetics of all GLVs present in grape must in the very early period of fermentation straight after yeast inoculation.

MATERIALS AND METHODS

Chemicals. The thiol calibration standards 3MH and 3MHA were purchased from Acros Organics (Geel, Belgium) and from Oxford Chemicals (Hartlepool, UK), respectively. GLVs hexanal (98%), (*E*)-2-hexen-1-al (98%), hexan-1-ol (≥99%), (*E*)-3-hexen-1-ol (97%), (*Z*)-3-hexen-1-ol (≥98%), and (*E*)-2-hexen-1-ol (≥96%) were all supplied by Sigma Aldrich (St. Louis, MO, USA). Deuterated internal standards 3-mercapto-1-$^2H_2$-hexan-1-ol (3MH-d2) and 3-mercapto-1-$^2H_2$-hexyl acetate (3MHA-d2) were synthesized at the University of Auckland,[31] and $^2H_{11}$-hexan-1-ol (hexan-1-ol-d11) was supplied by CDN Isotopes (Quebec, Canada). 4-Hydroxymercuribenzoic acid sodium salt (*p*HMB, 95%), butylated hydroxyanisole (BHA), L-cysteine hydrochloride hydrate (99%), DOWEX (1 × 2, Cl^--form, strongly basic, 50−100 mesh), and sodium hydrosulfide hydrate (NaSH·*x*H_2O, 69.3% NaSH) were purchased from Sigma Aldrich. Ethyl acetate (≥99.7, Fluka, Castle Hill, NSW, Australia) and dichloromethane (for gas chromatography, SupraSolv, Merck, Darmstadt, Germany) were used as solvents for thiol extraction. 5,5′-Dithiobis(2-nitrobenzoic acid) (99%) was sourced from Acros Organics (Geel, Belgium). All other remaining basic chemicals not mentioned here were purchased from Sigma Aldrich in their purest form possible. Helium (instrument grade) and nitrogen (food grade) were supplied by BOC Gases NZ Ltd. (Blenheim, New Zealand). All water used for thiol extraction and gas chromatographic analysis was processed by a Millipore water purification system (Merck Millipore, Billerica, MA, USA).

Media. All grape juices used in this study were machine harvested in 2010 and supplemented with antioxidants according to industry standards (~50 mg/L SO_2 and ~50 mg/L ascorbic acid). After settling and racking-off the grape solids, the musts were collected, homogenized, immediately frozen, and stored at −20 °C prior to usage. The origins and key properties of all four grape juices are listed in Table 1. Yeast peptone sucrose (YPS) medium consisted of 1% bacto-yeast extract, 2% bacto-yeast peptone, 10% sucrose, 1.5% tartaric acid, and 0.7% malic acid and had a pH of 3.14. The MS300 medium contained sugars, mineral salts, vitamins, amino acids, ammonium chloride, and ergosterol in amounts resembling real grape must and was prepared as described in detail by Clement et al.[32]

Table 1. Properties of Sauvignon Blanc (SB) Grape Juices from the Year 2010

grape juice	origin	°Brix	total acidity[a]	pH	YAN[b]
SB1	Sancerre, eastern Loire Valley, France	20.6	6.3	3.33	209
SB2	Languedoc-Roussillion, Gruissan, France	21.8	6.5	3.31	200
SB3	Languedoc-Roussillion, Gruissan, France	21.6	6.6	3.29	205
SB4	Lower Wairau, Marlborough, New Zealand	22.4	10.9	3.15	294

[a]Expressed in g/L of tartaric acid. [b]Yeast available nitrogen.

Yeast. All yeast strains used in this study belong to the species *Saccharomyces cerevisiae*. Vin13, Zymaflore X5, and Lalvin K1 (V1116) were purchased from Anchor Yeast, South Africa; Laffort, France; and Lallemand, Canada, respectively. The active dry yeasts were rehydrated according to the manufacturer's recommendations and inoculated at a concentration of 5 × 10^6 cells per milliliter.

Fermentation and Sampling. The initial H_2S-spiking experiment (Figure 1) was performed in duplicate at 21 °C with no agitation by using 2-L high-density polyethylene bottles fitted with airlocks and filled with 1.8 L of grape must. The monitoring of GLVs during commercial operation was performed in a 500 hL temperature-controlled stainless steel tank without using mechanical agitation. All other fermentations were conducted under temperature-controlled conditions with agitation (150 rpm) in triplicate by using 220-mL glass fermentors with a working volume of 200 mL. For the analysis of GLVs, samples were withdrawn and either immediately centrifuged

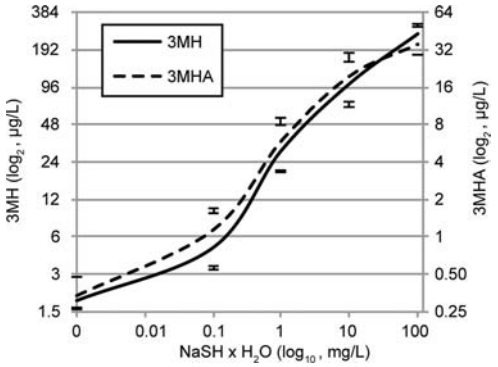

Figure 1. Impact of increasing amounts of NaSH·xH$_2$O (0.1−100 mg/L) in noninoculated SB grape juice on the synthesis of volatile thiols 3MH and 3MHA during fermentation with Anchor Vin13 yeast. Error bars = standard deviation (n = 4). Error bars above curves correspond to 3MHA values; error bars below curves correspond to 3MH values.

(4000g for 10 min, Figure 2) or filtered (0.45 μm, Figures 4 and 6) and frozen (−20 °C) prior to analysis. To minimize unwanted degradation, all NaSH·xH$_2$O stock solutions were freshly prepared in water immediately before spiking of the medium.

Gas Chromatography−Mass Spectrometry (GC-MS) Instrumentation and Compound Identification. All juice, must, and wine samples were analyzed on an Agilent 7890A GC (Santa, Clara, CA, USA), which was coupled to an Agilent 5975C mass spectrometer and was fitted with a COMBI PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). For conditioning and cleaning of the solid phase microextraction (SPME) fibers, a PAL SPME fiber conditioning station was used and operated at 250 °C under constant N_2 flow (6 mL/min). An Agilent HP-Innowax capillary column (60 m × 250 μm × 0.25 μm) was employed for volatile thiol analysis (for both the mercury salt and the head space method) as well as for the analysis of C6-alcohols and -aldehydes. The transfer line connecting the GC to the MS was held at 250 °C, and the ion source of the mass-selective detector was working in electron impact ionization mode set to 70 eV at 230 °C and a quadrupole operating temperature of 150 °C.

Detected compounds were identified by comparing mass spectra and retention times with purchased authentic standards, which were analyzed separately in diverse model media, as well as by comparing the obtained mass spectra with the ones reported in the Mass Spectral Library of the National Institute of Standards and Technology (NIST, version 2.0f, build Jul 23, 2008). The molecule was considered as positively identified only when both mass spectra and retention times matched those of authentic samples.

Methods for the Analysis of Volatile Thiols. For the data presented in Figure 1, volatile thiols analysis was performed by employing a method originally developed by Tominaga et al.,[33] which utilizes the reversible thiol-binding properties of the mercury salt (pHMB) combined with dichloromethane extraction and subsequent quantification via GC-MS. Further development of this method led to the following protocol. Five milliliters of 1 mM pHMB and 0.5 mL of 2 mM BHA were added to 50 mL of wine together with 50 μL of an internal standard mixture, resulting in a concentration of 1670 ng/L of 3MH-d2 and 170 ng/L of 3MHA-d2 in the wine sample. After pH adjustment to 7 with a 1−10 N NaOH solution, the sample was loaded onto a previously activated (0.1 M HCl) and prerinsed (ultrapure water) strongly basic anion exchange column containing 4.5 mL of DOWEX resin. Samples were then slowly percolated through the resin (one drop/5 s), and the column was washed with 0.1 M sodium acetate buffer (pH 6) before the DOWEX-bound thiols were finally released from the resin by eluting them (one drop/7 s) with 50 mM L-cysteine−HCl solution (400 mg in 50 mL of 0.1 M sodium acetate buffer) adjusted to pH 6. After addition of 0.5 mL of ethyl acetate, the obtained eluate was extracted twice with 4 and 2 mL of dichloromethane. At each extraction, the lower organic phase was recovered,

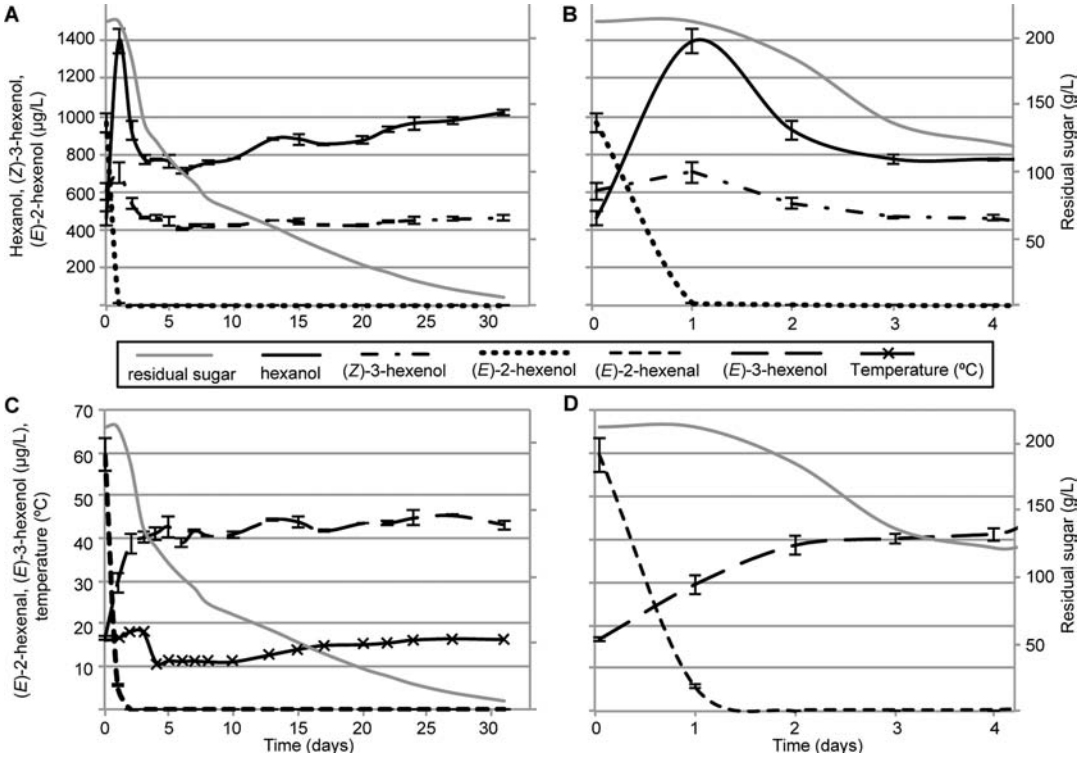

Figure 2. Evolution of hexan-1-ol, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol (A+B), (E)-2-hexenal, and (E)-3-hexen-1-ol (C+D) during fermentation of SB must with Zymaflore X5 at commercial scale. A and C show the data for the whole ferment, while B and D show the same data for the first four days. Single 500 hL ferment; bottom-sampled. Temperature values in C valid for A, B, and D as well. Error bars = standard deviation of three measurements.

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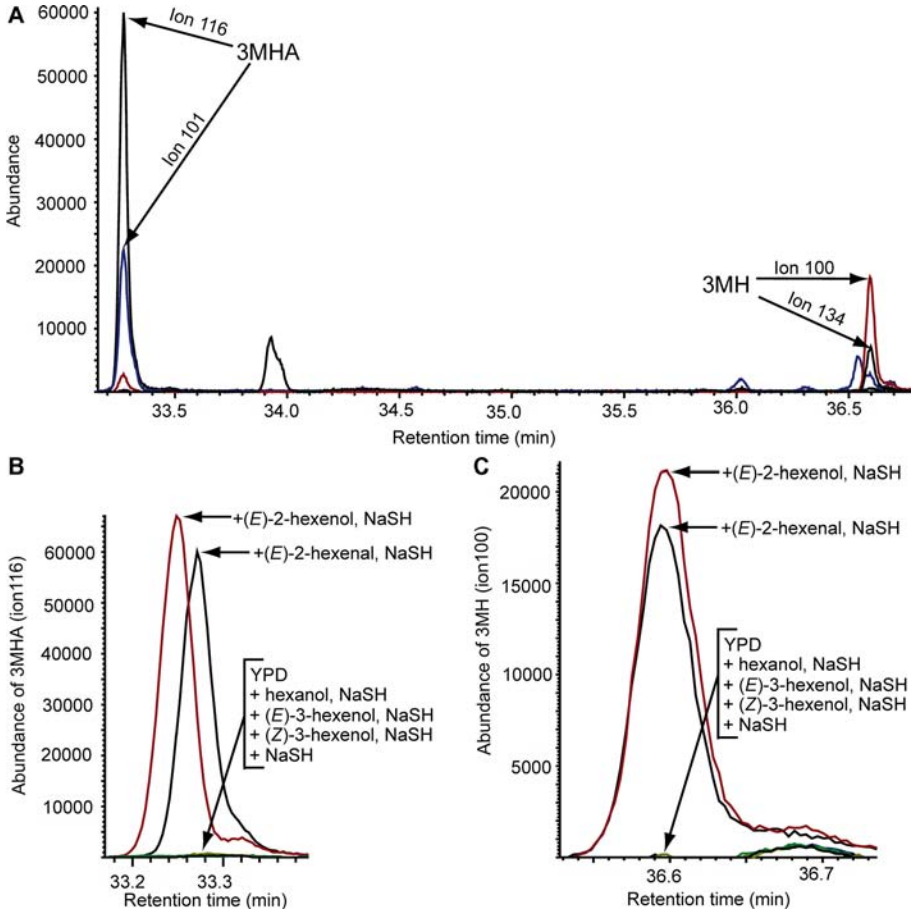

Figure 3. (A–C) 3MH and 3MHA peaks in a chromatogram derived by using HS-SPME GC-MS analysis. (B, C) Only samples spiked with either (*E*)-2-hexenal (1.5 mg/L) or (*E*)-2-hexen-1-ol (10 mg/L) exhibited (B) 3MHA and (C) 3MH peaks when fermented in the presence of 50 mg/L $NaSH{\cdot}xH_2O$.

dried over anhydrous sodium sulfate, filtered through silanized glass wool, and then concentrated under a flow of nitrogen to ~50 μL prior to GC-MS analysis. The injector inlet of the GC-MS was operated at 240 °C with helium as carrier gas at 112 kPa and a flow of 14.8 mL/min with the split vent set to 12 mL/min 1.5 min after injection. One microliter of sample was injected in pulsed splitless mode and was delivered onto the column with a constant helium flow rate of 0.8 mL/min. The initial oven temperature (50 °C for 5 min) was ramped to 115 °C at a rate of 3 °C/min, increased to 150 °C at 40 °C/min, held for 3 min and subsequently further raised to 173 °C/min at 3 °C/min, to finally reach the bake-out temperature of 250 °C (70 °C/min), which was held for 20 min, before the cycle finished by dropping to 50 °C again. Thiols were analyzed in selected ion monitoring (SIM) mode by using following quantifier/qualifier ions, respectively: 116/101 (3MHA), 118/103 (3MHA-d2), 134/100 (3MH), and 136/102 (3MH-d2). A colorimetric method developed by Riddles et al.[34] (using 5,5′-dithiobis(2-nitrobenzoic acid) as a reagent, which reacts with the free sulfhydryl groups in a mol to mol ratio) was used to determine the concentration of the thiol standards needed to generate the calibration curves. Standard curves were obtained with 10 calibration points by adding increasing quantities of the reference standards to 50 mL of a low-thiol SB wine (40–2200 ng/L for 3MHA; 200–12000 ng/L for 3MH). The linear regressions for all thiols were found to be very good, with r^2 (correlation coefficient)-values in the 0.99 range and recoveries around 100%. The relative standard deviation (RSD) was always lower than 10% for both compounds. High-thiol samples were diluted with the appropriate amount of water, in order to stay within the generated calibration ranges.

Due to the exceptionally high concentrations of 3MH and 3MHA encountered in the YPS medium (Figure 3, Supporting Information Figure 1), thiol analysis for this experiment was carried out by employing a head space–solid phase microextraction (HS-SPME) approach using a StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (2 cm, 50/30 μm, 23-gauge) supplied by Sigma Aldrich, rather than using the elaborate and time-consuming mercury method described above. Samples were analyzed in 20-mL SPME vials filled with 3 g of NaCl to which 5 mL of analyte was added and diluted 1 in 2 with deionized water. Sample vials were preincubated for 5 min at 40 °C with continuous shaking set to 250 rpm before the fiber was fully exposed to the sample for 40 min without shaking. Volatiles were then desorbed from the fiber in the GC inlet for 5 min at 240 °C with helium as a carrier gas at a flow rate of 15 mL/min and a pressure of 197 kPa, transferred onto the column, and separated at a constant flow rate of 1 mL/min using the following temperature gradient: 35 °C for 3 min, increasing at 4 °C/min to 200 °C, where the temperature was held for 10 min, before it was ramped up to 230 °C for a 10 min column bake-out. After each run, the SPME fiber was thermally cleaned and reconditioned at 250 °C for 10 min under constant N_2 flow (6 mL/min) in a fiber conditioning station. For identification and quantification of the generated 3MHA and 3MH in the YPS samples, deuterated internal standards 3MHA-d2 and 3MH-d2, which were quantified using the 5,5′-dithiobis(2-nitrobenzoic acid) method described above, were separately and sequentially added in increasing amounts (μg/L) to the samples: (2, 10, 20, 50, 100, 200) and (10, 50, 100, 250, 500, 1000), respectively. A peak area ratio of 1, between the added internal deuterated standard and the generated volatile thiols in the samples, indicated the absolute amount produced during fermentation. A good linear response could be observed with r^2-values in the 0.99 range and RSDs generally

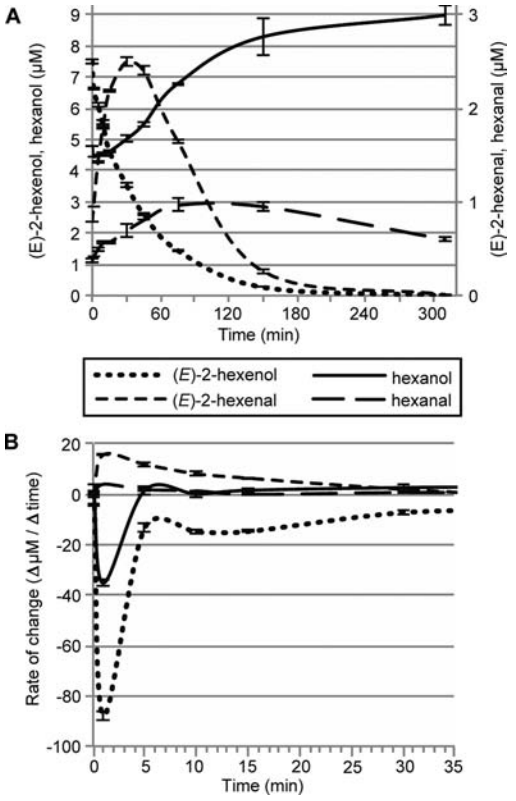

Figure 4. (A) Evolution of (*E*)-2-hexen-1-ol, (*E*)-2-hexenal, hexan-1-ol, and hexanal in French SB grape juice (SB1) during the first 5 h after yeast inoculation. (B) Rate of change during the first 35 min after yeast inoculation. The initial concentrations of (*E*)-3-hexen-1-ol (~0.15 μM) and (*Z*)-3-hexen-1-ol (~0.8 μM) did change only marginally and are therefore not included here. n = 4; error bars = standard deviation.

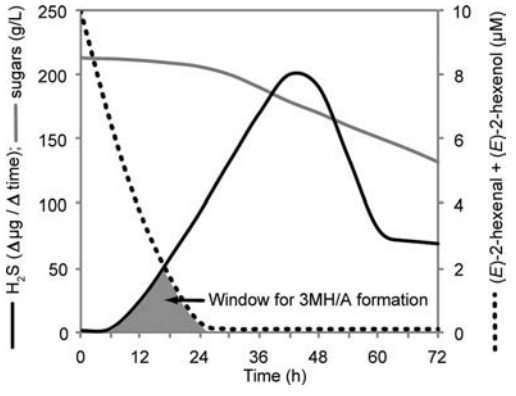

Figure 5. Kinetics of C6-thiol precursors, hydrogen sulfide, and sugars during a typical wine fermentation. The shaded area depicts the time window when C6-thiol precursors and hydrogen sulfide are present at the same time. (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, and sugar kinetics are based on actual data at commercial scale. The estimated evolution of hydrogen sulfide is derived from data presented by Thomas et al., [37] Mendes-Ferreira et al.,[38] and Ugliano et al.[39,44]

smaller than 10% for both compounds. Thiols were analyzed in SIM mode by using the same ions as listed in the *p*HMB method.

GC-MS Analysis of GLVs. An HS-SPME approach was developed for the analyses of GLVs in juice and wine. Sample (3.5 mL) and deionized water (3.5 mL) were transferred into a 20-mL SPME vial previously filled with 2 g of NaCl before 25 μL of internal standard hexan-1-ol-d11 (280 mg/L) was added. Adsorption of the headspace

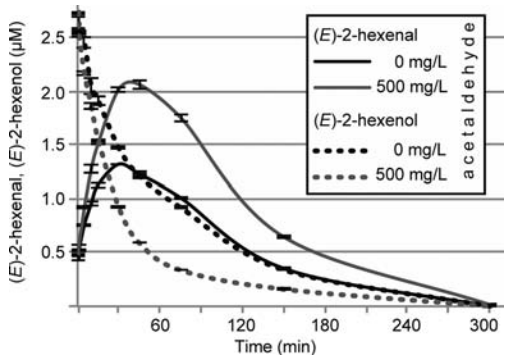

Figure 6. Effect of acetaldehyde on the conversion of (*E*)-2-hexen-1-ol into (*E*)-2-hexenal in SB grape must during the first 5 h after yeast inoculation. n = 4; error bars = standard deviation.

volatiles, including all C6-alcohols and -aldehydes, to the SPME fiber was facilitated by sample preincubation at 40 °C for 5 min with 500 rpm continuous agitation followed by exposing the fiber to the sample for 10 min without agitation. After desorption of the analyte into the GC inlet (240 °C, helium flow 15 mL/min, 197 kPa) in splitless mode for 10 min, column chromatography was performed at a constant helium flow rate of 1 mL/min using the following temperature program: an initial 5 min at 40 °C, increasing at 2 °C/min to 100 °C, before the temperature was further raised after 5 min to 230 °C (40 °C/min), where it was held for 10 min to thermally clean the column of any residues. The SPME fiber was thermally cleaned and reconditioned after each GS-MS run as previously described in the SPME thiol method. After compound identification and retention time determination of the various GLVs using full scan mode, quantification of the C6-alcohols and -aldehydes was carried out in SIM mode by using the following ions, with the quantifier ions listed first: 56/39/57 for hexanal, 83/42/57/69 for (*E*)-2-hexen-1-al, 69/55/56 for hexan-1-ol, 67/69/82 for (*E*)-3-hexen-1-ol, 67/69/82 for (*Z*)-3-hexen-1-ol, 57/67/82 for (*E*)-2-hexen-1-ol, and 76/64/62 for the internal standard hexan-1-ol-d11. Although the effect of different matrixes on the calibration curves was found to be minimal, standard addition curves were generated in either MS300, aqueous model medium (10% sucrose, 10% (v/v) ethanol, pH-adjusted to 3.15 with tartaric acid), SB grape juice, or SB wine, depending on the matrix of the samples analyzed. The 8-point calibration curves were generated by separately adding increasing quantities (in μg/L) of the following reference standards to the appropriate calibration medium: hexanal (10−1000), (*E*)-2-hexen-1-al (10−1000), hexan-1-ol (20−3000), (*E*)-3-hexen-1-ol (5−250), (*Z*)-3-hexen-1-ol (10−1000), and (*E*)-2-hexen-1-ol (10−3000). The standard addition functions were linear for all C6-compounds with r^2-values in the 0.99 range and recoveries around 100%. RSDs were lower than 10% for all GLVs.

Statistical Analysis. The conversion rates at peak level in Table 3 were analyzed in R[35] (version 2.15.1) by using one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference test. Tests for normality and homoscedasticity were performed by employing the Shapiro−Wilk and Fligner−Killeen test, respectively. All other statistical data were obtained in Microsoft Excel for Mac 2011.

Table 2. Efficiencies of H_2S-to-3MH/A Conversion in Grape Must SB4

range of H_2S addition (μM)	3MH/A increase compared to control (nM)	increase of 3MH/A (pM) per nM of H_2S added
0.00−0.12	27.5	22.3
0.12−12.36	239.1	19.3
12.36−123.62	859.3	7.0
123.62−1236.18	2104.9	1.7

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Table 3. Conversion Rates of (*E*)-2-Hexenal into (*E*)-2-Hexen-1-ol (lines 1−3) and Vice Versa (lines 4−9) in Spiked Synthetic (MS300) and Nonspiked Natural SB Grape Juice (SB 1−3)[a]

			(*E*)-2-hexen-1-ol (μM)		(*E*)-2-hexenal (μM)			(*E*)-2-hexen -ol/-al ratio		
medium	yeast	*T* (°C)	initial	peak	initial	peak	conversion at peak (%)	initial	peak	peak time (min)
MS300	K1	21	0	**0.86**	3.78	1.46	22.7 a	0.0	0.59	20
MS300	K1	5	0	**0.70**	3.18	1.00	21.9 a	0.0	0.70	75
MS300	K1	21	0	**3.09**	10.51	4.27	29.5 b	0.0	0.72	30
MS300	K1	21	21.43	7.76	0.47	**7.63**	33.4 C	45.41	1.02	45
MS300	K1	21	10.31	4.42	1.29	**4.08**	27.1 B	7.97	1.08	45
SB1	K1	21	7.50	3.54	0.79	**2.49**	22.8 A	9.56	1.42	30
SB3	K1	21	3.39	1.42	0.22	**1.05**	24.4 BA	15.20	1.35	45
SB2	Vin13	21	2.68	1.48	0.46	**1.32**	32.2 C	5.83	1.12	30
SB2 + acetaldehyde	Vin13	21	2.72	0.59	0.48	**2.03**	57.0 D	5.67	0.29	45

[a]Bolded numbers = peak levels of compound arising, $n = 4$, relative standard deviations of all values <10%. Different letters in the conversion column indicate statistically significant differences between the means (lower case letters = (*E*)-2-hexen-1-ol group, $p < 0.01$; capital letters = (*E*)-2-hexenal group, $p < 0.001$). Acetaldehyde was added at 500 mg/L.

RESULTS AND DISCUSSION

(*E*)-2-Hexenal and (*E*)-2-Hexen-1-ol Exhibit Immense Thiol-Forming Potential in Grape Juice. One reason for the restricted conversion of (*E*)-2-hexenal into 3MH and eventually 3MHA during the fermentation of grape must, as reported by Subileau et al.,[15] could have been the limited availability of a suitable sulfur donor such as hydrogen sulfide (H_2S). In order to study the intrinsic 3MH/A potential of (*E*)-2-hexenal in SB grape juice, we added increasing amounts (0.1−100 mg/L) of sodium hydrosulfide hydrate (NaSH·*x*-H_2O), which releases H_2S under the acidic conditions prevailing in grape juice, to machine-harvested and commercially processed SB grape juice (Table 1, SB4) prior to yeast inoculation. Fermentation was initiated after 6 h of incubation, and volatile thiols were measured at the end of fermentation. Figure 1 shows that supplying H_2S to grape juice results in large 3MH/A increases in the wine. This result provides the first direct experimental evidence for H_2S as a precursor to thiols. It is worth noting that the thiol-forming potential of grape juice is huge when excess H_2S is available. For example, the addition of 100 mg/L NaSH·xH_2O to SB grape juice prior to fermentation increased total volatile thiol synthesis during fermentation by 135-fold, leading to unprecedented amounts of 3MH (257 μg/L) and 3MHA (35 μg/L), which resulted in a combined total of 2121 nM 3MH/A in the finished wine. In contrast, SB wines that are regarded as having "high thiols" contain 7 to 18 μg/L of 3MH and 0.6 to 2.5 μg/L of 3MHA, 14 to 38 times less than encountered here.[1,2,36] One has to note that 100 mg/L of NaSH·xH_2O releases about 42 mg/L H_2S, which is an extreme treatment and lies well above the cumulative amount of H_2S normally produced by yeast during the fermentation of grape must. In contrast, the addition of 0.01−10 mg/L of NaSH·xH_2O, which releases between 0.004 and 4.2 mg/L of H_2S, roughly falls within the natural cumulative range found during the fermentation of wine.[37−39] This means that the associated 3MH/A increases (from 15.7 to 875 nM) encountered when NaSH·xH_2O was spiked to these levels could be, at least in theory, achievable under normal winemaking conditions. Table 2 illustrates that the H_2S-to-3MH/A conversion rate was dependent on the amount of H_2S added to the grape juice: increasing H_2S supplementation led to decreasing conversion efficiencies. This decrease could indicate that either the H_2S reaction partner(s) were becoming increasingly exhausted in the grape must and/or that the arising amounts of the toxic H_2S increasingly inhibited the yeast enzymes responsible for thiol formation.

In order to pinpoint the reaction partner(s) of the added H_2S, we monitored the evolution of various C6-compounds before yeast inoculation with Zymaflore X5 and during fermentation of SB grape juice (SB4) in a 500 hL fermentation tank (Figure 2). It was surprising to see that both (*E*)-2-hexenal and (*E*)-2-hexen-1-ol were almost completely metabolized by yeast during the first 24 h after inoculation, although the conversion of sugar had not yet commenced and the grape must had not been mechanically mixed in the tank (Figure 2A−D). To the best of our knowledge, this is the first time that this rapid conversion has been reported under winemaking conditions at commercial scale. Although the study by Joslin and Ough[28] had indicated this conversion occurred under aerobic conditions in separately spiked buffer medium at −1.1 °C and Herraiz et al.[29] reported a vanishing of (*E*)-2-hexen-1-ol at day 2 of fermentation at laboratory scale (their first sampling point), none of these studies investigated both compounds simultaneously and under commercial winemaking conditions. Armed with this knowledge, we spiked must SB4 with 50 mg/L NaSH·xH_2O when 50% of the sugar had been fermented and therefore (*E*)-2-hexenal and (*E*)-2-hexen-1-ol were no longer present in the must. The subsequent thiol analysis at the end of fermentation revealed no elevated 3MH/A content compared to the control wine (data not shown), indicating that one or both of these C6-compounds were the likely reaction partners of H_2S. To further substantiate this hypothesis, we spiked synthetic YPS medium individually with either (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, or hexan-1-ol at concentrations 5 times higher than expected in a high-GLV-containing SB juice from New Zealand, added 50 mg/L NaSH·xH_2O, stirred the spiked medium for 60 min, and inoculated it with Anchor Vin13 yeast. Samples were analyzed for thiols using an HS-SPME GC-MS approach, after fermentation had completed. 3MH and 3MHA peaks were obtained only for the medium spiked with (*E*)-2-hexenal and (*E*)-2-hexen-1-ol (Figure 3B,C). A double blind sensory evaluation of the fermentation products (diluted 1:100 in water) by six wine professionals confirmed unanimously that only the (*E*)-2-hexenal- and (*E*)-2-hexen-1-ol-spiked samples expressed a strong tropical scent (data not shown).

These observations led us to conclude that both (*E*)-2-hexenal and (*E*)-2-hexen-1-ol were the main reaction partners

of H_2S in grape juice, and therefore, for the first time, (*E*)-2-hexen-1-ol had been identified as a new additional 3MH/A precursor.

(*E*)-2-Hexenal Has a Higher Thiol Conversion Rate in Model Medium Than (*E*)-2-Hexen-1-ol. To determine the thiol yields generated in the YPS ferments, which were supplemented with 50 mg/L $NaSH{\cdot}xH_2O$ and spiked with either 1.5 mg/L (*E*)-2-hexenal or 10 mg/L (*E*)-2-hexen-1-ol, known quantities of deuterated 3MH-d2 and 3MHA-d2 were added in increasing amounts to the samples to create a calibration curve using the HS-SPME thiol method (Supporting Information Figure 1). This revealed that an addition of 100 μM (*E*)-2-hexen-1-ol yielded 1.15 μM 3MHA and 8.26 μM 3MH in the YPS ferments, which equals a molar conversion of 9.4%. In contrast, 15.3 μM (*E*)-2-hexenal was converted into 9 μM total thiols, a much more efficient conversion yield of 58.8%. It has to be noted that GC-MS analysis of the synthetic (*E*)-2-hexen-1-ol used in this experiment uncovered a 1% impurity of (*E*)-2-hexenal. Theoretically this impurity could have accounted for 0.59 μM, or 6.25%, of the total thiols formed and therefore reduces the (*E*)-2-hexen-1-ol thiol conversion rate to 8.8%. In any case, these thiol conversion rates in simple model medium with its high H_2S content do not reflect real winemaking conditions and only indicate that (*E*)-2-hexenal is more efficiently used as a 3MH/A precursor than (*E*)-2-hexen-1-ol when H_2S is in excess. It should be noted that a reaction of (*E*)-2-hexen-1-ol with H_2S would immediately result in the direct formation of 3MH without the need for any enzymatic yeast activity, whereas a reaction of (*E*)-2-hexenal with H_2S would result in the formation of 3-mercaptohexenal, which would need to be reduced by yeast alcohol dehydrogenases to form 3MH during fermentation. However, no 3MH could be detected in the noninoculated YPS medium spiked with (*E*)-2-hexen-1-ol (10 mg/L) and H_2S (50 mg/L), nor could we identify any 3-mercaptohexenal in the control YPS medium spiked with (*E*)-2-hexenal and H_2S but not inoculated with yeast (data not shown). These observations imply that the formation of 3MH/A via the H_2S pathway requires yeast activity and suggest that it is not driven by straightforward chemical reactions.

(*E*)-2-Hexen-1-ol Is Converted into (*E*)-2-Hexenal in SB Grape Must during the First Hours after Yeast Inoculation. Encouraged and puzzled by these results, we decided to investigate the kinetics of (*E*)-2-hexenal and (*E*)-2-hexen-1-ol in grape juice (SB1) and in spiked MS300 model medium during the first hours after yeast inoculation. We were intrigued to see that, only 30 min after yeast inoculation, at least 22.8% of the more abundant (*E*)-2-hexen-1-ol originally present in the grape juice was converted into its lesser abundant (*E*)-2-hexenal, tripling its concentration (Figure 4A; Table 3, line 6). Both (*E*)-2-hexenal and (*E*)-2-hexen-1-ol were almost completely metabolized and converted by yeast, mainly into hexan-1-ol and possibly hexanal, after about 3 h. These kinetics were mirrored in less complex MS300 medium that had been simultaneously spiked with amounts of C6-compounds similar to the ones naturally occurring in the French SB1 grape juice used in Figure 4 (see Table 3, compare lines 5 and 6). This finding supported our previous observation that (*E*)-2-hexen-1-ol does not directly react with H_2S to form 3MH, but most likely needs to be first transformed by yeast into (*E*)-2-hexenal before it can be further converted into 3MH/A in the presence of H_2S. Furthermore, this interconversion may also explain why (*E*)-2-hexen-1-ol exhibited a lower thiol conversion rate than (*E*)-2-hexenal in the H_2S-spiked YPS medium reported earlier. The concentrations of (*E*)-3-hexen-1-ol (~15 μg/L) and (*Z*)-3-hexen-1-ol (~80 μg/L) changed only marginally in the first 5 h after yeast inoculation and are therefore not further reported here. The very fast decrease of (*E*)-2-hexen-1-ol and hexan-1-ol during the first minute after yeast addition in the grape juice (Figure 4B) does not allow us to distinguish whether these compounds enter the yeast cell via an energy-requiring transport mechanism or simply diffuse across the plasma membrane. Since it has been suggested that the structurally related fusel alcohols translocate by passive diffusion,[40] it is conceivable that the GLVs might be able to do this as well. However, no conclusive data could be found on this in the literature, and it is not known if passive diffusion might also occur for the slightly polar (*E*)-2-hexenal. What is known is that (*E*)-2-hexenal has a proven inhibitory and fungicidal activity against *Saccharomyces cerevisiae*,[41] which is probably one of the reasons that yeast metabolizes it, together with its related alcohol (*E*)-2-hexen-1-ol, into the less toxic and less reactive hexan-1-ol.

(*E*)-2-Hexen-1-ol and (*E*)-2-Hexenal Can Be Interconverted. The results presented in Figures 4 and 6 clearly show that in grape juice and in the presence of yeast (*E*)-2-hexen-1-ol can be converted into (*E*)-2-hexenal. However, Joslin and Ough[28] indicated in individually spiked buffer medium that this conversion could go both ways. We confirmed this observation of interconversion in model medium MS300 for both compounds (Table 3). When only (*E*)-2-hexenal was present in the MS300 medium, yeast converted at least 21.9−29.5% of the (*E*)-2-hexenal into (*E*)-2-hexen-1-ol to reach a (*E*)-2-hexen-ol/-al ratio between 0.59 and 0.72, before both of the compounds were further converted by yeast presumably mainly into hexan-1-ol and hexyl acetate (Table 3, lines 1−3).[30] When (*E*)-2-hexen-1-ol exceeded the level of (*E*)-2-hexenal at the beginning of fermentation, yeast favored the conversion of (*E*)-2-hexen-1-ol into (*E*)-2-hexenal until a (*E*)-2-hexen-ol/-al ratio between 1.02 and 1.42 was reached (Table 3, lines 4−8). A decrease in temperature from 21 °C down to 5 °C delayed the (*E*)-2-hexenal to (*E*)-2-hexen-1-ol conversion peak 3.75-fold and roughly doubled the time needed until both thiol precursors were metabolized, but did not influence the final conversion rate at peak level (Table 3, lines 1 and 2).

These results confirm that yeast is able to interconvert the compounds (*E*)-2-hexen-1-ol and (*E*)-2-hexenal. In combination with the different efficiencies with which the two compounds form 3MH/A above, and based on the chemical properties of these two compounds, we interpret this to mean that (*E*)-2-hexen-1-ol acts as a precursor to thiols by first being converted to (*E*)-2-hexenal by yeast and that this is the main compound that reacts with H_2S and/or other thiol donors. This would mean that depending on the initial concentration in the grape juice, (*E*)-2-hexen-1-ol can have opposing effects on the formation of 3MH/A: (a) if (*E*)-2-hexen-1-ol exceeds the amount of (*E*)-2-hexenal in the grape juice (ol/al ratio > ~1), it can increase 3MH/A synthesis or (b) if initial (*E*)-2-hexen-1-ol concentrations are lower than that of (*E*)-2-hexenal in grape juice (ol/al ratio < ~1), yeast favors the conversion of (*E*)-2-hexenal into (*E*)-2-hexen-1-ol, leading to a potential decrease in 3MH/A. This could at least partially explain why Subileau et al.[15] were able to demonstrate only a minor 3MH/A contribution of (*E*)-2-hexenal: the related (*E*)-2-hexen-1-ol occurred only in insignificant quantities and was therefore

diminishing the already low quantities of (*E*)-2-hexenal (16 μg/L) in their grape juice even further.

Direct Thiol Formation from (*E*)-2-Hexenal and (*E*)-2-Hexen-1-ol: A Timing Problem. The rapid catabolism of both (*E*)-2-hexenal and (*E*)-2-hexen-1-ol in the very early stages of fermentation straight after yeast addition is most likely another reason that (*E*)-2-hexenal has been estimated to be very inefficiently used by yeast to form 3MH/A under experimental conditions in which H_2S is less abundant.[15] Although most yeast strains are capable of producing H_2S during grape must fermentation,[42,43] the synthesis of H_2S does not normally commence prior to the conversion of sugar.[37−39,44] At this time, most of the 3MH-forming reaction partners (*E*)-2-hexenal and (*E*)-2-hexen-1-ol have already been converted into the nonreactive hexanal and hexan-1-ol. In other words, the chances that the unsaturated C6-thiol precursors and their sulfur donor H_2S are present at the same time are small and short-lived. This relationship is illustrated in Figure 5. Winter et al.[45] observed that active dry yeast produced more 3MH/A during fermentation when it was supplemented with a nutrient mix during the yeast rehydration phase compared to a nonsupplemented control. Interestingly, this thiol increase of the nutrient-spiked yeast was accompanied by an earlier onset and increased initial production of H_2S. Furthermore, Harsch et al.[46] reported that yeast strains separately deleted in genes *MET17*, *CYS4*, and *CYS3* exhibited increased 3MH/A production in SB grape must. These three genes encode for enzymes responsible for three successive steps in the sulfur amino acid biosynthesis: the incorporation of inorganic H_2S into *O*-acetylhomoserine to form homocysteine and its conversion to cystathionine and then to cysteine. It is of no surprise that single-gene deletions of two of these genes, *MET17* and *CYS4*, are known to increase H_2S production.[47] Combined, these three studies seem to support our finding that the formation of volatile thiols via the H_2S−C6 pathway can be an important contributor to total 3MH/A synthesis during fermentation of grape must. However, given the ubiquitous presence of C6-compounds in grape juices across all varieties and the common potential for the formation of H_2S during fermentation, this does not explain why the ability to generate high amounts of 3MH/A seems limited to only a few varieties, most notably SB. It could be that variety-specific viticultural and harvesting practices positively influence (*E*)-2-hexenal and (*E*)-2-hexen-1-ol concentrations in the corresponding grape juices and therefore can contribute to an increased 3MH/A synthesis during fermentation under certain conditions. This hypothesis is certainly worth further study.

The data presented here show that, at least in some SB juices, H_2S is the limiting factor in the formation of 3MH/A via the H_2S−C6 thiol pathway (Figure 1). To be utilized in thiol production, H_2S has to be synthesized very early in the fermentation process in order to be able to react with the fast disappearing (*E*)-2-hexenal and (*E*)-2-hexen-1-ol (Figure 5). It may be that the contribution of the H_2S−C6 thiol pathway to total volatile thiol production during commercial winemaking is typically minor, but occasionally, when H_2S is in abundance very early in fermentation, can become the predominant thiol-contributing pathway. The extent to which this happens under commercial conditions clearly needs further study.

Differences in juice composition are the major factor influencing concentrations of 3MH/A in wine[14,48,49] and are also important for the production of H_2S during fermentation.[42,43,50] However, these parameters have not been compared directly in the same juices, particularly the degree of H_2S production early in fermentation. Although some juice factors (e.g., nitrogen content) are known to affect both H_2S production[51] and thiol yields,[45,46,52,53] the reported data are not always consistent, and there appears to be a high degree of variation between yeast strains in these responses.[39,43,46,52−54]

Acetaldehyde Increases (*E*)-2-Hexen-1-ol to (*E*)-2-Hexenal Conversion Ratio in SB Grape Must. It is known that acetaldehyde production by wine yeast during the early stages of fermentation is positively correlated with the duration of the yeast lag-phase.[55] It was thought that by increasing the lag-phase the degradation of (*E*)-2-hexen-1-ol and (*E*)-2-hexenal could be delayed and therefore the chances that both compounds coincide with early H_2S production could be increased (Figure 5). To test this hypothesis, 500 mg/L of acetaldehyde was added to French SB juice (Table1, SB2) prior to inoculation with wine yeast Vin13. Figure 6 compares the C6-evolution of the supplemented ferments with nonspiked control fermentations. Contrary to our expectation, the addition of acetaldehyde increased the (*E*)-2-hexen-1-ol degradation rate in the first 45 min after yeast inoculation, which led to a more efficient (*E*)-2-hexen-1-ol to (*E*)-2-hexenal conversion rate and hence increased the concentration of (*E*)-2-hexenal at peak level by 54% (Table 3, compare line 8 with 9). This observation is promising, since we have shown in this work that most likely (*E*)-2-hexen-1-ol has to be converted into (*E*)-2-hexenal first before it can be used by yeast to form 3MH/A when a sulfur donor is present. Cheraiti et al.[55] reported that some active dry yeast strains could produce up to 40 mg/L of acetaldehyde even during the rehydration phase, which opens up an opportunity to actively control and increase the concentration of (*E*)-2-hexenal during the early stages of fermentation.

The main role of enzymes belonging to the alcohol dehydrogenase family (ADH) is controlling the interconversion between ethanol, the key metabolite in the energy metabolism of yeast, and acetaldehyde.[56] However, these enzymes are not specific and can act on a variety of substrates, including (*E*)-2-hexen-1-ol and (*E*)-2-hexenal.[19] ADH1p seems to be the main enzyme responsible for the reduction of the aldehyde into the corresponding alcohol, whereas ADH2p does the inverse reaction but is only found in aerobically grown yeast cells.[56] Therefore, the aerobic conditions, prevailing in grape must straight after inoculation with yeast, should favor the conversion of (*E*)-2-hexen-1-ol into (*E*)-2-hexenal, whereas later on in fermentation, once the oxygen is depleted, this reaction is most likely reversed. This could also explain why the addition of acetaldehyde to the grape juice led to an increased conversion of (*E*)-2-hexen-1-ol into (*E*)-2-hexenal. Acetaldehyde is known to be able to bind free SO_2 in must and could have therefore led to a less reductive environment favoring *ADH2* activity.[57]

This study demonstrates the huge 3MH/A-forming potential of two GLVs in grape juice when a sulfur donor, such as H_2S, is present. We identified for the first time (*E*)-2-hexen-1-ol as a new precursor for 3MH/A and showed that it most likely needs to be enzymatically converted into (*E*)-2-hexenal before it can be transformed by yeast into 3MH/A. However, this conversion can potentially go both ways and is mainly driven by the initial (*E*)-2-hexen-1-ol to (*E*)-2-hexenal ratio in the grape juice. An initial ratio greater than 5.67 favored the conversion of (*E*)-2-hexen-1-ol into (*E*)-2-hexenal in our studies, but the exact turning point (probably about 1) of the

conversion remains to be established. These findings open up a new door of opportunities to increase and control the formation of 3MH and its acetyl ester before and during must fermentation. Furthermore, since GLVs are ubiquitous compounds in many plants and fruits, the formation of 3MH/A is not necessarily just limited to grape-based alcoholic beverages, but could possibly be used for the creation of novel beverages derived from fermented GLV-containing ingredients (e.g., beer[12,58]). Measures to exploit this newly discovered 3MH/A-forming potential of (*E*)-2-hexenal and (*E*)-2-hexen-1-ol should focus on enlarging the window of opportunity for the formation of volatile thiols, as illustrated in Figure 5. For example one could try to find ways to increase (*E*)-2-hexenal and (*E*)-2-hexen-1-ol levels in the grape must by experimenting with different viticultural methods (e.g., influence of defoliation), grape harvesting regimes (e.g., influence of damaged leaves and stalks in must), and perhaps limiting the use of ascorbic acid and SO_2 before grape crush (more oxygen available needed for the breakdown of C18 polyunsaturated fatty acids by LOX). More specifically, one could screen for yeast with delayed (*E*)-2-hexenal and (*E*)-2-hexen-1-ol catabolic properties, develop methods to positively influence the (*E*)-2-hexen-1-ol to (*E*)-2-hexenal conversion (like that described here with the addition of acetaldehyde), and most importantly find legal ways to increase the biological production of H_2S very early in fermentation (e.g., ref 45).

■ ASSOCIATED CONTENT

Supporting Information

Figures depicting the quantification of 3MH and 3MHA in YPS medium. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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3MH, 3-mercaptohexan-1-ol; 3MHA, 3-mercaptohexyl acetate; 3MH/A, 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate; SB, Sauvignon blanc; LOX, lipoxygenase; HPL, hydroperoxide lyases; GLVs, green leaf volatiles; 3MH-d2, 3-mercapto-1-$^2H_2$-hexan-1-ol; 3MHA-d2, 3-mercapto-1-$^2H_2$-hexyl acetate; hexan-1-ol-d11, $^2H_{11}$-hexan-1-ol; *p*HMB, 4-hydroxymercuribenzoic acid sodium salt; BHA, butylated hydroxyanisole; GC-MS, gas chromatography−mass spectrometry; SPME, solid phase microextraction; r^2, correlation coefficient; RSD, relative standard deviation; HS, head space; YPS, yeast peptone sucrose; YAN, yeast available nitrogen; ADH, alcohol dehydrogenase

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