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Rafael Jiménez-Lorenzo, Vincent Farines, Jean-Marie Sablayrolles, Carole Camarasa, Audrey Bloem. New Insights into the Origin of Volatile Sulfur Compounds during Wine Fermentation and Their Evolution during Aging. *Fermentation*, 2022, 8 (4), 10.3390/fermentation8040139 . hal-03671995

HAL Id: hal-03671995

<https://hal.inrae.fr/hal-03671995>

Submitted on 27 Jun 2022

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Article

New Insights into the Origin of Volatile Sulfur Compounds during Wine Fermentation and Their Evolution during Aging

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Abstract: Volatile sulfur compounds (VSCs) are associated with unpleasant reductive aromas and are responsible for an important reduction in wine quality, causing major economic losses. Understanding the origin of these compounds in wine remains a challenge, as their formation and further evolution during winemaking can involve both chemical and biological reactions. Comparing the VSCs profile (i) of fermenting synthetic grape juices supplemented with a selected VSC (eight compounds tested) and incubated in presence or absence of yeast, and (ii) during storage of wines under an accelerated aging procedure, allowed us to elucidate the chemical and metabolic connections between VSCs during fermentation and aging. Yeast metabolism, through the Ehrlich pathway and acetylation reactions, makes an important contribution to the formation of compounds such as methionol, 3-methylthiopropionate, 3-methylthiopropylacetate, 3-mercaptopropanol, 2-mercaptoethanol and thioesters. By contrast, chemical reactions are responsible for interconversions between thiols and disulfides, the formation of thiols from thioesters or, more surprisingly, the formation of ethylthiopropanol from methionol during fermentation. During aging, variations in heavy VSC concentrations, such as an increase in 3-methylthiopropylacetate and a decrease in ethyl-3-methylthiopropionate formation, were evidenced. Overall, this study highlights that it is essential to consider both yeast metabolism and the high chemical reactivity of VSCs to understand their formation and evolution during winemaking.

Keywords: fermentation; volatile sulfur compound; *Saccharomyces cerevisiae*

Citation: Jiménez-Lorenzo, R.; Farines, V.; Sablayrolles, J.-M.; Camarasa, C.; Bloem, A. New Insights into the Origin of Volatile Sulfur Compounds during Wine Fermentation and Their Evolution during Aging. *Fermentation* **2022**, *8*, 139. <https://doi.org/10.3390/fermentation8040139>

Academic Editor: M. Margarida Baleiras-Couto

Received: 1 February 2022

Accepted: 2 March 2022

Published: 23 March 2022

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1. Introduction

The aroma or bouquet of wine is a major determinant of its quality, guiding product acceptance or rejection by consumers. This characteristic is the result of the fine-tuned balance between several thousands of volatile compounds originating from grapes (varietal aromas), produced during fermentation (fermentative aromas) or during maturation (aging aromas). The origin of some families of odorants, including ethyl esters, acetate esters or terpenes, has been widely studied, as they positively contribute to the diversity, uniqueness and freshness of wine. However, other volatile molecules with a negative impact on wine sensory properties are also produced or released during winemaking. In particular, reductive off-odors, a critical defect in wines, have been reported as being mostly due to the presence of volatile sulfur compounds (VSCs) produced by yeasts during fermentation. In general, these molecules, which can account up to 10% of the odorous molecules in wine [1], have a low perception threshold (in the ng/L range) and are associated with unpleasant notes of rotten eggs, cabbage, onion, garlic or rubber, all of which are undesirable in wines. Their accumulation, even in low concentrations, can result in wine spoilage and cause important economic losses to winemakers.

The sulfur-containing molecules responsible for reductive sensory character consist of hydrogen sulfide (H₂S) and compounds belonging to the thiol chemical family—with the exception of varietal thiols, which impart notes of grapefruit, passion fruit and boxwood

to the wine—as well as thioesters, sulfides—excluding dimethyl sulfur (DMS), that can positively influence wine sensory properties depending on its amount [2]—and disulfides. Various factors have been identified as modulating VSC formation at all stages of the wine-making process, related to (i) viticultural practices, including the use of sulfur-containing pesticides, (ii) the composition of the grape juice, including the availability of nutrients and pantothenic acid or the concentration in inorganic sulfur, (iii) the microbial community present in berries or during fermentation and (iv) the postfermentation technological operations (copper addition, aging procedure).

The mechanisms involved in the formation of H₂S have been well documented, with this molecule being released mainly as a result of an imbalance between its production by the sulfur assimilation pathway and its utilization for homocysteine formation. An alternative route involves cysteine catabolism [3]. However, many questions regarding the origin of other VSCs have yet to be addressed. Indeed, both chemical and enzymatic reactions have to be considered to understand the dynamics of VSC concentrations throughout fermentation and during aging. First, the catabolism of sulfur amino acids (methionine and cysteine) and their derivative homocysteine through the Ehrlich pathway is at the core of the formation of many VSCs by yeasts. However, the metabolic origin of other compounds remains poorly understood, as does the possibility of yeast-mediated conversion between VSCs. Due to the presence of a sulfur atom, VSCs are chemically very active nucleophilic molecules that can react with one another, particularly through the formation of disulfide bonds [4], or with other molecules in the medium, such as metal cations or polyphenols [5,6]. Consequently, the concentrations of VSCs can vary throughout the fermentation and aging processes because of these chemical conversions. Recently, a general picture of the chemical and biological pathways for the formation of 11 light VSCs (i.e., with boiling points below 90 °C) deriving from ethanethiol (EtSH) and methanethiol (MeSH) was proposed, highlighting the complexity of the connections between these molecules [7].

The aim of this work is to provide a comprehensive overview of the origin of sulfur-containing compounds and their evolution during fermentation and aging. In particular, the nature of the formation of 7 light and 11 heavy VSCs, chemical or biological, was addressed, and possible connections between molecules were investigated during the different steps of winemaking. It is difficult to elucidate the origin of VSCs from natural grape juice, for which the chemical composition is incompletely known. Thus, as a first approach, this study was conducted using a chemically defined synthetic must and a high VSCs producing strain.

2. Materials and Methods

2.1. Chemical and Standards

VSC standards were purchased from Sigma Aldrich (St Louis, MI, USA), viz. EtSH (75-08-1), DMS (75-18-3), diethyl sulfur (DES) (352-93-2), thiophene (TP) (110-02-1), S-methylthio acetate (SMTA) (1534-08-3), dimethyl disulfur (DMDS) (624-92-0), ethylthio acetate (ETA) (625-60-5), diethyl disulfur (DEDS) (110-81-6), methional (MAL) (3268-49-3), 2-mercapto ethanol (2ME) (60-24-2), 2-methylthio ethanol (2MTE) (5271-38-5), ethyl-3-methylthio propanoic acid (E3MTP) (13327-56-5), 3-methylthiopropyl acetate (3MTPAc) (16630-55-0), 3-mercapto ethanol (3MP) (19721-22-3), methionol (ME) (505-10-2), ethylthio propanol (ETP) (18721-61-4), 4-methylthio butanol (4MTB) (20582-85-8), 3-methylthio propanoic acid 3MTPA (646-01-5) and ethyl(methylthio) acetate (EMTA) (4455-13-4).

2.2. Strain and Culture Conditions

The *S. cerevisiae* commercial strain LMD17, provided by Lallemend SA (Montreal, QB, Canada) and maintained on YEPD agar plates (2% glucose, 1% yeast extract, 2% peptone, and 2% agar) at 4 °C, was used in this work. Precultures were obtained by inoculating 200 mL YEPD in 1L-erlenmeyer flasks with a single colony. After incubation for 24 h at 28 °C with 190 rpm agitation, the cell suspension was centrifuged (5 min, 4500 rpm,

4 °C), washed twice with sterile physiological water (9 g/L NaCl) and suspended in 50 mL physiological water.

2.3. Fermentations and Sampling Procedure

Fermentations were carried out in a synthetic must that mimics a natural grape juice [8], with some minor modifications: the carbon source was provided at a total concentration of 210 g/L as a 1:1 mixture of glucose and fructose, a final concentration of 200 mg/L of yeast assimilable nitrogen (YAN) as a mixture of ammonium and amino acids was used, and the anaerobic factors were replaced by β -phytosterols at a final concentration of 2 mg/L.

A total volume of 12 L of SM in a glass container was inoculated at 10^6 cells/mL and incubated at 22 °C with agitation (180 rpm) for 50 h to obtain cells in the stationary phase of wine fermentation. Then, the fermentation broth was divided into two equal aliquots. The first part was further used unchanged (condition in presence of cells) while cells were removed by centrifugation (4500 rpm, 10 min) from the second fraction (condition without cells). For each condition (with or without cells), 36 subfractions of 95 mL were then transferred to a 100 mL vial sealed with a rubber cap equipped with a needle to allow for CO₂ release. The subfractions were supplemented with a specific VSC (SMTA, ETA, MAL, ME, 3MTPA, 2ME, 3MP and 2MTE), added in excess (400 μ M) to promote interconversions, as previously described [7]. A control experiment without any VSC addition was performed. Each condition was carried out in triplicate. Samples were collected 24 h after spiking the media with VSCs and at the end of fermentation, when the rate of CO₂ production was lower than 0.01 g/L/h. Figure 1 summarizes the experimental design.

Accelerated aging experiments were conducted using three different media: a natural Viognier grape juice, the synthetic medium SM previously described and a synthetic medium enriched with cysteine and methionine. For this last condition, the nitrogen source consisted of 200 mg/L methionine, 200 mg/L cysteine and the required amount of the mixture of ammonium and amino acids for a final concentration of 200 mg N/L. Viognier grape juice containing initially 208 g/L of sugar and a 38 mg/L of YAN was supplemented with 162 mg/L of YAN as diammonium phosphate (DAP).

Fermentations were carried out in 1 L fermenters at 22 °C under agitation at 210 rpm with the strain LM17. At the end of fermentation, an accelerated aging procedure adapted from [9] was performed. Samples were centrifuged and 95 mL final wine was transferred to 100 mL vials. To create an anoxic environment, which was required to mimic wine aging in bottle, a flow of argon was applied to each vial for 10 min before sealing them with a rubber cap. Vials were stored in the dark at 50 °C for 21, 42 and 63 days. A control condition was maintained at room temperature for 63 days. All fermentations were conducted in triplicate.

2.4. VSCs Determination

The determination of light VSCs and the calibration procedure (EtSH, DMS, DES, SMTA, DMDS, ETA and DEDS) were carried out as previously described [10]. Briefly, 25 μ L of internal standard (TP) was added to 7 mL of synthetic wine or standard solutions in a 15 mL screw cap headspace vial. Analytes from the liquid samples were transferred to a gas phase using a Dynamic Headspace System (DHS) module equipped with a Thermal Desorption Unit (TDU) tube packed with Tenax TA (020810-005-00). VSCs were quantitated using a 7890 GC system (Agilent Technologies, Santa Clara, CA, USA), fitted with a 30 m \times 0.25 mm \times 0.1 μ m ZB WAX (Phenomenex, Torrance, CA, USA), and coupled with a 5975 single quadrupole mass spectrometry detector (Agilent). Maestro Software Control (Gerstel, Mülheim an der Ruhr, Germany) and Chemstation Software (Agilent Technologies, Santa Clara, CA, USA) were used to control the instrument and analyze the data, respectively. For quantification, the mass spectra were recorded in selected ion monitoring (SIM) using positive ion electron impact at 70 eV. The ion in bold for each compound was used for quantification, while the other ions were used as qualifiers. Standard curves were

obtained by plotting the sulfur response ratio of the sulfur compound and the internal standard against the concentration ratio.

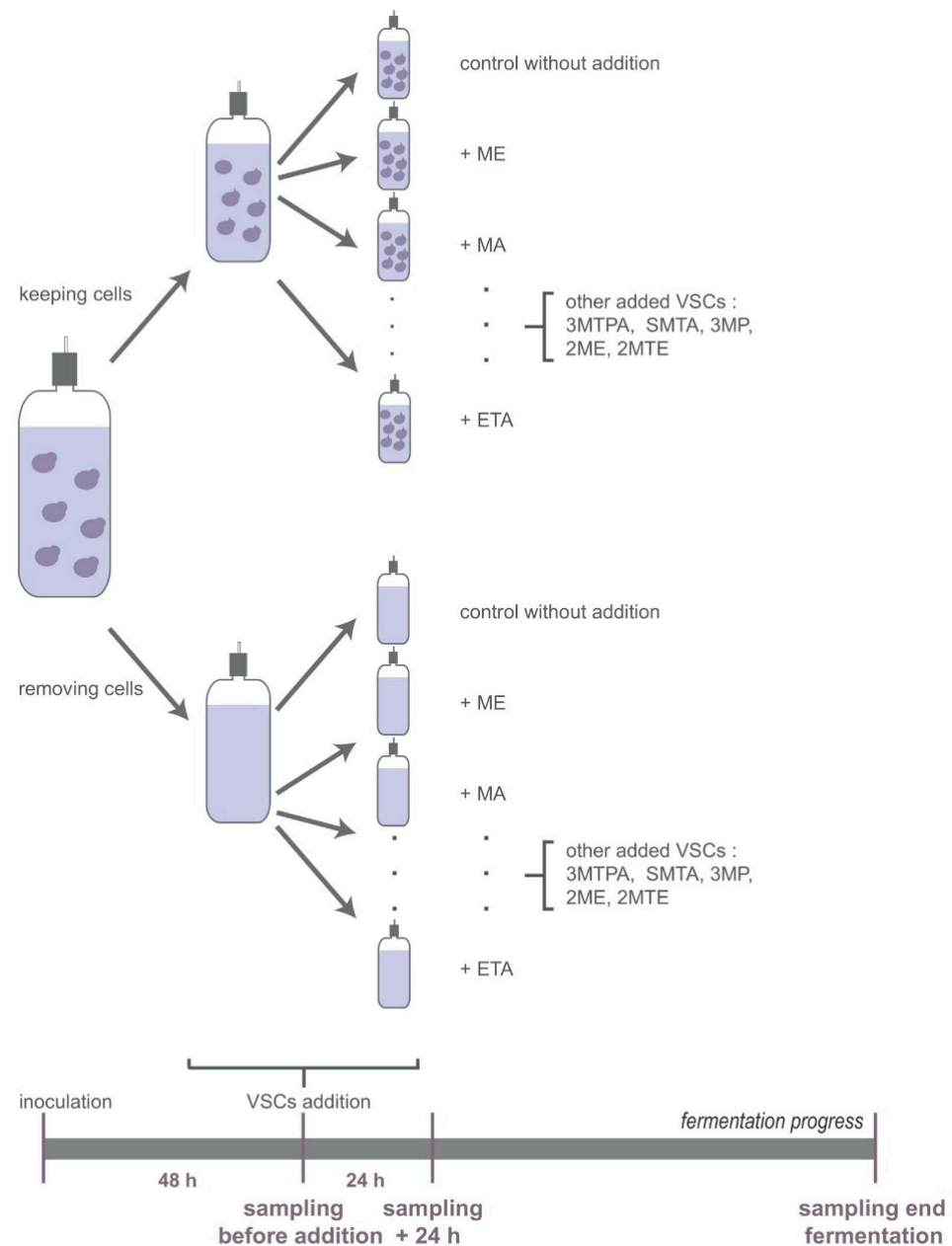


Figure 1. Experimental procedure implemented to analyze the metabolic and chemical connections between VSCs during fermentation. Cells were grown for 50 h in synthetic grape juice at 22 °C. The fermentation broth was divided into two fractions: one remained unchanged while cells were removed from the second by centrifugation. Then, 95 mL aliquots of each fraction were transferred to 100 mL sealed vials maintaining anaerobiosis and supplemented with one of the eight studied VSCs: SMTA, ETA, MAL, ME, 3MTPA, 2ME, 3MP and 2MTE. A control without VSC addition was carried out. Each of the 18 incubation conditions was performed in triplicate. Samples were analyzed after 24 and 234 (corresponding at the end of fermentation for assays in the presence of cells) hours of incubation.

Heavier sulfur compounds (2ME, 2MTE, E3MTP, 3MTPAc, 3MP, ME, ETP, 4MTB and 3MTPA) were extracted through double liquid–liquid extraction (DLLE), as previously detailed [8]. Samples were analyzed with a Trace 1300 GC system gas chromatograph (ThermoFisher Scientific, Waltham, MA, USA) equipped with a Thermo Scientific Triplus

RSH Sampler used in liquid mode, and coupled to a Thermo Scientific ISQ 7000 single quadrupole mass spectrometry detector (Thermo Scientific). Analyte separation was performed using a 30 m × 0.25 mm × 0.25 µm DB-FFAP, with a constant flow rate of 1.0 mL/min of helium as the carrier gas. For quantification, mass spectra were recorded in Selected Ion Monitoring (SIM) mode with positive ion electron impact set at 70 eV.

3. Results and Discussion

Thiols, thioethers, thioesters and disulfides are the main VSCs imparting reductive off-odors to wines. These molecules are formed during fermentation through yeast metabolism; however, as a result of their high reactivity, they can also be chemically interconverted or formed from precursors during fermentation and aging. To further investigate the origin of VSCs during winemaking, the *S. cerevisiae* strain LM17 was grown on synthetic must for 50 h to obtain cells in the stationary phase of alcoholic fermentation. The fermentation broth was then divided into two fractions, the cells being preserved in the first and removed by centrifugation in the second. Each batch was then divided into nine aliquots, eight of which were supplemented with 400 µM of a selected volatile compound, and the last one being used as a control. VSC profiles were measured after 24 h of incubation and at the end of fermentation in presence of yeast and with a similar incubation time for cell-free experiments (Figure 1; Table S1).

3.1. Elucidating the Origin of VSCs in Absence of Supplementation

To assess the relative contribution of chemical and enzymatic pathways, VSC formation without any supplementation was compared in the presence or absence of cells, after incubation times of 24 h and 234 h, which corresponded to the time required for sugar depletion during experiments with yeasts (Control in Figures 1 and 2). Most of the light VSCs (apart from SMTA) and three heavier compounds (MAL, E3MTP and 4MTB) were not detected under these conditions. The concentrations of eight of the nine detected compounds (ME, 3MTPAc, 3MTPA, ETP, 2ME, 3MP, 2MTE, SMTA) were substantially increased in the presence of cells. Conversely, their formation was not significantly enhanced during experiments without cells. These observations revealed that yeast metabolism is largely involved in the production of these VSCs during wine fermentation, extending the findings from [7,11] to a wider range of VSCs.

The formation of thioesters according to the experimental conditions conformed to different patterns. First, ETA concentration remained close to the initial level, i.e., corresponding to the production during the growth phase, in cell-free conditions, but was drastically decreased in the presence of cells (Figure 2E). These variations show that ETA is produced during the first stage of wine fermentation, likely through enzymatic conversion of EtSH or H₂S, as reported [6], and is further consumed by yeasts and used as a precursor to the biosynthesis of other VSCs during the second part of fermentation (no or low detection of this compound after 24 h and 234 h incubation with cells) or chemically converted, at a slow rate (strong decrease observed only after 234 h incubation without cells). SMTA also displayed a specific production profile (Figure 2B), with an increase in concentration after 24 h of incubation in the presence of cells (from 5.4 µg/L to 26.4 µg/L), and a subsequent drop (13.7 µg/L). The same pattern was observed in the absence of cells but to a lesser extent, with a production after 24 h incubation of 9.2 µg/L instead of 26.4 µg/L. These changes are in line with a transient accumulation of SMTA at the beginning of fermentation, mainly due to yeast metabolism with a minor contribution of chemical reactions, followed by a significant chemical or biological degradation of this compound.

Finally, a substantial production of 2ME was observed in the presence of cells (Figure 2C), consistent with a yeast-mediated synthesis from cysteine through the Ehrlich pathway [12,13]. Furthermore, a slow decrease in its concentration was found during the incubation of a cell-free sample, suggesting a chemical conversion of this compound.

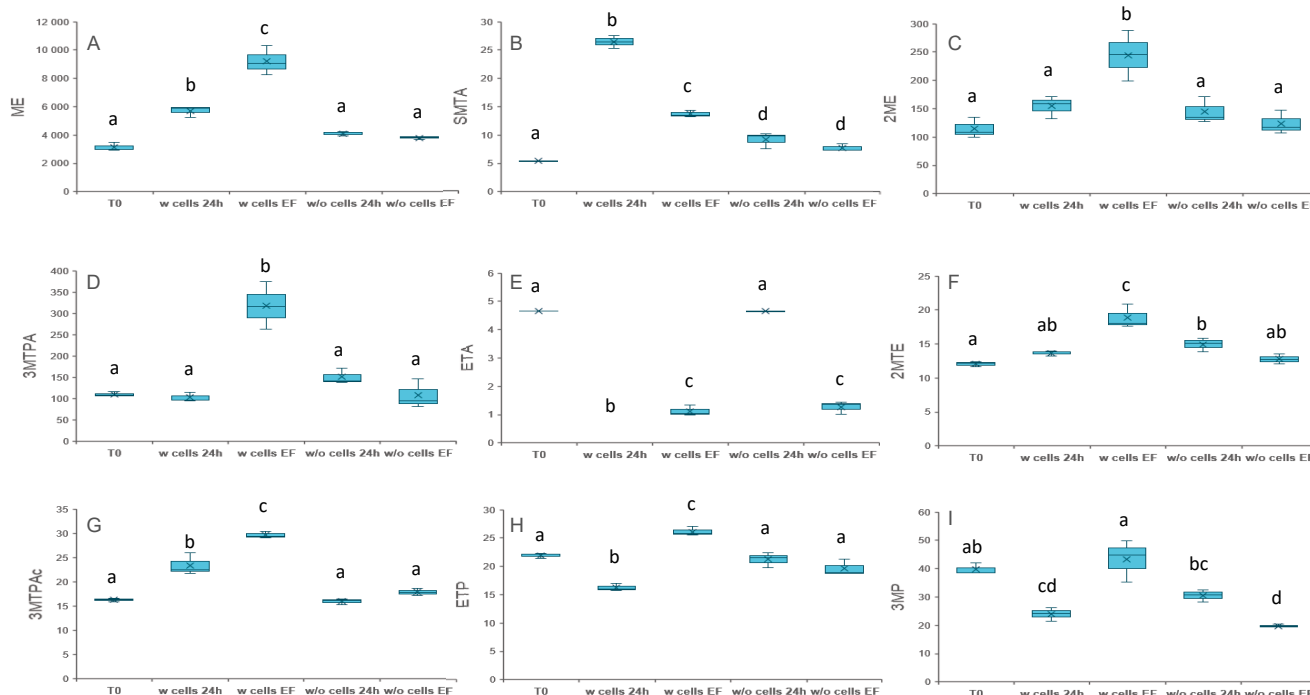


Figure 2. Evolution of VSC concentrations during incubation without supplementation. Concentrations expressed in $\mu\text{g/L}$. T0: start of the incubation; w cells 24 h: incubation in presence of cells for 24 h; w cells EF: incubation in presence of cells for 234 h (end of fermentation); w/o cells 24 h: incubation in absence of cells during 24 h; w/o cells EF: incubation in absence of cells during 234 h. Experiments were conducted in triplicate. The letters in capital refer to the different sulphur compounds: (A) ME, (B) SMTA, (C) 2ME, (D) 3MTPA, (E) ETA, (F) 2MTE, (G) 3MTPAc, (H) ETP, (I) 3MP. A one-way ANOVA test ($n = 3$, p -value < 0.05) was applied to evaluate the statistically significant differences between condition, depicted by letters: conditions sharing at least one letter are not significantly different. EF: end of fermentation.

3.2. Unravelling the Chemical and Biological Connection between VSCs

It is noteworthy that under control conditions, i.e., without VSC addition, some compounds were never detected, including EtSH, DMS, DMDS, DEDS and 4MTB. Supplementing the medium with a high concentration of sulfur-containing molecules should make it possible to further investigate the origin and pattern of formation of these latter molecules during wine fermentation, and more broadly, to unravel the possible chemical or metabolic connections between VSCs. With this aim, 400 μM thioesters (SMTA, ETA) or compounds deriving from methionine (MAL, ME, 3MTPA), homocysteine (3MP) and cysteine (2ME, 2MTE) catabolisms were added at the end of the growth phase and changes in VSC formation profile after incubation in the presence or absence of cells were then analyzed (experimental design summarized in Figure 1). An increased concentration of a compound during cell-free incubation with a selected VSC compared with the control conditions without addition indicates a chemical connection between the two molecules. Likewise, an increase in the content of a molecule following VSC addition, found in a larger quantity in the presence than in absence of cells, reflects that the added VSC is used as a precursor for the biological formation of this compound.

3.2.1. Addition of Compounds Deriving from Methionine Catabolism

An analysis of the changes in the profile of VSCs formation resulting from MAL addition first showed that this aldehyde was largely chemically reduced to ME (26.3 mg/L) or oxidized to 3MTPA (3.3 mg/L) (Figure 3A, purple). However, it is noteworthy that MAL is usually never detected in the medium during fermentation [14,15], as this transient metabolic intermediate produced intracellularly is highly reactive. Therefore, the extra-

cellular chemical formation of ME and 3MTPA from MAL produced by yeast and further excreted in the medium during fermentation remains an unlikely hypothesis.

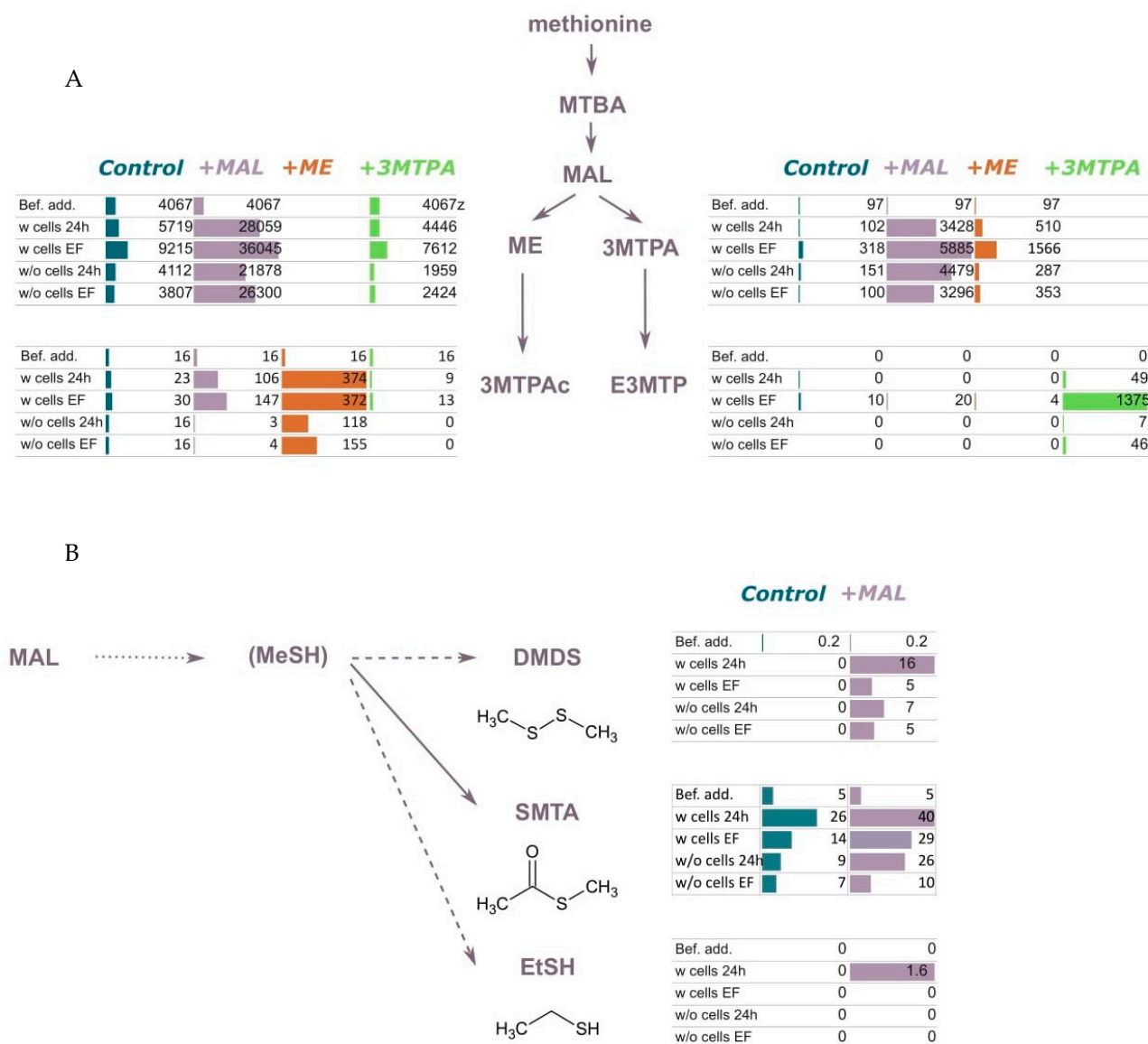


Figure 3. Variation of the concentrations of VSCs during incubation after spiking with compounds deriving from methionine catabolism. **(A)** Formation of compounds from the Ehrlich pathway. **(B)** Formation of light VSCs. Concentrations expressed in $\mu\text{g/L}$. **Control:** control without addition; **+MAL:** supplementation with $400 \mu\text{M}$ MAL; **+ME:** supplementation with $400 \mu\text{M}$ ME; **+3MTPA:** supplementation with $400 \mu\text{M}$ 3MTPA; Bef. add.: concentration just before spiking; w cells 24 h: incubation in presence of cells for 24 h; w cells EF: incubation in presence of cells for 234 h (end of fermentation); w/o cells 24 h: incubation in absence of cells during 24 h; w/o cells EF: incubation in absence of cells during 234 h. Experiments were conducted in triplicate and the mean values are reported. EF: end of fermentation.

A comparison of the VSC profiles obtained after incubation in the absence or presence of cells revealed a substantial biological conversion of MAL to ME, 3MTPA and 3MTPAc, with a respective production of 36 mg/L , 5.9 mg/L and $147 \mu\text{g/L}$ at the end of yeast fermentation supplemented with $400 \mu\text{M}$ MAL. In addition, the major change triggered by ME supplementation compared to control conditions (Figure 3A, orange versus blue) was a 12-fold increase in 3MTPAc formation in the presence of cells. Likewise, the 69-fold increase

in the production of E3MTP observed in the presence of cells was the sole impact on VSC formation of spiking the medium with 3MTPA (Figure 3A, green). These observations, emphasizing the leading role of yeast metabolism in the production of ME, 3MTPA, 3MTPAc and E3MTP, were expected, as it has been widely reported that MAL is the aldehyde precursor for the synthesis of ME and 3MTPA, through the Ehrlich pathway [14,15], and their further derivatives 3MTPAc and E3MTP, respectively. Interestingly, incubating cells in the presence of 400 μ M 3MTPA was the only experimental condition affecting E3MTP formation, with a slight increase (9.3 μ M) compared to the amount of added 3MTPA (data compared in μ M to reflect the stoichiometry of conversions). The increase in 3MTPA production from 2.7 μ M (control conditions) to 13 or 49 μ M, as a consequence of MAL or ME addition respectively, had no impact on E3MTP production. This result points to the low efficiency of 3MTPA esterification with ethanol. Along the same lines, the formation of 3MTPAc (2.5 μ M) represented only a minor fraction of its direct precursor ME when provided in excess (400 μ M). This finding is consistent with previous data [13] reporting a low capacity of *S. cerevisiae* to produce 3MTPAc, even in presence of excess ME, mainly due to the limiting activity of the enzyme involved in ME acetylation. Furthermore, these data revealed that, overall, the reduction of MAL to ME is favored at the expense of its oxidation to 3MTPA, which is in agreement with literature data [16]. These findings are consistent with the previously described absence or low detection of 3MTPAc in beer or wine, respectively [17]. This is likely related to yeast redox status maintenance during fermentation through the elimination of excess reduced cofactors produced during growth and energy production [18].

Another unforeseen finding regarding the connection between methionine derivatives was the increase in 3MTPA and MAL after the addition of 400 μ M ME, which was significantly higher in the presence of cells than in cell-free conditions, and was already observed after 24 h of incubation. Five alcohol dehydrogenases (Adhp1–3,5–6) have been reported to be potentially involved in the conversion of MAL to ME, and more generally, a total of 16 reductases have been identified as catalyzing the interconversions between aldehydes and alcohols in *S. cerevisiae* [15]. Furthermore, three aldehyde dehydrogenases (Aldp4–6) are involved in the oxidation of MAL in 3MTPA. Thus, when provided in excess, ME was likely oxidized in MAL by reversible alcohol dehydrogenases, which is further oxidized in 3MTPA. Conversely, 3MTPA cannot be reduced to ME by a biological pathway, as no change in ME formation was observed when the medium was supplemented with 3MTPA.

More surprising variations in VSCs profile were observed in response to the addition of MAL, first among which was the transient accumulation of EtSH in the presence of cells, detected after 24 h of incubation, but which was entirely dissipated at the end of fermentation (Figure 3B). In general, the presence of EtSH was only rarely observed in all the samples of the experimental design, and MeSH was never detected under any conditions in our experiments. This is most likely due to their low boiling points (35 °C and 6 °C, respectively) and the high chemical reactivity of the free function –SH in their structure. Likewise, DMDS concentrations of 16.1 and 7.2 μ g/L were quantified after 24 h of incubation in the presence or absence of cells, respectively, while this compound was not recovered in samples from control conditions. Lower contents (4.8 μ g/L) were measured at the end of fermentation with cells. Finally, adding MAL in the presence of yeasts resulted in an increase in the formation of thioesters compared to nonsupplemented conditions, in particular, of SMTA, with concentrations varying from 26.4 to 40.3 μ g/L and from 13.7 to 29.0 μ g/L after 24 h incubation and at the end of fermentation, respectively. The loss of the VSCs during the incubation period could be explained either by a low boiling point or by a CO₂ stripping mechanism, since losses were higher when the fermentative activity was maintained.

Overall, these results suggest a biologically-mediated conversion of MAL into DMDS, EtSH and thioesters, combined with the contribution of chemical reactions. In yeasts, MeSH has been described as a key centralized hub, involved in particular in the formation of EtSH, thioesters and disulfides through chemical and/or biological routes [7,19,20].

During winemaking, MeSH can be first chemically produced by Strecker degradation of methionine [21] or by the retro-Michael reaction from methional [22]. This compound is also biologically synthesized via yeast enzymes, in particular, by demethiolation of methionine, α -keto-4-methylthiobutanoic acid (KMBA) originating from methionine transamination or MAL [14,19,23,24]. Two genes, STR3 and CYS3, have been reported as being potentially responsible of these conversions in *S. cerevisiae* [25,26]. Consequently, the production of EtSH, DMDS, ETA and SMTA as a result of excess MAL addition could be explained by the transient formation of MeSH from MAL and the further conversion of this compound. In agreement with this hypothesis, DMDS was the most abundant compound detected after spiking the medium with MeSH [7].

More unexpectedly, 3MP formation was substantially increased as a consequence of 3MTPA spiking, by 20 $\mu\text{g/L}$ at the end of fermentation, regardless of the presence of cells. The mechanism responsible for this chemical conversion remains to be elucidated. Lastly, when ME was added to cell-free or cell-containing samples, an additional accumulation of ETP was found in the medium, likely due to a chemical substitution of the ME methyl group by an ethyl group.

3.2.2. Addition of Thioesters

Adding thioesters first triggered a set of chemical reactions consistent with the high chemical reactivity of these molecules due to their intrinsic electrophilicity [27] favoring nucleophilic attacks. Through this chemical reaction, added ETA and SMTA can be transformed into their associated thiols, EtSH and MeSH. In our experiments, incubating cell-free medium with excess ETA resulted in substantial EtSH accumulation (607 $\mu\text{g/L}$) (Figure 4A). Both these conversions were supported by the detection of DEDS (28.5 $\mu\text{g/L}$) and DMDS (24.0 $\mu\text{g/L}$) during cell-free incubations with 400 μM ETA and SMTA, respectively, as DEDS and DMDS have been reported to be formed by EtSH and MeSH oxidation through dimerization reactions [7,28,29]. The concentrations of DEDS and DMDS measured in the experiments with cells were lower than those obtained in cell-free modalities. As an example, DMDS concentration at the end of fermentation (in presence of yeasts), if spiked with SMTA, was 12 $\mu\text{g/L}$, while levels of around 24 $\mu\text{g/L}$ were measured during incubation in the absence of cells under the same conditions. In the same way, the presence of cells resulted in a 45% decrease in DEDS concentration during experiments supplemented with ETA. These observations can be explained by a stripping of these molecules due to the production of CO_2 by yeasts, by a capacity of yeast to further catabolize these compounds, or by the biological use of EtSH and MeSH, limiting their chemical oxidation to DEDS and DMDS. Consistent with the latter interpretation, it has been reported that a large panel of thioesters is produced through yeast metabolism, combining EtSH or MeSH with acyl-CoA [17,30,31].

The origin of DMS in wine is not yet fully understood. A chemical formation involving either SMM breakdown, dimethyl sulfoxide reduction [30] or MeSH methylation [7] appears to be the most likely source of DMS. A yeast-mediated production of this compound from S-methyl methionine (SMM) [14] or potentially by MeSH methylation [32] should also be considered. Finally, cysteine and glutathione have been reported as DMS precursors [17,33], even if the mechanisms responsible for these yeast-mediated conversions remain unexplained. However, the DMS produced during fermentation is, for the most part, removed by the release of CO_2 [34]. Adding ETA was the only treatment which led to a substantial production of DMS. A concentration of around 20 $\mu\text{g/L}$ DMS was produced after only 24 h of incubation, which remained stable in the cell-free assays, but significantly decreased in the presence of cells. This is in line with an early chemical formation of DMS from ETA and a further loss of this molecule by stripping due to CO_2 production under fermentation conditions. However, these data are quite surprising, as ETA derives from cysteine catabolism, while previous hypotheses regarding the origin of DMS in wine are consistent with a chemical or biological formation from methionine derivatives.

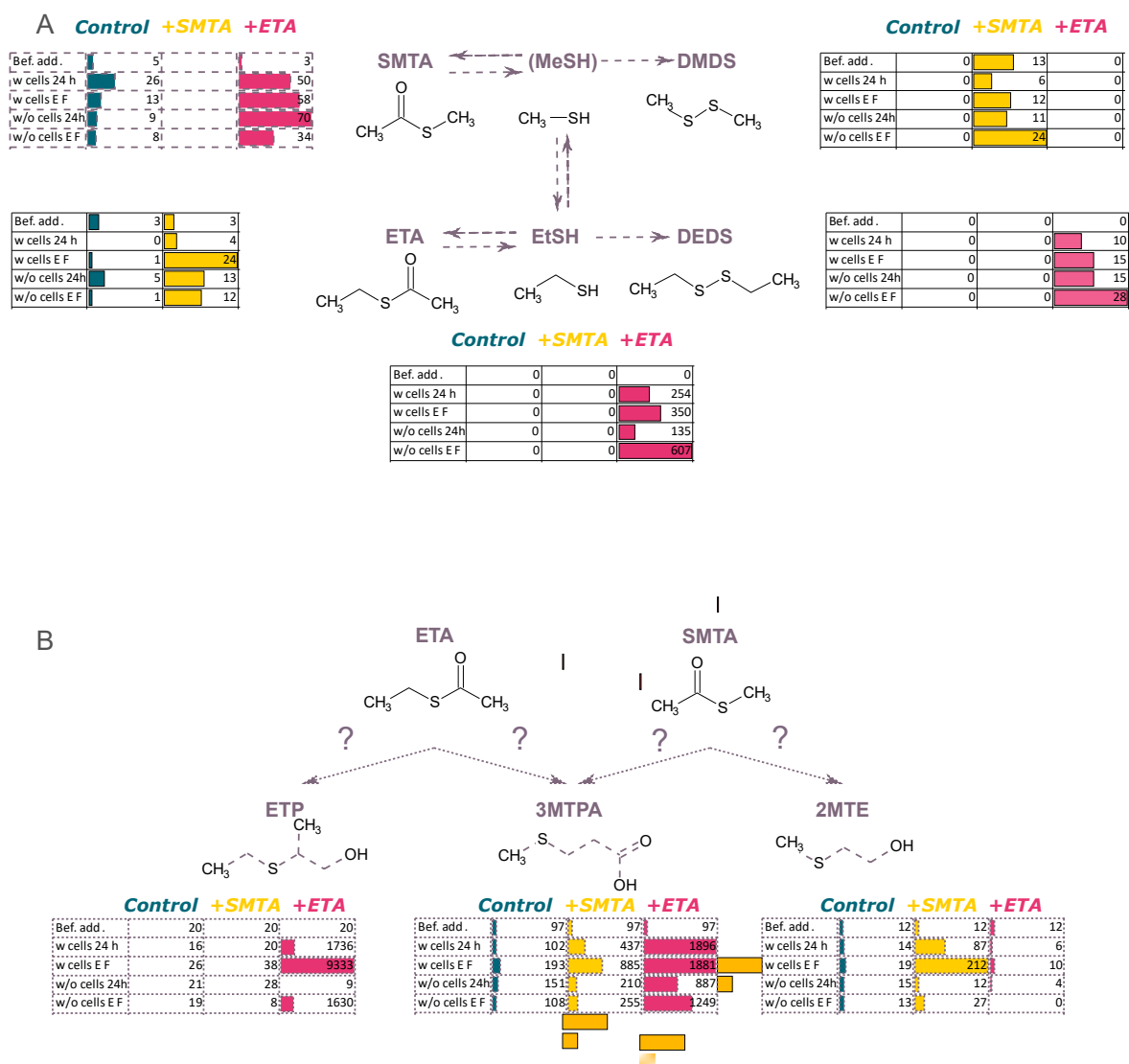


Figure 4. Variation of VSCs concentrations during incubation after spiking with thioesters. Concentrations expressed in $\mu\text{g/L}$. (A) VSCs deriving from MeSH and EtSH. (B) Formation of ATP, 3MTPA and 2MTE. **Control:** control without addition; **+SMTA:** supplementation with 400 μM SMTA; **+ETA:** supplementation with 400 μM ETA; **Bef. add.:** concentration just before spiking; **w cells 24 h:** incubation in presence of cells for 24 h; **w cells EF:** incubation in presence of cells for 234 h (end of fermentation); **w/o cells 24 h:** incubation in absence of cells during 24 h; **w/o cells EF:** incubation in absence of cells during 234 h. Experiments were conducted in triplicate and the mean values are reported. Raw data are available in supplementary data. EF: end of fermentation.

Spiking the medium with ETA or SMTA during cell-free incubation resulted in an increased formation of the other thioester, SMTA (+27 $\mu\text{g/L}$) or ETA (+12 $\mu\text{g/L}$), respectively (Figure 4A). These findings revealed the possibility of a chemical interconversion between ETA and SMTA, either directly or via their thiol counterparts, EtSH and MeSH. These findings complement recent data from the literature [7] that established a two-step chemical pathway for the transformations of ETA to MeSH and SMTA to EtSH, combined with exclusively biological conversions of MeSH to SMTA and EtSH to ETA. The conversion of ETA to SMTA was enhanced in the presence of cells, which was not the case for the reverse reaction (SMTA to ETA). The two acetylation steps are the only biological reactions reported as being involved in these interconversions [7]. As a consequence, a low availability of

EtSH, chemically produced from SMTA and further enzymatically converted in ETA, could explain that the presence of cells had no effect on the conversion of spiked SMTA to ETA.

Adding SMTA or ETA during fermentation also revealed yeast-mediated specific conversions of these molecules (Figure 4B). First, the formation of 3MTPA was substantially increased as a consequence of either thioesters supplementation, with greater increases in the presence of cells (+1563 and +568 µg/L with ETA and SMTA in comparison with control experiment without addition, respectively) compared to cell-free incubations (+1149 and +155 µg/L). Furthermore, it is noteworthy that the addition of thioesters had no effect on the formation of other compounds from the catabolism of methionine through the Ehrlich pathway. These observations indicate a biological formation of this acid directly from SMTA or ETA or from the catabolism of their chemically-produced derivatives, through a metabolic route independent from the Ehrlich pathway that remains to be identified.

2MTE production was specifically increased ten-fold compared to control conditions when fermenting yeast cells were incubated in presence of SMTA. Two different pathways have been proposed regarding the microbial formation of 2MTE: the methylation of 2-ME, synthesized from the degradation of cysteine by the Ehrlich pathway [11] and the reduction of 2-methylthioacetaldehyde, formed from 5-methylthioadenosine through the methionine salvage route [35,36]. Until now, this latter metabolic pathway has been only reported in bacteria (*Rhodospirillum rubrum* and *Rhodopseudomonas palustris*), and there is no experimental verification to support its activity in yeast. However, our results demonstrated a yeast-mediated conversion of SMTA to 2MTE that can be explained by a chemical or biological isomerisation of SMTA to 2-methylthioacetaldehyde, enzymatically reduced further to 2MTE. This biological reduction of SMTA extends knowledge on the metabolic network involved in yeast production of 2MTE. No variation in SMTA formation was found when the incubation medium was supplemented with 2MTE, in agreement with a one-directional conversion of SMTA to 2MTE.

Finally, a huge increase in ETP formation was found incubating cells in presence of ETA (9333 µg/L) compared to control conditions (27 µg/L), which was accompanied by a larger production of 3MP (104 µg/L with ETA spiking compared to 59 µg/L without spiking).

3.2.3. Addition of Compounds Deriving from Homocysteine and Cysteine Catabolism

Overall, the supplementation with metabolites from homocysteine or cysteine catabolism through the Ehrlich pathway (3MP, 2ME and 2MTE) had a limited impact on both the chemical and biological formation of other VSCs. In particular, adding 400 µM 3MP during fermentations or cell-free incubations did not trigger any significant changes in the VSC profile (Figure 5). This was expected, since 3MP has been reported to be the end product of homocysteine degradation by *S. cerevisiae* through the Ehrlich pathway, without sharing metabolic intermediates with the other routes involved in the formation of sulfur-containing molecules.

Regarding the experiments supplemented with 2ME and 2MTE, compounds deriving from cysteine catabolism through the Ehrlich pathway and further methylation of 2ME to 2MTE, only the formation of 3MP was affected (Figure 5). The pattern of variation of the production of 3MP according to the presence or absence of yeast was consistent with its chemical formation from 2MTE and biological production from 2ME.

3.3. Changes in VSCs Profile during Aging

VSCs include some chemically very reactive molecules such as mercaptans, which can react with one another or with wine compounds (including metal cations, oxidized polyphenols) or through photochemical and thermal reactions to form nonvolatile complexes [5,37–39]. Consequently, the VSC profiles of wines can evolve during their storage. To investigate these changes in VSC concentrations, an accelerated aging procedure was applied to three different types of wines: (i) a synthetic wine produced by growing *S. cerevisiae* on SM; (ii) a synthetic wine obtained by growing *S. cerevisiae* on synthetic medium contain-

ing an equimolar mixture of cysteine and methionine as the sole nitrogen source (Cys-Met wine) to enrich the wine in VSCs and facilitate analyses throughout the evolution of the aging process; and (iii) a Viognier wine, to assess the contribution of interactions between VSCs and varietal compounds of white wines. The evolution of VSCs during red wine aging, likely different due in particular to the presence of polyphenols, was not considered in our work.

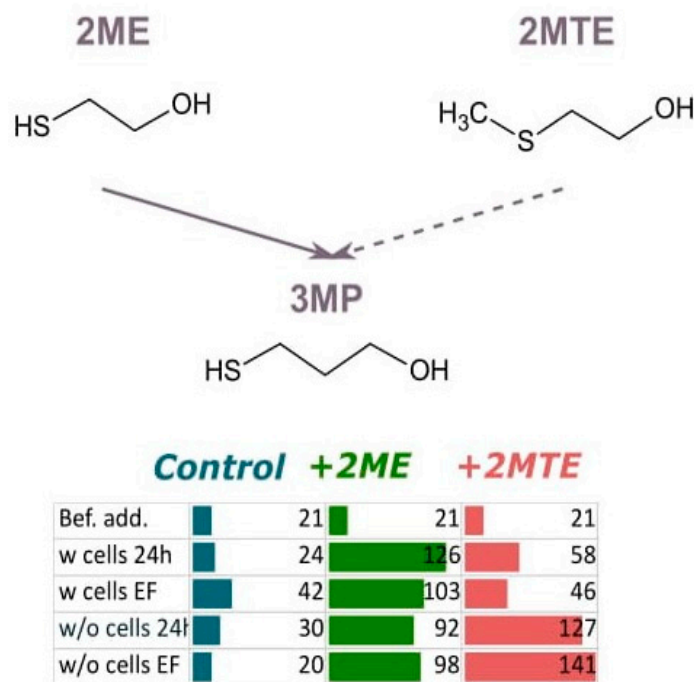


Figure 5. Variation of VSC concentrations during incubation after spiking with compounds deriving from cysteine catabolism. Concentrations expressed in µg/L. **Control:** control without addition; **+2ME:** supplementation with 400 µM 2ME; **+2MTE:** supplementation with 400 µM MTE; Bef. add.: concentration just before spiking; w cells 24 h: incubation in presence of cells for 24 h; w cells EF: incubation in presence of cells for 234 h (end of fermentation); w/o cells 24 h: incubation in absence of cells during 24 h; w/o cells EF: incubation in absence of cells during 234 h. Experiments were conducted in triplicate and the mean values are reported. Raw data are available in supplementary data. EF: end of fermentation.

The wine aging procedure, consisting of incubating the wines in darkness under anoxic conditions for 9 weeks at 50 °C, was developed previously to investigate the modifications of the profile of VSCs [40] or fermentative and varietal volatile carbon compounds [41] that occur during wine storage. Experiments were carried out in triplicate and the concentrations of VSCs were measured initially and after 3, 6 and 11 weeks of aging (Table S2).

Overall, important changes in VSC contents were found during wine storage, as the concentration of only 3 out of the 14 molecules detected (i.e., 2MTE, 4MTB and ETP) remained unchanged over time for the three wines. This observation is in line with a previous study [40], reporting stable concentrations of 2MTE and 4MTB in chardonnay aging under anoxic conditions, while these compounds were totally oxidized during wine storage in the presence of air. These findings are supported by the absence of chemical connections between 2MTE, 4MTB and ETP, produced at very low levels during fermentation, and other VSCs, as shown by spiking experiments.

In the literature, contradictory data have been reported regarding SMTA evolution during wine storage, which could be related to differences in the grape variety or aging procedures [39,42–44]. Applying an 11-week, accelerated aging procedure resulted in a progressive decrease in SMTA but also in ETA concentrations in both synthetic and natural wines (Figure 6). Furthermore, a substantial decrease in thioester content was found during

storage of wines enriched in VSCs, i.e., from 332 to 175 $\mu\text{g/L}$ and from 131 to 72 $\mu\text{g/L}$ for ETA and SMTA, respectively. Furthermore, under the latter condition, EtSH, initially present at a content of 22.5 $\mu\text{g/L}$, was totally eliminated after 6 weeks of storage at 50 °C. These changes could be explained by a chemical conversion of SMTA and ETA into their associated thiols, MeSH and EtSH, as previously discussed (Section 3.2.2.) and reported [45]. The absence of MeSH and EtSH in wine during storage could be due either to a further combination of these compounds with metal cations (copper, zinc, iron) to form stable complexes [5,37] or to their dimerization [44]. This model of evolution is supported by the increase observed in the DMDS content in the two synthetic wines (with and without VSCs enrichment) during the accelerated aging process, from 0 to 2.4 $\mu\text{g/L}$.

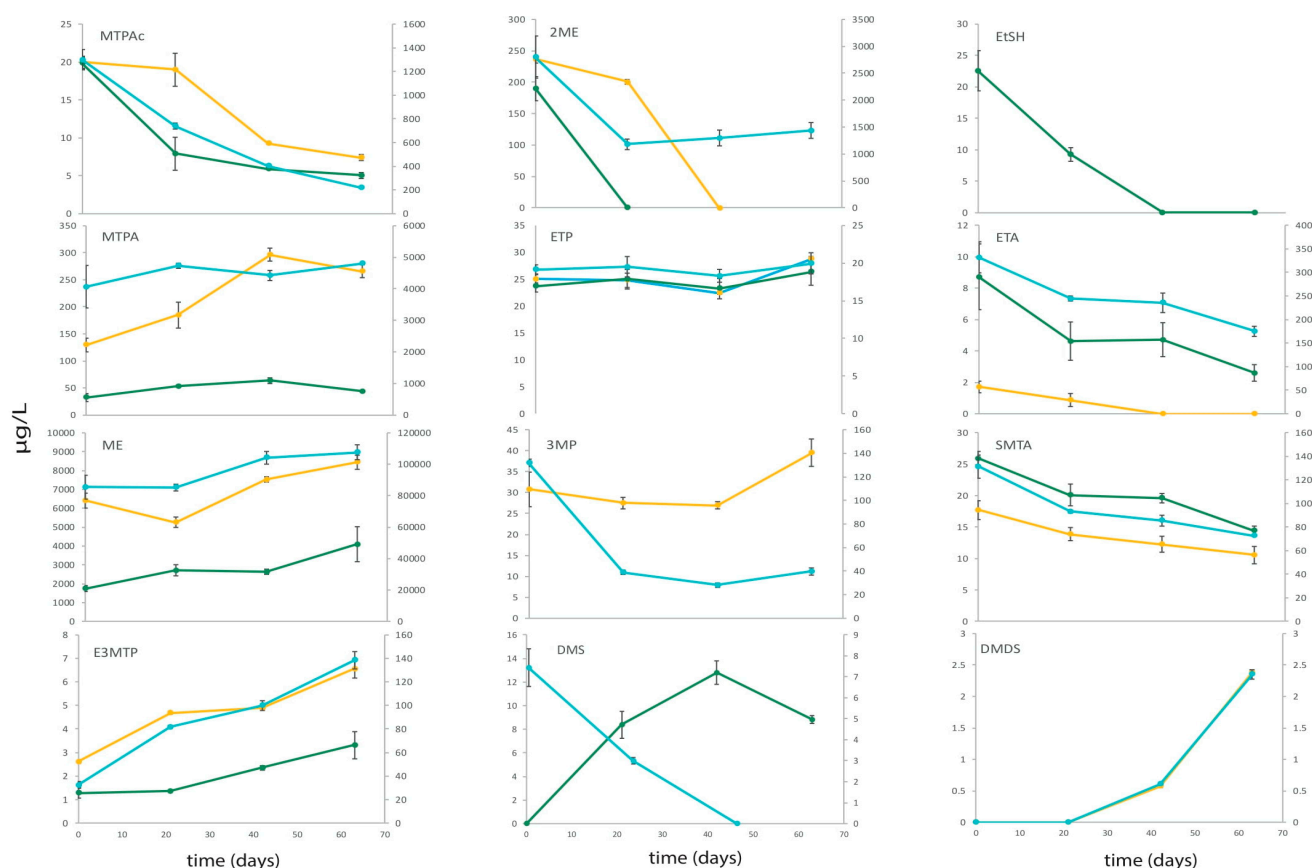


Figure 6. Evolution of VSC concentrations during aging. Concentrations expressed in $\mu\text{g/L}$. An accelerated aging procedure (incubation at 50 °C under anaerobiosis) was applied to synthetic wine (■), Viognier wine (■) and synthetic wine obtained with a synthetic grape enriched with methionine and cysteine (■). Samples were collected after 21, 42 and 63 days of incubation. Experiments were conducted in triplicate and mean values are reported. Raw data are available in supplementary data.

DMS was produced throughout Viognier wine storage, up to 12 $\mu\text{g/L}$, but not during the aging of synthetic wine. Furthermore, the profile of DMS concentration during the storage of a synthetic wine enriched with VSCs revealed a progressive decrease until exhaustion but no formation of this compound. An increase in DMS during natural wine aging has been widely reported in literature [39,42,43,46,47]. A chemical mechanism involving a slow hydrolysis of S-methylmethionine (SMM) from grapes has been suggested to explain this DMS formation [6,34,48]. This hypothesis of a chemical conversion of grape precursor at the origin of DMS is supported by the observations in our work, as DMS production during aging did not occur when SMM was not provided in the medium (in both synthetic wines).

Modifications in the profile of VSCs with higher boiling point during wine storage were also investigated. First, we observed a fast decrease in 2ME concentration until exhaustion in natural and synthetic wines after 3 or 6 weeks of accelerated aging, respectively. This finding reinforces the assumption, based on the comparison of VSCs in wines from different vintages, that the 2ME content in wines tends to decrease over time [49]. The possibility of 2ME dimerization to bis(hydroxyethyl)disulfite via the formation of disulfide bonds, which takes place in a wine medium [50,51], could be responsible for this evolution. For VSC-enriched wine, the decrease in 2ME concentration during the first 3 weeks of storage at 50 °C (from 2.8 to 1.2 mg/L) was followed by a slight increase, up to 1.4 mg/L, suggesting that, in this specific condition, in addition to being degraded, 2ME was produced through an independent chemical route, i.e., intermediates from the catabolism of methionine and cysteine may accumulate in VSC-enriched wines, as these amino acids were used as sole nitrogen sources and further chemically converted to 2ME during aging. The other short carbon chain mercaptan, 3MP, was not detected in Viognier wine, and displayed a pattern of evolution similar to that of 2ME in synthetic wines, but with a lower range of variation.

ME is the only VSC deriving from the Ehrlich degradation of methionine for which the behavior during aging has been documented in literature. Variable evolutions have been reported, with concentrations increasing or decreasing according to wine variety and aging conditions, i.e., duration, tanks, presence of SO₂. ME decrease was explained by its oxidation to methionol-S-oxide or other unidentified compounds, while an increase was supposed to be related to the degradation of methionine to methional via the Strecker reaction and subsequent reduction [42,43,49,51]. The overall increase in ME concentrations observed during the accelerated wine aging carried out in this study supported these interpretations, as the use of anoxic conditions likely prevented ME chemical oxidation. Moreover, larger variations were detected for the Cys-Met wine (+22 mg/L) produced using a methionine-enriched medium, compared to synthetic (+2.1 mg/L) and natural (+2.3 mg/L) wines, in line with a chemical formation of ME from methionine degradation during wine storage. The two-fold increase in the concentration of 3MTPA during the storage of synthetic and natural wines also corresponded well with a contribution of residual methionine as a precursor for the chemical formation of VSCs during aging. Finally, changes in the content in E3MTP and 3MTPAc of wine during aging, not investigated until now, were observed with an increase in E3MTP concentration, likely originating from the slow chemical combination of a small fraction of 3MTPA with ethanol. Conversely, 3MTPAc concentration decreased with aging, which could be explained by its hydrolysis. Volatile sulfur compounds are known to have a high impact on the organoleptic properties of wines. Some compounds are associated with reduced wine character, such as H₂S, methanethiol, DES, DMDS, DES, ethanethiol and carbon disulfur, imparting odors of cooked cabbage, onion, garlic, or rotten eggs [52]. Other compounds make positive contributions, as already described for DMS and MTPA, with an increase in fruitiness [22,53]. In our conditions, an increase in DMDS, methionol and E3MTP above their perception thresholds could contribute to the reduced character of wines. Conversely, an increase in DMS and 3MTPA, which contribute to a fruity character, could counterbalance the impact of the molecules involved in the reduced character. The perception thresholds are very different depending on the molecules, from µg/L to mg/L, so the impact of the different molecules and their interactions must be considered when assessing the overall positive or negative contribution of the evolution of VSC profiles during aging.

4. Conclusions

This work offers a comprehensive overview of the origin of VSCs during wine fermentation and their evolution during aging, examining the formation of 18 sulfur-containing molecules under laboratory conditions (chemically-defined must and wine, specific yeast strain, accelerated aging procedure). Yeast plays an important role in these productions through the catabolism of sulfur amino acids, methionine and cysteine through the Ehrlich pathway which is directly involved in the synthesis of ME, 3MTPA, 3MTPAc, E3MTP, 3MP

and 2ME. Other conversions are catalyzed by yeast, such as the acetylation of thiols (MeSH, EtSH) to thioesters (SMTA, ETA). Furthermore, during fermentation, in addition to these enzymatic productions, chemical reactions are responsible for interconversions between VSCs, e.g., between thiols and disulfides (DMDS, DEDS).

Thiols appear as a core component of VSC formation during wine fermentation, being produced from amino acids or heavy VSCs and then converted to light VSCs. However, experimental hurdles, due to their low boiling point, have limited our understanding of their role in VSC production. To overcome this problem, further experiments monitoring thiol concentrations in the headspace of fermenters are currently in progress.

Substantial changes in VSC profiles occur during wine storage, with either an increase or decrease in their concentration depending on the chemical reactivity of their sulfur group. These modifications can be individually beneficial or detrimental to wine quality, and should be considered with respect to the overall sensory perception of each wine.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation8040139/s1>, Table S1: Raw data of volatile sulfur compounds concentrations during spiking experiments; Table S2: Raw data of volatile sulfur compounds concentrations during accelerated wine aging experiments.

Author Contributions: Conceptualization, R.J.-L., V.F., J.-M.S., C.C. and A.B. Formal analysis, R.J.-L., V.F. and A.B.; Funding acquisition, C.C.; Investigation, R.J.-L.; Methodology, R.J.-L.; Project administration, C.C.; Supervision, J.-M.S., C.C. and A.B.; Validation, V.F.; Writing—original draft, R.J.-L.; Writing—review & editing, V.F., J.-M.S., C.C. and A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was conducted in the framework of RJL's PhD project as part of the within the Innovative Training Network Aromagenesis. This research was funded by the European Union's Horizon 2020 research and innovation programme, Marie Skłodowska-Curie grant agreement N° 764364.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data supporting the conclusions of this article are included in the manuscript.

Acknowledgments: We would like to acknowledge Pascale Fernandez for her technical support and Valérie Nolleau and Teddy Godet for their assistance in the analysis of sulfur compounds.

Conflicts of Interest: The authors declare no conflict of interest.

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