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# The contribution of varietal thiols in the diverse aroma of Italian monovarietal white wines

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ABSTRACT

Thanks to their low odor detection thresholds, free varietal thiols (VTs) play a key role in the primary aroma of wines, to which they confer an intense scent reminiscent of box tree, grapefruit, citrus fruits, passionfruit and cat urine odor. Excluding wines from a few VT-rich grapevine cultivars, VTs appear to be present in most cultivars at trace levels, although a comprehensive dataset is still missing. The low concentration of VTs combined with their high reactivity and matrix complexity make their determination in wines a challenging task. In this research an optimized liquid chromatography – tandem mass spectrometry (LC-MS/MS) method was validated and used for the quantification of 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA) and ethyl 3-sulfanylpropionate (E3SP) in 246 samples (vintage 2019) representative of 18 monovarietal Italian white wines. VTs were detected in all cultivars even though higher values of 3-SH were found in Lugana, Müller-Thurgau and Verdicchio cultivars. Müller-Thurgau wines showed the highest level of 4-MSP, that was mainly correlated to the odor descriptors of passionfruit and box tree/cat urine. The VTs composition of Müller-Thurgau was confirmed on a second set of 50 wines from different vintages. From a sensory perspective, the samples of Müller-Thurgau showed the best positive correlations between chemical variables and the odor descriptors thiol note, passion fruit and box tree/cat urine. These notes are significantly related to 4-MSP, suggesting that it could play a relevant olfactory role for the aroma of Müller-Thurgau wines. Sorting analysis allowed to group these wines according to their thiolic characteristics. The chemical variables and the odor descriptors attributable to the thiol notes are important for Müller-Thurgau and Lugana wines, while the contribution of thiol notes was sensorially negligible for the other wines.

## 1. Introduction

Wine aroma originates from a complex mixture of various compounds; some of these molecules derive directly from the grapes, while most of them are released and produced during wine fermentation or ageing (Polášková et al., 2008). Among these compounds, volatile thiols are odor-active molecules belonging to the broad category of Volatile Sulphur Compounds (VSCs) whose contribution to wine aroma is significant (Buettner and Schieberle, 2001). Volatile thiols, historically named mercaptans, are organic molecules containing a -SH group and

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are known to be potent odorants, playing a key role in food aroma, due to their broad presence and low Odor Detection Threshold (ODT) (McGorrin, 2011). Among the various volatile thiols present in wine, the so-called varietal thiols (VTs) are already present in grapes (usually in a bonded form) and are therefore considered as "varietal compounds" (Villano et al., 2017) even if the winemaking process in general, and the specific yeast strain activity in particular, can increase their content in finished wines (Swiegers and Pretorius, 2007). According to the huge bibliography proving the sensory importance of specific VTs for the aroma of different wines, the most powerful odor-active VTs in wine are 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH) and its ester 3-sulfanylhexyl acetate (3-SHA) (Roland et al., 2011). Thanks to its ODT of 3 ng/L in wine (Howell et al., 2004), 4-MSP (box tree and cat urine odor) is the most potent thiol odorant in wine as well as the spearhead of Sauvignon blanc aroma (Darriet et al., 1995; Vermeulen et al., 2006). 3-SH is mostly associated with the grapefruit odor (ODT: 60 ng/L) and, despite being present in many wines at concentrations higher than 4-MSP, it is not always perceptible (Flamini et al., 2010). 3-SHA is the esterification product of 3-SH and acetic acid, and is related to the passionfruit scent (ODT: 4 ng/L) (Tominaga et al., 1996). Finally, ethyl 3-sulfanylpropionate (E3SP) is an ethyl ester with an olfactory threshold significative higher than other VTs (ODT: 500 ng/L) which is known to contribute to the olfactory bouquet of aged champagne wines (Tominaga et al., 2003a).

The role of the 3 most representative VTs in wine aroma (4-MSP, 3-SH, and 3-SHA) have been extensively evaluated, especially in the last 30 years, and the importance of their contribution have been cross-confirmed by chemical and sensory analysis. The above-mentioned compounds have been recently included in the list of 35 wine aroma vector within the citric-green category (Ferreira et al., 2022). 4-MSP was identified as a powerful modulator of Scheurebe aroma using aroma extract dilution analysis (AEDA) coupled to GC-O (Guth, 1997). The role of this VT was further assessed a few years later in Maccabeo wines by reconstitution study and sensory analysis (Escudero et al., 2004) where it demonstrated its importance, especially concerning regional identification. Due to its more frequent presence above the ODT, 3-SH was deeply investigated; for instance, some article published by Ferreira et al. demonstrated its importance for the enhancement of fruity and citric nuances in Grenache aroma by AEDA and omission/reconstitution studies (Ferreira et al., 2002). More recently, a similar experimental design (aroma reconstitution and omission studies coupled to GC-O) was used for the characterization of Petit Manseng key odorants which underlined the significative role of 3-SH (Lan et al., 2021). 3-SH was also identified to be crucial in Syrah aroma because of its modulation role for the fruity nuance; Geffroy et al. highlighted a significative decrease of this attribute by omission experiments (Geffroy et al., 2020). Finally, a similar approach was extended to 3-SHA to study its contribution in Sauvignon Blanc aroma (Benkwitz et al., 2012); AEDA and omission/reconstitution experiments demonstrated the importance of this VT. To complete the scenario of thiols contribution a comprehensive sensory study that covers the role of 5 major VTs in white wine aroma was published by Mateo-Vivaracho et al.: this research covered 130 different samples of 6 cultivars and underlined how 4-MSP, 3-SH, and 3-SHA impact odor profile giving a relevant contribution to fresh, tropical, green and fruity nuances (Mateo-Vivaracho et al., 2010).

Despite their important contribution to wine aroma, there are several issues that make the quantification of VTs a challenging task (Chen et al., 2019). First, the VTs content in wine is usually detected in parts per trillion (ng/L), meaning that an enrichment technique and a sensitive instrumentation are required by most analytical protocols (Hart, Jolly, & Ndimba, 2019; McGorrin, 2011). In addition, wine is a very complex and highly variable matrix, where the content of many compounds can heavily affect the measurement of trace analytes (Bonnaffoux et al., 2018; Lyu et al., 2021; Spence and Wang, 2018). Finally, the thiols of interest are reactive molecules whose content can be affected by several reactions, impacting on their final concentration in wine (Liem-Nguyen et al., 2015; Ugliano et al., 2011); the-SH group is known to be the most reactive functional group available in non-synthetized products (Petri et al., 2020). A broad variety of protocols, based on many different instrumentations and sample preparation strategies, have been described in the last years, although it is possible to group them into two main approaches: GC-MS based and LC-MS based methods (Wang et al., 2020). The most recent GC-MS based methods involve time-consuming sample preparation to allow a proper enrichment and isolation of the analytes: most of them work through a derivatization to enhance their response to mass spectrometers and to increase their stability that represents the main problem for their quantification (Schneider et al., 2003; Schoenauer and Schieberle, 2019). These procedures often require large volumes of sample (Darriet et al., 1995), long preparation and chromatographic separation times; in addition, conventional methods often need toxic derivatizing agents (Tominaga et al., 1998) and hazardous solvents in contrast to the Green Analytical Chemistry (GAC) rules (Armenta et al., 2019; Płotka-Wasylka et al., 2021), or requires some specific instrumentations (e.g., Purge Trap injectors, HS – SPME) (Musumeci et al., 2015). On the other hand, LC-MS methods are increasing in popularity thanks to a simplified sample preparation (Mayr et al., 2015), an improved sensitivity provided by electrospray sources, and the possibility of analyzing free and bonded forms in the same run (Tonidandel et al., 2021). Since VTs are not directly detectable by ESI-MS, derivatization is mandatory for their detection (Liu et al., 2014). Even though many derivatizing agents have been successfully used for VTs analysis (Capone et al., 2015), 2-phenyl-1,2-benzisoselenazol-3-one (Ebselen) is the one that showed the best selectivity, efficiency, versatility, and stability (Quintanilla-Casas et al., 2015; Vichi et al., 2013, 2015, 2014).

This study was performed within the context of the D-Wines PRIN project titled "The aroma diversity of Italian white wines" and aimed at investigating the origin of flavour characteristics of 18 Italian white wines representative of the most important national productions from a chemical, biochemical, and sensory perspective. In this frame, the specific goal of this research was to quantify the varietal thiols 4-MSP, 3-SH, 3-SHA, and E3SP in the 18 monovarietal wines, and to test their olfactory impact by descriptive and sorting sensory analyses, for the first time. To do this, a simple, fast, and robust LC-MS method, based on that proposed by Román et al. (Román et al., 2018), was optimized and validated.

## 2. Materials and methods

### 2.1. Solvents and standards

All chemicals, including salts and solvents used for the extraction and LC-MS analysis (LC grade) were purchased from Merck KGaA (Darmstadt, Germany). Plastic syringes and the 0.22 μm cartridge filter were supplied by Millex-GV (Millipore, Tullagreen, Ireland). Analytes (4-MSP, 3-SH, and 3-SHA) and internal standards (4-Methoxy-α-toluenethiol) were all bought from Merck KGaA (Darmstadt, Germany) at the highest purity available (≥95%) except for Ethyl 3-sulfanylpropionate (E3SP), which came from abcr GmbH (Karlsruhe, Germany). The chemical structure of the analytes and internal standards are reported in Fig. 1. Finally, Ebselen, the derivatizing agent of choice, also came from Merck KGaA (Darmstadt, Germany).

### 2.2. Wine samples

White wines, sample set #1. A first sampling consisted of 246 monovarietal white wines (vintage 2019) from 18 Italian grape cultivars collected in 9 Italian regions. For each variety, between 8 and 21 different commercial wines, all produced without wood refining, were collected from the main geographical areas of production: 21 Lugana (Veneto, LUG); 17 Gewürztraminer (Trentino Alto Adige, GWR); 16 Cortese (Piemonte, CRT), 15 Erbaluce (Piemonte, ERB); 14 Albana

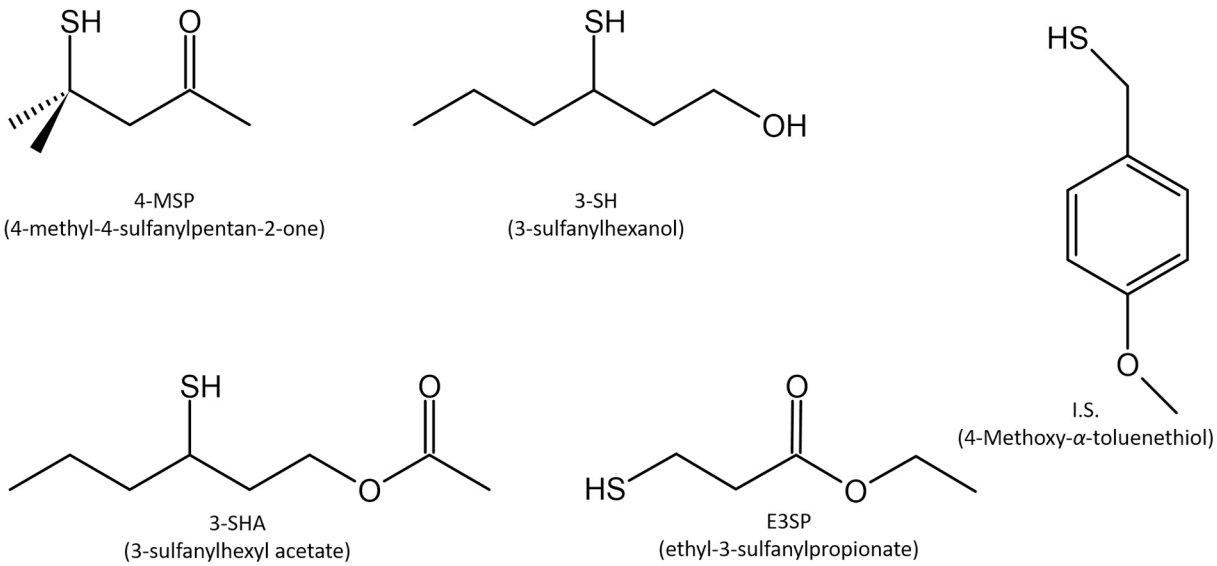


**Fig. 1.** Free volatile thiols of interest (4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA) and ethyl 3-sulfanylpropionate (E3SP)) and the molecule used as an internal standard.

(Emilia-Romagna, ALB); 14 Garganega (Veneto, GAR), 14 Ribolla Gialla (Friuli Venezia Giulia, RIB); 14 Vermentino (Sardegna, VEM); 13 Arneis (Piemonte, ARN); 13 Falanghina (Campania, FAL); 13 Greco di Tufo (Campania, GRE); 13 Müller-Thurgau (Trentino Alto Adige, MLR); 13 Pallagrello (Campania, PAL); 12 Fiano (Campania, FIA); 12 Nosiola (Trentino Alto Adige, NSL); 12 Pinot grigio (Friuli Venezia Giulia /Veneto/Trentino Alto Adige, PG); 11 Verdicchio (Marche, VERD); 8 Vernaccia (Toscana, VER). The bottles were stored at cellar temperature (4 °C) until analysis (performed in 10 months after sampling).

Müller-Thurgau, sample set #2. An additional sample set of 50 Müller-Thurgau commercial wines (12 produced in 2019 and 38 in 2020) was also analyzed. The bottles were stored at cellar temperature (4 °C) until analysis (performed in 4 months after sampling).

### 2.3. Extraction procedure

The extraction method for LC-MS/MS, developed by Román et al., 2018 was used with some modifications that increased both productivity and performance. The main modification adopted was the use of acetonitrile instead of ethanol for a better and faster phase separation which did not produce precipitates due to its lower polarity. 4-Methoxy-α-toluenethiol instead of 1-hexanethiol was adopted as internal standard, reproducing the procedure purposed by Vichi et al., 2015. The upgraded protocol is reported below.

First, for the extraction phase, a salt mixture was prepared weighing 12 g of anhydrous Magnesium sulphate, 4 g of Sodium chloride, 1.5 g of Sodium citrate dibasic sesquihydrate, 3 g of dehydrate tribasic Sodium citrate in a 50 mL screw cap plastic falcon tube.

Separately, 35 mL of wine sample, 35 μL of internal standard solution (4-methoxy-α-toluenethiol 100 μg/L) and 5 mL of acetonitrile were mixed into a 50 mL glass flask. The prepared solution was then transferred into the plastic falcon containing the salt mixture and stirred for 10 min at 60 rpm in an orbital shaker. The organic and aqueous phases contained in the falcon tube were then separated by centrifugation (4500 rpm, 5 °C, 5 min); 2 mL of the organic phase were transferred into a 4 mL amber vial and spiked with 150 μL of Ebselen ethanol solution (600 mg/L). The obtained mixture was stirred for 5 min at 60 rpm in an orbital shaker to perform the derivatization process, filtered with a 0.22 μm cartridge filter, and stored at −20 °C until analysis by LC-MS.

### 2.4. LC-MS/MS conditions

The separation was performed with an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) using an Acquity UPLC BEH C18 (1.7 μm, 2.1 mm × 50 mm) column (Waters corporation, Milford, MA, USA) at 40 °C. 10 μL was the injection volume. The mobile phase consisted of water + 0.1% formic acid (A) and methanol + 0.1% formic acid (B). Elution was performed at 0.45 mL/min with the following gradient: 0 – 0.25 min at 0% B, 0.25 – 6.5 min increase to 91% B, 6.5 – 6.51 min increase to 100% B, 6.51 – 8 min hold 100% B, 8 – 8.5 decrease to 20% B, and 8.5 – 11 min hold 20% B. An integrated valve was scheduled to release the analytes into the mass spectrometer only from 5 to 7 min in order to keep source and analyzer free from dirt.

An AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA) was operated in positive ion multiple reaction monitoring (MRM) mode using a Turbo V ion source with the following settings: Curtain Gas (CR) 35 °C, IonSpray Voltage (IV) 5500 V, Temperature 250 °C, Collision Gas (CAD) *Medium*, Ion Source Gas 1 (GS1) 50 psi, and Ion Source Gas 2 (GS2) 60 psi. Each period was scheduled with 600 cycles of 0.2 s cycle time each. The signal was acquired only in the analyte elution window (from 5 to 7 min). The detailed settings for the MS/MS method are summarized in Table 1. MultiQuant and Analyst from AB Sciex LLC (Framingham, MA, USA) were used for data acquisition and elaboration, respectively.

### 2.5. Calibration

Calibration curves were acquired by submitting equal-to-real spiked samples prepared using a deodorized commercial white wine (Tavernello, Caviro, Faenza, Italy) to the whole analytical process. Deodorization was performed by stirring the wine with a commercial food-grade charcoal (Geosorb, Bordeaux, France) at 100 g/L. Curves were calculated interpolating 11 calibration points from 0.5 ng/L to 1000 ng/L highlighting fitting at lower levels (weighting 1/x).

### 2.6. Sensory analysis

#### 2.6.1. Descriptive analysis

Twelve panelists (22–50 years old; 5 males, 7 females) were recruited among students and researchers of the University of Naples Federico II (Department of Agricultural Sciences, Division of Vine and Wine Sciences), and selected based on their interest, availability, and

**Table 1**
Instrumental operating settings for each analyte: Quanitifer transition (Q), Qualifier transition (q), Parent (Q1) and product (Q3) ions, Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE), and Collision Cell Exit Potential (CXP). 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), ethyl 3-sulfanylpropionate (E3SP) and 4-Methoxy-α-toluenethiol (IS) were considered after derivatization with ebselen (ebs).

| Analyte | Retention time (min) | Q1 | Q3 | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|
| 4-MSP-ebs (Q) | 5.51 | 408 | 276 | 40 | 14 | 15 | 20 |
| 4-MSP-ebs (q1) | 5.51 | 408 | 310 | 40 | 14 | 15 | 20 |
| 4-MSP-ebs (q2) | 5.51 | 408 | 184 | 40 | 14 | 15 | 20 |
| E3SP-ebs (Q) | 5.61 | 432 | 362 | 100 | 15 | 31 | 20 |
| E3SP-ebs (q1) | 5.61 | 432 | 318 | 96 | 15 | 42 | 20 |
| 3-SH-ebs (Q) | 5.63 | 410 | 276 | 5 | 12 | 12 | 23 |
| 3-SH-ebs (q1) | 5.63 | 410 | 156 | 5 | 12 | 12 | 23 |
| 3-SH-ebs (q2) | 5.63 | 410 | 196 | 5 | 12 | 12 | 23 |
| IS-ebs (Q) | 6.06 | 430 | 276 | 15 | 13 | 19 | 17 |
| IS-ebs (q1) | 6.06 | 430 | 196 | 15 | 13 | 19 | 17 |
| IS-ebs (q2) | 6.06 | 430 | 121 | 15 | 13 | 19 | 17 |
| 3-SHA-ebs (Q) | 6.23 | 452 | 276 | 34 | 11 | 5 | 8 |
| 3-SHA-ebs (q1) | 6.23 | 452 | 196 | 34 | 11 | 5 | 8 |

sensory abilities. They were all expert wine tasters with previous experiences in performing descriptive sensory analysis tests. All procedures were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments, or comparable ethical standards. Participation was on a voluntary basis and, prior to the experiments, tasters were required to sign an informed consent form disclosing the type of research, voluntary participation and agreement to taste/smell reference solutions and wines. All data were collected anonymously.

The panel training and the sensory assessment of wine samples were performed as previously described (Pittari et al., 2020), with slight modifications.

Panel training: panelists were trained to recognize, discriminate and describe olfactory stimuli by identifying 62 odor standards, selected from the literature (Campo et al., 2008; Nanou et. al., 2020; Sáenz-Navajas et al., 2011) as representative of different odor families and descriptors of white wines (Table 1S). During the first 4 training sessions, panelists were provided with a list of 16 white wine odor families (fruity, citric, exotic fruit, dried fruit, floral, vegetal, balsamic, spices, woody, aromatic herbs, sweet odors, undergrowth, lactic, thiolic, mineral, off-odors). In each session, from 13 to 17 odor standards, belonging to 2 to 5 different odor families were presented to the panelists. Panelists were asked to smell each standard (served in covered disposable 80 mL plastic cups), and to recognize the corresponding odor family/ies or specific descriptors based on their knowledge, expertise, and previous descriptive training experiences. At the end of each training session, the perceived sensations were discussed with the participants to prevent overlapping and redundancies among terms, to help their memorization, and to generate a consensual vocabulary.

To train panelists in evaluating the intensity of odor descriptors, a fifth session was dedicated to a ranking test using aqueous solutions of isoamyl acetate at 100, 250, 400, 600, and 800 μg/L.

Finally, 4 further sessions were carried out to familiarize the panel with the procedure and real wines. For this purpose, 20 wines picked among the samples under investigation (5 per session, from each grape cultivar, 35 mL served in black glasses coded with three-digit codes and presented in a randomized order across panelists) were assessed using the same evaluation procedure as run-through prior to the real analytical sessions. Subjects were asked to smell wines and assess the perceived odors on the following numerical category scale: 1 = very low, 2 = low, 3 = medium, 4 = high, and 5 = very high, with half values allowed. They were provided with water and required to wait at least 20 s between samples. Each session was followed by a discussion on the odor families/descriptors used, as well as on the use of the scale.

Sensory descriptive assessment: the sensory analysis was performed on the 246 wines composing the first sample set. A total of 492 samples (246 wines*2 replicates) were assessed during 41 sessions (12 wines/session) according to a full randomized design per replicate. In the very few cases of unavailability, recovery additional sessions were organized within the two following days. Samples (35 mL) were served in black glasses identified by three-digit codes and presented in a randomized order among panelists. Wines were evaluated in individual booths at room temperature (19 ± 2 °C).

The consensually generated vocabulary including 16 odor families previously mentioned, as well as more specific odor descriptors, was employed. The perceived odor intensity was evaluated using the numerical category scale, as had happened during training. Panelists were asked to smell each wine sample, to recognize the perceived odor families/descriptors and to score the corresponding intensities on the scale above mentioned.

The aim of this research was to investigate the contribution of varietal thiols to the aroma of Italian monovarietal white wines. Therefore, only results regarding odor families or specific descriptors previously reported in the literature as correlated to varietal thiols were considered here, and namely: thiolic, box tree/cat urine, citric, grapefruit, tropical fruit, and passion fruit (Roland et al., 2011).

### 2.6.2. Sorting analysis

Twelve enologists (28–62 years old; 6 females, 6 males) belonging to Assoenologi association (Milan, Italy), with extensive experience in Italian wines tasting, participated in sorting analysis of Müller-Thurgau wines. In previous studies, sorting has been already applied to highlight aroma similarity/dissimilarity among wines (Johnson et al., 2013; Piombino et al., 2004). In our study, samples were presented in transparent coded glasses (ISO 3591, 1977) with plastic covers, and in randomized order. Panelists were requested to sort samples into as many groups as they wanted, based on ortho- and retro- nasal aroma similarity. After grouping, the panelists were also asked to assign the most characteristic descriptors to each group based on a pre-defined list of descriptors from the aromatic compounds identified in MLR wines and reported in literature (Moio, 2016; Nicolini et al., 1996; Versini et al., 1995). Furthermore, panelists were also allowed to formulate new descriptors.

## 2.7. Data analysis

A Principal Component Analysis (PCA) was conducted using XLSTAT (version 2019.6, Addinsoft, Paris, France) and carried out on the correlation matrices (Pearson, $p < 0.05$) between the concentrations of VTs and the mean intensities of the olfactory descriptors – rated by the 12 panelists – for each wine.

The analysis of variance (ANOVA) and the visualization of chemical results were performed using SPSS V19 (IBM Statistics).

Wines from Sorting were mapped by means of Multidimensional Scaling (MDS) using Kruskal'stress parameter to evaluate the efficiency of approximation of the distances between the products. Agglomerative Hierarchical Clustering (AHC) was carried out for grouping samples whereas relationships between wines and thiolic descriptors were analyzed by regression analysis carried out on wines' MDS configuration and number of citations by enologists for each descriptor. All analyses were performed with XLSTAT (version 2021 3.1., Addinsoft, New York, USA).

## 3. Results and discussion

### 3.1. Method validation

Since the aim of the method was to develop a high-throughput protocol suitable for the analysis of a considerable number of samples, it was validated in terms of limit of detection (LOD) and quantification (LOQ), linearity range, intra-day and inter-day repeatability, and evaluating recoveries in real samples spiked with a known amount of VTs. All validation data are reported in Table 2.

Validation data clearly highlighted the suitability of the method for its stated purpose. For all VTs, the LODs are lower than their Odor Detection Thresholds (ODT), the LOQs are close to ODTs for 4-MSP and 3-SHA, and lower for 3-SH and E3SP, and the linearity range covers the amount detected in most of the samples with excellent precision ($R^2 >$ 0.995). These results can be considered satisfactory especially considering that no enrichment steps were applied, thus making this procedure fast and straightforward. Intra-day repeatability was assessed repeating the whole procedure 6 times for the calibration level at 100 ng/L whereas inter-day repeatability was evaluated extending the same program for 2 weeks (3 repetitions per week). The relative standard deviation (RSD%), ranging from 2.33 to 11.32% intra-day, and from 7.81 to 14.78% inter-day, demonstrates good stability and robustness, especially considering the low analytes' concentration. Since this method is very similar to the one from which it was developed, the evaluation of repeatability using only one central level can be considered satisfactory. Finally, recoveries were evaluated by spiking an untreated white wine sample with 10, 100, and 1000 ng/L of the four VTs; values ranged between 92.0% and 121.7% for all analytes demonstrating reliability and robustness.

### 3.2. Volatile thiols in Italian white wines

The four major VTs investigated in this study were quantified in the 246 monovarietal white wines coming from the 18 varieties (Sample set #1) using the method described above. The results are given in Table 2S, providing for the first time a comprehensive overview of the highly diversified Italian white wines scenario. From a varietal point of view, VTs were observed in at least one sample of each cultivar, implying that, in the context of Italian white wines, these compounds can be considered rather ubiquitous. Quantitative variations across cultivars, as well as within the same cultivar, were rather large, reflecting the complex array of factors that can determine the VTs content of wines, such as the pedoclimatic characteristics of the vineyard, the management of the winery pre-fermentative steps, the yeast strain used in fermentation, and the levels of oxygen exposure of the wines after fermentation and after bottling (Coetzee and du Toit, 2012). These results are in strong agreement with the observations by Mateo et. al. (Mateo-Vivaracho et al., 2010) concerning VTs variations across Sauvignon blanc wines as well as for other cultivars; they also agree with those by Capone et al., 2017 for Chardonnay wines. To highlight the complexity of VTs occurrence in wines further, it is also worth mentioning that the relative ratios of the four analyzed VTs differed, so that some cultivars, or even individual samples, appeared to enjoy a closer association, thanks to the high occurrence of, for example, 3-SH or 3-SHA, whereas others could be characterized more by the content of 4-MSP. As such, it can be argued that, even within the same cultivar, it is possible to obtain different VTS aroma profiles. A content of VTs above their ODTs for many wines means that it would be possible for winemakers to adopt techniques able to valorize their contribution. Indeed, VTs play a relevant sensory role, as will be discussed later.

The content of 4-MSP in the wines of sample set #1 is plotted in Fig. 2. The richest cultivar was Müller-Thurgau with a content ranging from n.d. (not detected) to 35.1 ng/L, with 3 samples showing values above 20 ng/L, and an average concentration of 10.9 ng/L. As for the other cultivars, some samples with relatively high values were found, although the contents of 4-MSP were in these cases below 10 ng /L, a value close to the Müller-Thurgau average content.

4-MSP was the first VT identified in Sauvignon blanc wines (Darriet et al., 1995) and it is also the one with the lowest ODT, equal to 3 ng /L (in wine); in this cultivar the reported concentration ranges from 0 to 400 ng/L. However, also other cultivars, such as Scheurebe, Macabeo, Gewürztraminer, Riesling, Muscat, Colombard, Petit Manseng and Tokay wines (Roland et al., 2011), were reported to contain detectable amounts of 4-MSP, while it is the first time, to our knowledge, that this compound is found in Müller-Thurgau.

As for 3-SH and 3-SHA, significant contents in these compounds were observed in several cultivars. The highest 3-SH content was observed in 3 Lugana wines (Fig. 3), with a maximum value of 2782 ng/L and an average value of the 21 samples analyzed of 1347 ng/L. Several studies concerning this cultivar confirm the presence of VTs, which is also strongly affected by the winemaking method (Luzzini et al., 2021; Mattivi et al., 2012). Another cultivar rich in 3-SH is Verdicchio, with an average content of 898 ng/L and a maximum value of 1659 ng/L.

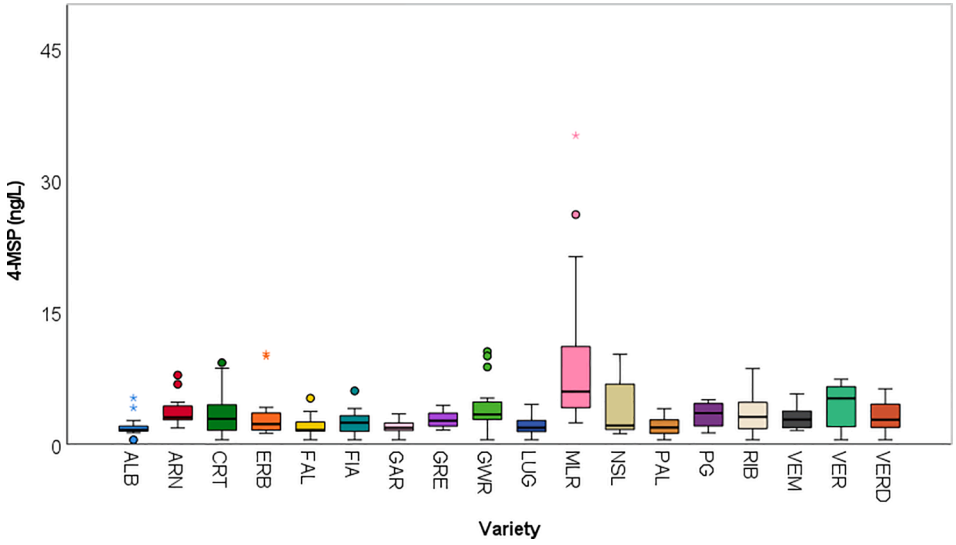


**Fig. 2.** 4-methyl-4-sulfanylpentan-2-one (4-MSP) distribution in the Italian cultivars (ng/L): Albana (ALB), Arneis (ARN), Cortese (CRT), Erbaluce (ERB), Falanghina (FAL), Fiano (FIA), Garganega (GAR), Greco di Tufo (GRE), Gewürztraminer (GWR), Lugana (LUG), Müller-Thurgau (MLR), Nosiola (NSL), Pallagrello (PAL), Pinot Grigio (PG), Ribolla (RIB), Vermentino (VEM), Vernaccia (VER), and Verdicchio (VERD).

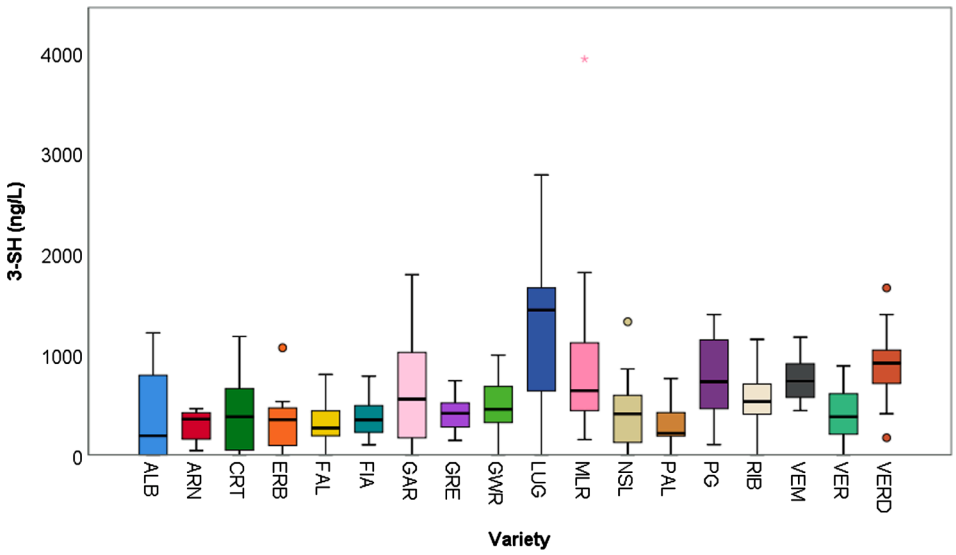


**Fig. 3.** 3-sulfanylhexan-1-ol (3-SH) distribution in the Italian cultivars (ng/L): Albana (ALB), Arneis (ARN), Cortese (CRT), Erbaluce (ERB), Falanghina (FAL), Fiano (FIA), Garganega (GAR), Greco di Tufo (GRE), Gewürztraminer (GWR), Lugana (LUG), Müller-Thurgau (MLR), Nosiola (NSL), Pallagrello (PAL), Pinot Grigio (PG), Ribolla (RIB), Vermentino (VEM), Vernaccia (VER), and Verdicchio (VERD).

Verdicchio is closely related to Lugana (Ghidoni, 2010), as indicated by the genetic and ampelographic analyses, and provides wines that often possess thiolic notes (Luzzini et al., 2021). However, the wine showing the highest amount of 3-SH was again a sample of Müller-Thurgau (3933 ng/L), while the average of all the 13 samples was 1014 ng/L, in line with previously found values for this variety (Tonidandel et al., 2021).

3-SHA is formed by the esterification of 3-SH with acetic acid during fermentation. The final content of this compound depends on many factors, including the balance between the activities of the enzymes that promote esterification and those involved in the hydrolysis, but it is also affected by the yeast strain used (Swiegers et al., 2006). This ester is produced above the equilibrium during the fermentation and is relatively quickly hydrolyzed to 3-SH during the storage. Usually this compound amounts to 10% of the 3-SH content in young wines (Cutzach et al., 1999), although in this study this proportion was not always found. Results did not indicate the presence of monovarietal wines particularly rich in this compound (Fig. 4), although some samples showed to contain some 3-SHA but mostly below 4 ng/L, a value lower than the ODT. Only the sample of Müller-Thurgau with the highest concentration of 3-SH (3933 ng/L) contained 133 ng/L of 3-SHA.

3-SH and 3-SHA are more ubiquitous than 4-MSP and have been identified in many varieties; however, Sauvignon blanc is the cultivar where these compounds reach concentrations close to 20 μg/L for 3-SH and greater than 2 μg/L for 3-SHA (Coetzee and du Toit, 2012).

An unexpected unknown peak was detected at 5.44 min, responding to the same MRM transitions of 3-SH but eluted a few seconds before. Vichi et al. (who worked in similar chromatographic conditions) attributed it to ethyl 3-sulfanylpropionate (E3SP) by HRMS analysis (Vichi et al., 2015). This compound is a thiolester known to confer a pleasant fruity, grapy, rhubarb flavour. Concord grape and related wines are products where parts-per-million-level amounts of this compound were detected in 1983 by Kolor et. al. (Kolor, 1983). Nowadays E3SP is mostly known because of its significant role in the odor of Munster and

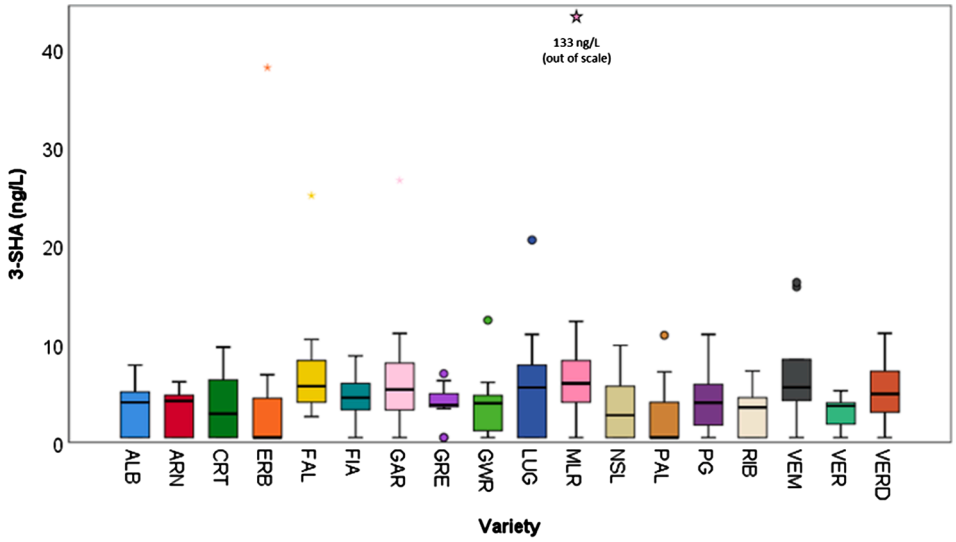


**Fig. 4.** 3-sulfanylhexyl acetate (3-SHA) distribution in the Italian cultivars without the highest point of 1 MLR wine: (133 ng/L) Albana (ALB), Arneis (ARN), Cortese (CRT), Erbaluce (ERB), Falanghina (FAL), Fiano (FIA), Garganega (GAR), Greco di Tufo (GRE), Gewürztraminer (GWR), Lugana (LUG), Müller-Thurgau (MLR), Nosiola (NSL), Pallagrello (PAL), Pinot Grigio (PG), Ribolla (RIB), Vermentino (VEM), Vernaccia (VER), and Verdicchio (VERD).

Camembert cheese (Sourabié et al., 2008). Its presence in wine was evaluated by Tominaga et. al. who detected a relevant amount of thiolesters (comprising E3SP) in aged sparkling wines, which they confirmed have a considerable odor contribution (Tominaga et al., 2003a).

Unfortunately, with the presented chromatographic setting, E3SP had the same retention time of 3-SH and contributed to its measured concentration. To overcome this issue, E3SP was added as a target analyte by repeating the whole extraction procedure, comprising MS optimization, method validation, calibration, and measurement of all wine samples. The analytical results (Fig. 5) showed that E3SP was present in very low concentrations only, well below its ODT (200 ng/L, Tominaga et al., 2003a) for most samples, excepting two Lugana wines; because of these results, the E3SP aroma contribution could be considered negligible. Even though E3SP was identified in low concentrations, the 3-SH areas were corrected by subtracting the E3SP contribution from the 3-SH signal in order to obtain a reliable quantitation. Finally, the peak at 5.44 min for which this focus was set and whose structure remains unknown, was semi-quantified using the 3-SH calibration curve (Fig. 1S).

### 3.3. Volatile thiols in Müller-Thurgau wines

In consideration of the obtained results, which highlighted how the 4-MSP content in wines of the Müller-Thurgau cultivar was particularly high, and that the thiolic olfactory notes were independently highlighted also by sensory analysis (following section), an in-depth study of other wines of this cultivar was done (sample set #2).

Müller-Thurgau, a crossing of Riesling × Madeleine Royale, is a variety cultivated mainly in Germany, Austria and in some areas of northern Italy, where it finds a favorable habitat in the Trentino Alto Adige region (North East Italy).

Fig. 6 shows the contents of 4-MSP, 3-SH, 3-SHA and E3SP in the 50 Müller-Thurgau wines. The average content of 4-MSP was 9 ng/L and the 2019 samples contained less 4-MSP than the 2020 samples. Additionally, 3 wines from 2019 vintage contained more than 10 ng/L of 4-MSP, while in the 2020 (n = 38) 16 wines showed a content greater than 10 ng /L. The 3-SH content was very similar in both vintages with an average value of 491 ng/L in 2019 wines and 514 ng/L in 2020 wines. For the 3-SHA, it was observed that the content in 2020 wines was higher, with an average value of 13.4 ng/L, while in 2019 wines the values were always below 5 ng/L. Even if the 2019 wines are not exactly the same as those of 2020, it can be assumed that the lower contents in 4-MSP and 3-SHA in the 2019 samples are ascribed to the loss of these compounds over time.

It is known that thiolic notes can rapidly decrease in just several months in the bottle. VTs are highly reactive molecules, which easily react with trapped oxygen in presence of trace amounts of metals such as iron and copper (Lopes et al., 2009); in addition, due to their nucleophilicity, VTs are also involved in polymerization reactions (Nikolantonaki et al., 2010).The loss of varietal thiols has been linked to several reactions, such as polyphenolic oxidation catalyzed by metals and also acid-catalyzed hydrolysis at wine pH (Herbst-Johnstone et al., 2011; Tominaga et al., 2003b; Ugliano et al., 2009). 3-SHA and 3-SH are both susceptible to oxidative degradation even if it was observed that the 3-SHA is the less stable varietal thiol and decreases constantly while 3-SH is more stable over the time (Herbst-Johnstone et al., 2011; Ugliano et al., 2009).

### 3.4. Descriptive sensory analysis

In order to investigate if the detected VTs compositions could have a sensory role on the aroma profile of the various Italian white wines, a PCA was performed (on the results from descriptive sensory analysis): the variables were the VTs and the wine odor descriptors; observations were the 246 wines from the 2019 vintage (sample set #1). Seven components explained more than 80% of the total variance (82,5%), while the first 6 components satisfied the Kaiser's criterion. After the examination of loadings, the first 3 components showing most of the variance (52%) were displayed in Fig. 7 as the most informative. The first dimension (explaining 27.21% of variance) could be denoted as thiolic/non thiolic. Indeed, the Italian white wines are separated along F1 according to the sensory impact of the detected VTs (Fig. 7a). The Müller-Thurgau and Lugana samples show the best positive correlations with both the chemical variables and the odor descriptors ascribable to VTs; in contrast, most of the other wines are on the opposite side of F1, suggesting that the corresponding VTs compositions (Table 2S) do not directly impact the odor profile of these wines with their own distinctive olfactory notes. F2 (explaining 12.50% of variance) is mainly drawn on the chemical variable E3SP in the positive direction, which is non-correlated to any of the sensory variables but shows a significant correlation with 3-SH and even with 3-SHA. The third PC, explaining 11.88% of variance (Fig. 7b), is mainly representative of the sensory dimension including citrus and tropical fruit odors. Based on the

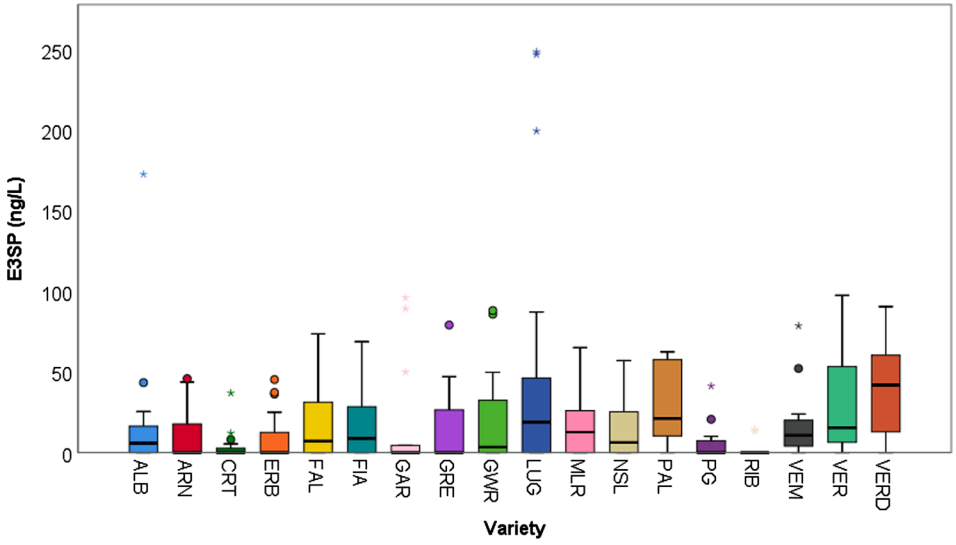


**Fig. 5.** Ethyl 3-sulfanylpropionate (E3SP) distribution in the Italian cultivars Albana (ALB), Arneis (ARN), Cortese (CRT), Erbaluce (ERB), Falanghina (FAL), Fiano (FIA), Garganega (GAR), Greco di Tufo (GRE), Gewürztraminer (GWR), Lugana (LUG), Müller-Thurgau (MLR), Nosiola (NSL), Pallagrello (PAL), Pinot Grigio (PG), Ribolla (RIB), Vermentino (VEM), Vernaccia (VER), and Verdicchio (VERD).

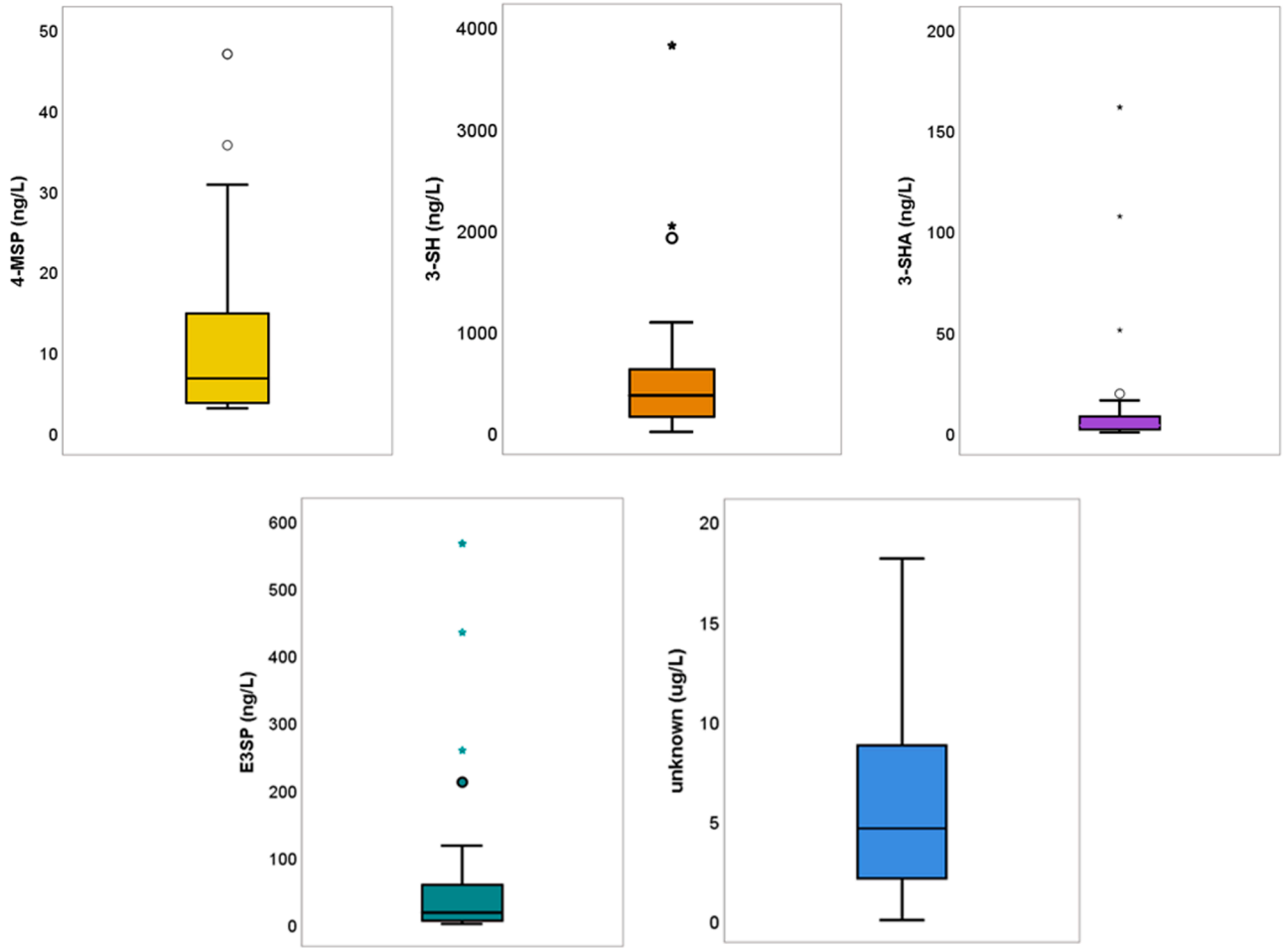


**Fig. 6.** 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA), ethyl 3-sulfanylpropionate (E3SP), and unknown compound distribution in Müller-Thurgau wines.

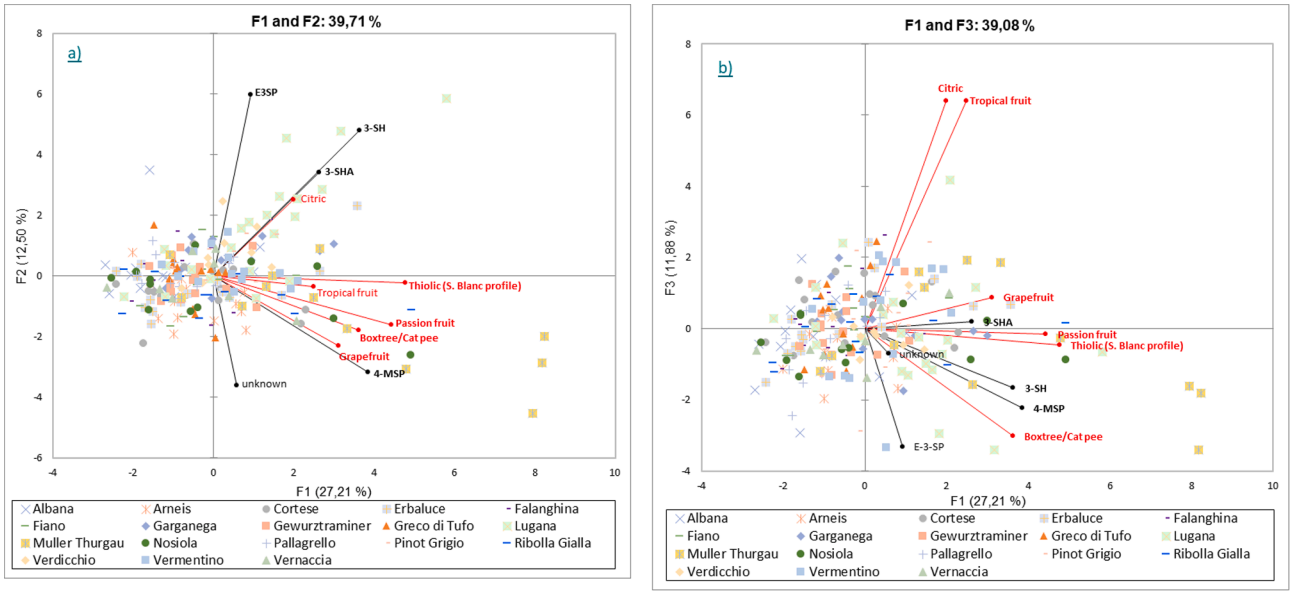


**Fig. 7.** Principal Component Analysis (PCA) biplots carried out on the correlation matrices (Pearson, $p < 0.05$) between the concentrations of VTs and the mean intensities of ascribable olfactory descriptors – rated by the 12 panelists – for each of the 246 wines from the 2019 vintage; (a) PC1 vs PC2, (b) PC1 vs PC3. Variables in bold are significantly correlated with one of the PCs, based on the r coefficient of correlation matrix (Pearson, $p = 0.05$).

correlation matrix coefficients (Pearson, $p = 0.05$), none of the sensory variables are correlated to E3SP and to the unknown compound. Differently, the 3 descriptors thiolic, passion fruit and box tree/cat urine are all significantly correlated to 4-MSP, 3-SH and 3-SHA, with 4-MSP showing the best coefficients (r, 0.441 to 0.372) thus indicating that it is the main chemical driver of Müller-Thurgau wines' VTs aroma. Finally, grapefruit results weakly but significantly correlated to both 4-MSP and 3-SH, citrus fruit only to 3-SH, and tropical fruit only to 3-SHA. It was previously found that in Chardonnay wines high 4-MSP contents gave a pungent box hedge/cat-urine-like aroma and that the sensory scores for passionfruit or box tree/cat urine aroma continued to increase as the concentration of 3-SHA increased (Capone et al. 2017, and references therein).

Our results add further evidence, showing that VTs can contribute to different sensory characters, as they change in concentration moving from general fruity to citrus, to tropical fruit, to cat urine/box tree. This is not surprising as, even at low concentrations, 4-MSP, 3-SH and/or 3-SHA are, among volatile sulfur compounds, relevant molecules involved in the distinctive olfactory character of diverse tropical fruits, such as Guava (*Psidium guajava*), Passion fruit (*Passiflora edulis*) (Fedrizzi et al., 2012) and more (Cannon and Ho, 2018).

From sorting analysis data, Müller-Thurgau wines were analyzed to highlight similarity among samples in terms of "thiolic character". The resulting two-dimensional MDS configuration (Fig. 8), with a stress value of 0.181, indicates an acceptable representation of the original data. Wines were scattered over the map in which AHC analysis identified two groups: MT2, MT3, MT7, MT8, MT9, MT11, MT12 (group 1) and MT1, MT4, MT5, MT6, MT10 (group 2). Interestingly, the first group was described by '"thiolic characters" such as broom, box tree, grapefruit, citric, tomato leaf and a general "thiolic"; moreover, apart from broom, all descriptors were highly correlated among them and with F1. Wines belonging to second group were not defined by specific thiolic terms.

## 4. Conclusions

In this study, a very detailed overview of the thiol content of 246 monovarietal white wines from 18 Italian grape cultivars was carried out, plus a second set of 50 Müller-Thurgau wines.

From a varietal point of view, VTs were observed in at least one sample of each cultivar and can be considered rather ubiquitous. It should be noted that Müller-Thurgau was in this survey the only cultivar that differs for its high 4-MSP content. 3-SH was instead more ubiquitous, as it was present in almost all cultivars, however the richest cultivar was Lugana followed by Müller-Thurgau and Verdicchio. There was great variability within the individual cultivars, certainly attributable to agronomic (clones, vineyard management) and technological (vinification, yeast, closure) variables. The 3-SHA content was low, probably because the wines had been analyzed 12–14 months after bottling. Injection of ethyl 3-sulfanylpropionate (E3SP) in order to identify a compound that elutes before 3-SH and which responds to the same transition made it possible to observe the almost perfect co-elution of this compound with 3-SH; the E3SP was therefore quantified, and the 3-SH area was appropriately corrected. It was observed that E3SP exceeded the olfactory threshold of 200 ng/L only in 2 samples. An additional compound eluting before 3-SH remained unidentified but it did not appear to be related to any specific cultivar. The analysis of a further sampling of 50 Müller-Thurgau wines confirmed the high presence of 4-MSP in these wines.

PCA showed that 4- MSP could play a key role in the aroma of Müller-Thurgau wines, which, unlike all the others, showed concentrations significantly higher than 10 ng/L (OAV ranging from 1 to 12), imparting a distinctive "Thiolic character" with passion fruit, box tree/cat urine and grapefruit odors, in which also the 3-SH (OAV ranging from 3 to 66)

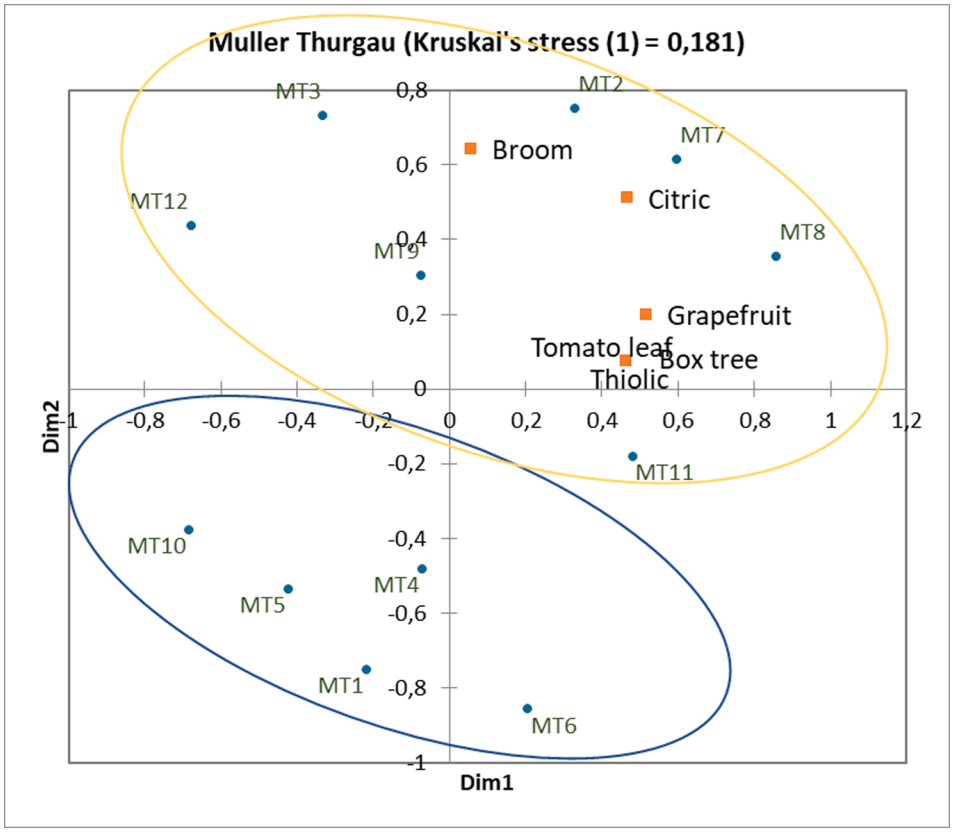


**Fig. 8.** Sorting Analysis: projection of aromatic descriptors and Müller Thurgau wine (MT1-MT12) samples in the Multidimensional Scaling map (dimensions 1 and 2).

**Table 2**
Calibration and method validation data for all analytes (4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH), 3-sulfanylhexyl acetate (3-SHA) and ethyl 3-sulfanylpropionate (E3SP)). Values were expressed in ng/L. Limit of detection (LOD) was estimated as the lowest calibration point which qualifier transition gives a Root mean square (RMS) S/N greater than 3. Repeatability was evaluated at 100 ng/L for 6 replicates within the same day (intra-day) and within two weeks (inter-day).

| Analyte | | Calibration | | | | | | Repeatability RSD% at 100 ng/L (N = 6) | | Recovery on spiked samples (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acronym | ODT (ng/L) | LOD | LOQ | Intercept | Slope | Linearity range | $R^2$ | Intra-day | Inter-day | 10 ng/L | 100 ng/L | 1000 ng/L |
| 4-MSP | 4.0 | 2.0 | 5.0 | −0.031 | 0.019 | 5–1000 | 0.9995 | 2.69 | 12.52 | 121.3 | 92.0 | 104.8 |
| E3SP | 200.0 | 4.0 | 10.0 | 0.0014 | 0.001 | 10–1000 | 0.9966 | 2.33 | 7.81 | 114.6 | 102.5 | 103.7 |
| 3-SH | 60.0 | 4.0 | 10.0 | 2.820 | 0.011 | 10–1000 | 0.9969 | 11.32 | 14.78 | 117.2 | 103.4 | 108.0 |
| 3-SHA | 4.0 | 2.0 | 5.0 | −0.005 | 0.001 | 5–1000 | 0.9989 | 2.43 | 8.95 | 121.7 | 102.3 | 114.5 |

is likely involved. In agreement with descriptive analysis performed by trained wine experts, sorting task performed by enologists showed that these descriptors were well represented in the set of analyzed wines.

Except for Lugana, only a few samples of the other monovarietal wines were correlated to VTs and their related descriptors. Results also suggest that the levels of 3-SH and 3-SHA could modulate the citrus/grapefruit and tropical fruit odors, respectively. Finally, E3SP was not correlated to sensory descriptors, likely because it is always detected far below its ODT. Future analyses of further data obtained on the same sample-set, in the frame of the D-Wines project, are likely to help with further understanding of these findings and of the olfactory role played by VTs in respect also to other VOCs, in the wines from Italian white grape cultivars.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111404.

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