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Occurrence, biogenesis and sensory impact of methyl salicylate in Lugana wines

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ABSTRACT

Lugana wines are produced in the winemaking regions of Veneto and Lombardia, employing Turbiana grapes grown in the proximity of the Garda Lake. Methyl salicylate (MeSA) has been reported as a potentially impactful compound in Lugana wines. The aim of this study was to evaluate the occurrence of MeSA in commercial Lugana wines, elucidate its formation during winemaking and aging, and assess its potential contribution to Lugana aroma. MeSA was quantified in a total of 93 samples including Lugana, Verdicchio (another Italian white wine produced in central Italy using Turbiana grapes locally referred to as Verdicchio), as well as other Italian white and red wines such as Corvina, Corvinone, Garganega, Sangiovese, Pinot nero, Pinot grigio. The results showed that Lugana showed an average concentration of MeSA of 50.6 µg/L, Verdicchio of 33.4 µg/L, while the rest of the wines showed concentration below 5 µg/L. These results indicate that MeSA can be considered a varietal marker of Lugana wines, as well as more in general of wines obtained from the Turbiana/Verdicchio varietal cluster. Concentration of free MeSA in Lugana grape must is however low, and experimental fermentations showed that MeSA was largely formed by yeast activity during alcoholic fermentation, which hydrolyzes the glycosidic precursors of MeSA present in the must to release the aglycone. Additional MeSA can be formed in wine during aging by acid hydrolysis of the glycosidic precursors. Finally, the olfactory threshold of MeSA in a commercial Lugana was estimated by BET method at 38 µg/L, suggesting that MeSA could play a role in Lugana aroma.

KEYWORDS: Methyl salicylate, Lugana, Turbiana, glycosidic precursors, Italian wines

INTRODUCTION

Lugana is an Italian white wine produced in northern Italy, in the areas of the Lombardia and Veneto regions surrounding the southern coast of the Garda Lake. The name Lugana refers to a wine made with Turbiana grapes, which according to the DOC appellation regulation have to be employed at minimum of 90 % for the production of DOC Lugana wines (Consorzio Tutela Lugana DOC). Turbiana grapes are also known as Trebbiano di Soave or Trebbiano di Lugana (Costacurta *et al.*, 2003; Consorzio Tutela Lugana DOC) and are genetically identical to Verdicchio (Ghidoni *et al.*, 2010), another Italian variety cultivated in the Marche region, in Central Italy. It is believed that the grape was originally cultivated by peasants from the province of Verona in northern Italy, which is due to the plague migrated to the Marche in the 15th century, taking the vine with them (Scienza, 2015).

Recent studies on Lugana wines indicate that they are characterized by rather high concentrations of methyl salicylate (MeSA) (Carlin *et al.*, 2019a, Fracassetti *et al.*, 2020; Slaghenaufi *et al.*, 2021). MeSA has a characteristic aroma of wintergreen oil, and its odour threshold in water was reported at 40 µg/L (Buttery *et al.*, 1990). Other studies indicated MeSA as characterized by spicy and minty odours, potentially contributing to the odour of Cognac (Ferrari *et al.*, 2004), and tea (Wang *et al.*, 2008). As MeSA was reported in Lugana wines at concentrations up to hundreds of micrograms per litre, (Carlin *et al.*, 2019a; Fracassetti *et al.*, 2020; Slaghenaufi *et al.*, 2021) it may contribute to Lugana wine aroma, although specific threshold data is missing. Relatively high levels of MeSA can also occur in Lugana wines as glycosidic precursors, (Carlin *et al.*, 2019b; Slaghenaufi *et al.*, 2021), from which in theory MeSA could be generated by either enzymatic or acid hydrolysis. In winemaking, a significant increase in MeSA concentration was observed by using the non-*Saccharomyces* yeast *Lachancea thermotolerans* (Beckner Whitener *et al.*, 2015).

The aims of this study were to evaluate MeSA occurrence and its impact in Lugana wines and to elucidate its biogenesis during wine production.

MATERIALS AND METHODS

1. Reagents

Octan-2-ol (97 %), MeSA (≥ 99 %), glucose (99.5 %), casein peptone, yeast extract and phosphate citrate buffer were supplied by Sigma-Aldrich (Milan, Italy). Dichloromethane (≥ 99.8 %) and methanol (≥ 99.8 %) were furnished by Honeywell (Seelze, Germany).

2. Wines

Ninety-three wines both white and red, from different regions and varieties have been analyzed. Main information such as vintage, type and production region are reported in table 1. Verdicchio, Pinot nero and Pinot grigio and Lugana samples were of commercial origin, whereas Garganega, Sangiovese, Corvina and Corvinone were from experimental trials.

3. MeSA analysis

MeSA has been extracted and analyzed as described by Slaghenaufi *et al.* (2020) with minor modification. Fifty milliliters of sample were added with 20 µL of internal standard solution (2-octanol at 42 mg/L in ethanol) and diluted with 50 mL of distilled water. The solution was then loaded on a BOND ELUT-ENV, SPE cartridge, containing 1 g of sorbent (Agilent Technologies. USA), previously activated with 20 mL of methanol and equilibrated with 20 mL of water. The cartridge was then washed with 15 mL of water. Free MeSA was eluted with 10 mL of dichloromethane, and then concentrated under gentle nitrogen stream to 200 µL prior to GC injection. Glycosylated MeSA was eluted with 20 mL of methanol, the solvent was then evaporated under vacuum to dryness. The extract was then dissolved in 5 mL of citrate buffer (pH 5) to which 200 µL of AR2000 enzyme preparation (DSM, Brussels, Belgium, prepared at 70 mg/mL in citrate buffer) was added and incubated at 37 °C for 24 h under shaking (150 rpm).

GC–MS analysis was carried out on an HP 7890A (Agilent Technologies) gas chromatograph coupled to a 5977B quadrupole mass spectrometer, equipped with a Gerstel MPS3 auto sampler (Müllheim/Ruhr, Germany). Separation was performed using a DB-WAX UI capillary column (30 m × 0.25, 0.25 µm film thickness, Agilent Technologies) and helium as carrier gas at 1.2 mL/min of constant flow rate. GC oven was programmed as follow: started at 40 °C for 3 min, raised to 230 °C at 4 °C/min and maintained for 20 min. Transfer line was set at 200 °C. Mass spectrometer operated in electron ionization (EI) at 70 eV with ion source temperature at 250 °C and quadrupole temperature at 150 °C. Mass spectra were acquired in single ion monitoring mode (quantitation ion m/z 152, qualifier ions m/z 120, 92).

Calibration curve was prepared using seven concentration points and three replicate solutions per point in model wine (12 % v/v ethanol, 3.5 gr/L tartaric acid, pH 3.5). Twenty µL of internal standards 2-octanol (42 mg/L in ethanol), were added to the solution. SPE extraction and GC-MS analysis were performed as described above for the samples. Calibration curves were obtained using Chemstation software (Agilent Technologies, Inc.) by linear regression, plotting the response ratio (analyte peak area/internal standard peak area) against concentration ratio (analyte added concentration/internal standard concentration). Method characteristics are reported in Supplementary S.1.

4. Fermentation

Turbiana grapes from the 2018 vintage were harvested between 20 and 22 °Brix with three different vineyards within the Lugana appellation. Compositional characteristics of the three grape batches are given in Supplementary material For each batch, five kilograms of grapes were destemmed, crushed in plastic bags and pressed under inert atmosphere using nitrogen. Three litres of the obtained juice was added with 2 /hL of pectolytic enzyme (Lafazym CL, Laffort,

TABLE 1. Wine variety, code, number of samples analyzed, wine type, region of production and vintages.

Wine[a]	Code	Number of samples	Type	Region	Vintages
Lugana*	LU	26	White	Veneto/Lombardia	2014, 2016, 2017, 2018, 2019
Garganega+	GA	3	White	Veneto	2019
Verdicchio*	VERD	17	White	Marche	2004, 2007, 2013, 2014, 2015, 2016, 2017, 2018
Pinot grigio*	PG	10	White	Veneto/Trentino A.A.	2016, 2017, 2019
Corvina+	COA	17	Red	Veneto	2016, 2018, 2019
Corvinone+	CONE	10	Red	Veneto	2018, 2019
Sangiovese+	SG	5	Red	Toscana	2015
Pinot nero*	PN	5	Red	Trentino A.A.	2014, 2016, 2017, 2018

[a]Indicates wine variety or commercial name. *according to appellation regulation made at least 85 % of the variety indicated); +100 % monovarietal made with the variety indicated.

France) then stored at 4 °C overnight. After that, 2.1 L of the supernatant with a final turbidity of 200 NTU ± 10 NTU were transferred into 3L glass containers. Musts were then inoculated with commercial yeast (Zymaflore VL3, Laffort, France) and fermented at 16 °C. At the end of fermentation wine samples were kept at 4 °C overnight to separate yeast lees. Potassium metabisulphite was added in order to reach 30 mg/L of free SO_2. Tartaric stabilization was done by placing wines at -4 °C for one week, after that samples were bottled with 30 mg/L of free SO_2 in crown cap sealed bottles and stored at 16 °C until analysis. All fermentations were conducted in triplicate.

5. HPLC fractionation of Lugana must

Ten kilograms of Turbiana grapes were harvested in 2018 at 21.9 °Brix. Grapes were destemmed and crushed manually, and then pressed with a vertical stainless-steel basket press. The free juice was collected and added with potassium metabisulphite (50 mg/L) and polyvinylpyrrolidone (0.5 g/L). The must was kept at 4 °C overnight and then centrifugated using an Avanti J-25 (Beckman Coulter, Pasadena, CA, USA) at 4420 g 30 minutes). In preparation for the resting cells experiment (see next section) the precursor fraction of the must was fractionated by loading the centrifuged must onto a column (diameter 3 cm) filled with 10 g of polystyrene divinylbenzene resin (BOND ELUT-ENV, Agilent Technologies. USA) previously activated with 100 mL of methanol and equilibrated with 200 mL of water. After loading, the column was washed with 100 mL of water followed by 100 mL of dichloromethane. Precursors were eluted with 200 mL of methanol. The methanolic extract was then evaporated to dryness and dissolved with 2 mL of a solution of water/methanol (1:1, v/v) prior to HPLC injection. The HPLC fractionation was performed using a Jasco LC-2000 Plus system (JASCO, Inc., Easton, MD, U.S.A.), consisting of a LC-Net II/ADC system controller, AS-2055 autosampler with an injection loop of 100 µL, PU-2085 quaternary gradient pumps, CO-2060 column ovens, and MD-2010 diode array. A Vydac 218TP C18 (Grace, Columbia, U.S.A.) 250x10 mm, 5 µm column was used. The flow rate was set a 4 mL/min. The mobile phase was consisted of a binary gradient of 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B). Elution was performed with a flow rate of 4 mL/min and the following gradient program (v/v): starting at 5 % of solvent B for 3 minutes, 5-40 % in 25 minutes, 40-80 % in 10 minutes, hold for 10 min. The column was re-equilibrated for 5 minutes before the next injection. Eluent was collected each 2 minutes using a Biofrac fraction collector (Biorad, Hercules, California, U.S.A.). Each fraction was evaporated to dryness and resuspended with 0.5 mL of phosphate-citrate buffer (pH 5).

6. Incubation of methyl salicylate precursors with yeast resting cells

As described by Ugliano *et al.* (2006) with slight modification (Slaghenaufi *et al.*, 2020), yeasts were growth in YPD medium and incubated at 30 °C, under shaking (150 rpm) until 4 × 107 cells/mL was reached. The yeast culture was centrifugated (4000 rpm for 10 min at 4 °C) and then washed twice with 0.9 % NaCl. The obtained pellet was dissolved in 5 mL of sterile 0.05 M phosphate−citrate buffer (pH 5) with 7.5 % glucose. Fifty microliters of studied compound were added: in one case it was a MeSA solution (1 mg/L in ethanol), in the other Lugana must fractions obtained as described above were added. This cell suspension was then transferred to a 10 mL glass tube and incubated at 30 °C, under orbital stirring (150 rpm) for 72 hours. Three different control samples were performed: non-inoculated without compound addition, inoculated without compound addition, and non-inoculated with compound addition. All assays were performed in triplicate.

7. Wine model aging

The protocol used for model aging of wine was described by Slaghenaufi and Ugliano (2018). Briefly, the samples were adjusted to 30 mg/L of free SO_2 and placed in 115 mL glass vial crimped leaving 2 mg/L of headspace oxygen. Vials were then sealed with Araldite glue and vials were then placed at 40 °C for 1 month. Model aging was carried out in duplicate for each biological replicate. Additional

information concerning the wines is given in Suppementary material

8. Determination of odour detection threshold

Detection threshold was determined in commercial white wine by the three-alternative forced choice (3-AFC) method (3AFC ISO 13301, 2002) using ascending concentration of MeSA in order to obtained concentration levels of 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 µg/L respectively. A neutral white wine (12.5 % vol, total acidity 5.4 g/L, free SO_2 20 mg/L, total SO_2 110 mg/L) was used for the test, containing 0.21 µg/L of MeSA. The sensory panel was consisted of 19 judges, 8 females and 11 males. Aged between 21 and 55 years. All were wine-science researchers, university teaching staff or enology students, familiarized with wine tasting and the procedure for measuring detection thresholds. The test was done in ISO glasses. For each concentration the judges received three glasses encoded with three-digit random numbers, two of which containing the reference wine and one the wine with the addition of MeSA. The judges were asked to smell all three glasses and indicate which was perceived different. The odour threshold of MeSA was determined using the Best Estimate Threshold (BET) method (Meilgaard *et al.*, 1999). For each panellist the individual BET value was calculated as the geometric mean between the last concentration missed and the first concentration detected. The odour threshold was determined by the geometric mean of the individual BETs.

9. Statistical analyses

For the section of the study concerning the influence of wine type on MeSA content, one-way analysis of variance (ANOVA) was applied to the variable type of wine using XLSTAT 2017 (Addinsoft SARL, Paris,France).

RESULTS

1. Occurrence of MeSA in wines

Occurrence of MeSA has been evaluated in various white and red wines of different origin (Table 2). Analysis of 23 commercial Lugana wines, all from different estates, indicated that, on average, MeSA concentration was 50.6 µg/L, ranging from 8.66 to 191 µg/L. Verdicchio wines also showed relatively high MeSA content, ranging from 6.7 to 207 µg/L with an average value of 33.4 µg/L. Compared to the various other wines of different origins analysed, MeSA levels in Lugana and Verdicchio wines were significantly higher (Figure 1), as all the other samples showed concentrations of one or two orders of magnitude lower than Lugana and Verdicchio. The lowest concentrations were found in Corvinone and Sangiovese wines, in average 0.56 µg/L and 0.48 µg/L respectively, but in no case values were higher than 3 µg/L. Analysis of the variance (ANOVA) showed significance differences ($p < 0.0001$) between wine types.

In light of these results, MeSA appears to be a good marker of Lugana and Verdicchio wines, confirming the observations of other authors on smaller sample sets (Carlin *et al.* 2019a; Carlin *et al.*, 2019b; Fracassetti *et al.*, 2020; Slaghenaufi *et al.* 2021). Even though Lugana and Verdicchio come from genetically identical varieties (Vantini *et al.*, 2003), Lugana wines seemed to be generally richer in MeSA than Verdicchio, confirming recent data on a smaller wine set (Slaghenaufi *et al.*, 2021). This suggests that the formation of MeSA could be influenced by various factors related to geographic origin such as pedoclimatic conditions, specific local grape biotypes, viticultural and oenological practices. Poitou *et al.* (2021) also reported a major influence of grape Esca disease on MeSA content of red wines, although in our case the large number of samples analyzed of different type and origin strongly advocates for a varietal origin of MeSA in the case of Lugana and Verdicchio wines.

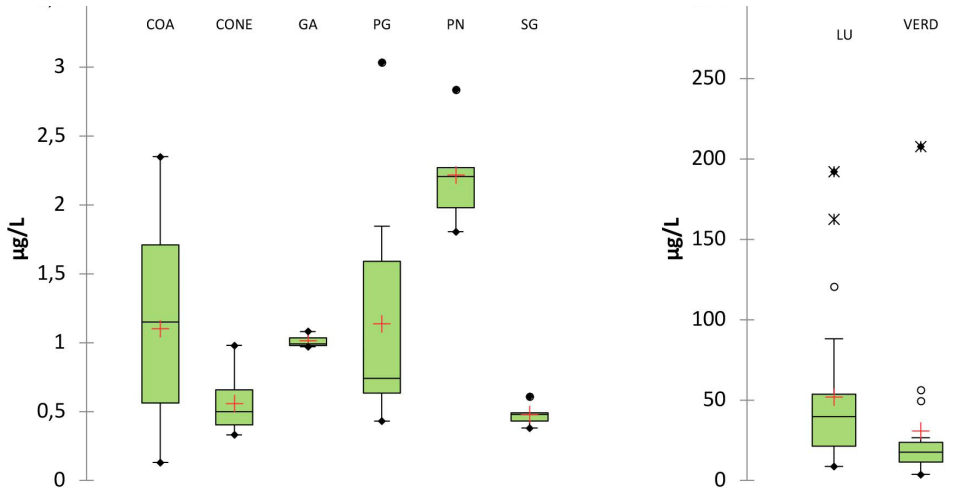

FIGURE 1. Concentration of free MeSA in commercial wines. Corvina (COA), Corvinone (CONE), Garganega (GA), Pinot grigio (PG), Pinot nero (PN) Sangiovese (SG), Lugana (LU), Verdicchio (VERD).

TABLE 2. Concentration (means ± standard deviation) of free MeSA, in different wines.

table 2 (1/2)

Wine[a]	Region	Wine type	Vintage	Aging time (years)	MeSA (µg/L)
Lugana	Veneto	white	2018	2	19.0 ± 1.5
Lugana	Veneto	white	2016	4	37.7 ± 0.7
Lugana	Veneto	white	2017	3	20.8 ± 0.8
Lugana	Veneto	white	2017	3	192 ± 10
Lugana	Veneto	white	2018	2	26.7 ± 1.9
Lugana	Veneto	white	2016	4	49.8 ± 1.5
Lugana	Veneto	white	2014	6	8.7 ± 0.69
Lugana	Veneto	white	2016	4	78.3 ± 7.8
Lugana	Veneto	white	2017	3	22.5 ± 0.9
Lugana	Veneto	white	2017	3	88.1 ± 6.2
Lugana	Veneto	white	2017	3	48.8 ± 2.9
Lugana	Veneto	white	2017	3	83.8 ± 8.4
Lugana	Veneto	white	2019	1	16.2 ± 1.6
Lugana	Veneto	white	2018	2	26.4 ± 0.5
Lugana	Veneto	white	2019	1	10.9 ± 1.0
Lugana	Veneto	white	2018	2	41.8 ± 2.5
Lugana	Veneto	white	2019	1	24.5 ± 2.7
Lugana	Veneto	white	2016	4	50.8 ± 2.5
Lugana	Veneto	white	2017	3	30.3 ± 1.5
Lugana	Veneto	white	2018	2	18.9 ± 0.8
Lugana	Veneto	white	2017	3	162 ± 5
Lugana	Veneto	white	2018	2	51.0 ± 6.1
Lugana	Veneto	white	2019	1	54.3 ± 2.7
Verdicchio	Marche	white	2017	3	7.54 ± 0.23
Verdicchio	Marche	white	2016	4	17.4 ± 0.3
Verdicchio	Marche	white	2017	3	13.8 ± 1.0
Verdicchio	Marche	white	2017	3	26.5 ± 1.6
Verdicchio	Marche	white	2017	3	19.1 ± 1.0
Verdicchio	Marche	white	2018	2	56.4 ± 1.1
Verdicchio	Marche	white	2017	3	11.3 ± 0.4
Verdicchio	Marche	white	2013	7	12.6 ± 1.7
Verdicchio	Marche	white	2015	5	14.8 ± 1.8
Verdicchio	Marche	white	2013	7	6.7 ± 0.47
Verdicchio	Marche	white	2014	6	3.73 ± 0.19
Verdicchio	Marche	white	2007	13	207 ± 8
Verdicchio	Marche	white	2014	6	49.4 ± 4.4
Verdicchio	Marche	white	2004	16	20.9 ± 1.5
Garganega*	Veneto	white	2019	1	0.99 ± 0.01
Garganega*	Veneto	white	2019	1	0.97 ± 0.03
Garganega*	Veneto	white	2019	1	1.08 ± 0.11
Pinot grigio	Veneto	white	2019	1	0.43 ± 0.07
Pinot grigio	Veneto	white	2019	1	0.57 ± 0.14

table 2 (2/2)

Wine[a]	Region	Wine type	Vintage	Aging time (year)	MeSA (µg/L)
Pinot grigio	Veneto	white	2019	1	0.68 ± 0.21
Pinot grigio	Veneto	white	2019	1	0.68 ± 0.08
Pinot grigio	Veneto	white	2019	1	0.62 ± 0.14
Pinot grigio	Veneto	white	2019	1	0.86 ± 0.18
Pinot grigio	Veneto	white	2019	1	0.8 ± 0.01
Corvina*	Veneto	red	2018	2	0.13 ± 0.01
Corvina*	Veneto	red	2018	2	0.22 ± 0.02
Corvina*	Veneto	red	2018	2	0.91 ± 0.05
Corvina*	Veneto	red	2018	2	2.24 ± 0.16
Corvina*	Veneto	red	2018	2	1.15 ± 0.12
Corvina*	Veneto	red	2019	1	0.17 ± 0.02
Corvina*	Veneto	red	2019	1	0.4 ± 0.05
Corvina*	Veneto	red	2019	1	0.56 ± 0.01
Corvina*	Veneto	red	2019	1	1.71 ± 0.17
Corvina*	Veneto	red	2019	1	0.65 ± 0.02
Corvinone*	Veneto	red	2018	2	0.41 ± 0.02
Corvinone*	Veneto	red	2018	2	0.56 ± 0.03
Corvinone*	Veneto	red	2018	2	0.33 ± 0.04
Corvinone*	Veneto	red	2018	2	0.34 ± 0.02
Corvinone*	Veneto	red	2018	2	0.69 ± 0.07
Corvinone*	Veneto	red	2019	1	0.46 ± 0.05
Corvinone*	Veneto	red	2019	1	0.4 ± 0.02
Corvinone*	Veneto	red	2019	1	0.98 ± 0.07
Corvinone*	Veneto	red	2019	1	0.86 ± 0.09
Corvinone*	Veneto	red	2019	1	0.54 ± 0.06
Sangiovese*	Toscana	red	2015	5	0.38 ± 0.02
Sangiovese*	Toscana	red	2015	5	0.49 ± 0.05
Sangiovese*	Toscana	red	2015	5	0.61 ± 0.04
Sangiovese*	Toscana	red	2015	5	0.43 ± 0.03
Sangiovese*	Toscana	red	2015	5	0.48 ± 0.03
Pinot nero	Trentino	red	2016	4	1.80 ± 0.11
Pinot nero	Trentino	red	2017	3	2.83 ± 0.20
Pinot nero	Trentino	red	2018	2	2.20 ± 0.22
Pinot nero	Trentino	red	2014	6	2.27 ± 0.27
Pinot nero	Trentino	red	2018	2	1.98 ± 0.14

[a]: denotes the main variety used in the commercial blend according to the current regulation (minimum 85 % in all cases). Asterisk denotes experimental or commercial unblended samples (100 % monovarietal).

2. Biogenesis and stability of MeSA during winemaking

2.1. Formation of MeSA during fermentation

Little is known about the origin of MeSA in wine, in particular whether it is already present in grapes or if it is formed during fermentation or wine aging. MeSA exist also as a glycosidic precursor, and six different glycosidic precursors of MeSA have been identified in wine (Carlin *et al.*, 2019b). MeSA precursors concentration was quantified in our sample set, varying from 0.20 to 166 µg/L (Figure 2). As in the case of free MeSA, significant differences ($p < 0.0001$) were found between wine types. The highest glycosidically-bound MeSA concentration was found in Lugana, in average 71.7 µg/L, followed by Verdicchio 47.0 µg/L, Sangiovese 4.58 µg/L, the rest of the wines showed concentration below 3 µg/L.

In spite of the high content of free and glycosylated MeSA of Lugana wines, analysis of different Lugana musts carried out in our laboratory indicated that the concentrations

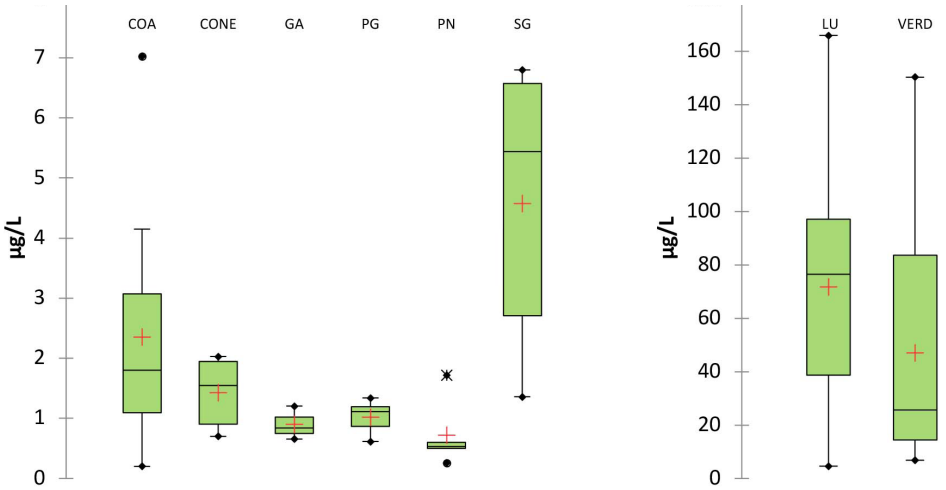

FIGURE 2. Concentration of glycosidically-bound MeSA in commercial wines. Corvina (COA), Corvinone (CONE), Garganega (GA), Pinot grigio (PG), Pinot noir (PN), Sangiovese (SG), Lugana (LU) Verdicchio (VERD).

of free MeSA found in musts were quite low, around 1.37 µg/L ± 0.14 (average of 6 samples), indicating that the majority of free MeSA is not already present in grapes but is formed during winemaking.

Formation of MeSA during the alcoholic fermentation was therefore investigated by vinifying three different Lugana musts and monitoring MeSA formation over time. The three musts A, B and C had respectively 301 ± 15, 294 ± 21 and 317 ± 20 µg/L of glycosylated MeSA. The results (Figure 3) showed that MeSA concentration increased during fermentation, suggesting that yeast could be involved in MeSA biogenesis. In these experimental conditions appeared that MeSA was mainly formed during the first part of fermentation in which about half of the sugars were metabolized by yeasts. In the first 2 days of fermentation the concentrations found were quite similar among the samples, whereas differences emerged in the second part of the fermentation with must C providing approximately twice as much MeSA of must A. Considering that fermentation kinetics and content of glycosylated MeSA of the three musts were very similar these differences might reflect variations in type of precursors present and/or different pathways, so that these aspects were further investigated.

2.1.1. Investigations on possible MeSA precursors

Yeasts are able to de-novo synthetize benzenoid compounds (Martin *et al.*, 2016) although de-novo synthesis of MeSA from yeast was excluded as increases in MeSA concentration were not observed fermentations made in our laboratory using the same yeast but grape varieties other than Turbiana or Verdicchio (data not shown). Two other formation pathways are however possible, one involving the esterification of salicylic acid with methanol (Deng *et al.*, 2017; Zhao *et al.*, 2016; Sá *et al.*, 2017; Bhardwaj *et al.*, 2017), the other through hydrolysis of glycosidic precursors by yeast.

In order to investigate the contribution of these pathways on MeSA formation during fermentation, experiments using resting cells were performed (Slaghenaufi *et al.*, 2020). When cells were incubated in the presence of salicylic acid, neither MeSA nor the other salycilate ester, ethyl salicylate, were observed in the samples. Conversely other volatile compounds related to fermentation such as fatty acids, higher alcohols, ethyl ester of fatty acids, and higher alcohol acetates were observed, indicating that yeast metabolism was active in the incubation buffer, in agreement with the presence of glucose. Accordingly, esterification of salicylic acid by yeast during alcoholic fermentation to form MeSA was excluded.

Concerning MeSA formation from glycosidic precursors, Carlin *et al.* (2019b) reported the existence of different glycosides of MeSA in grapes, mostly glucosides or disaccharides including an xylopyranoside, apiopyranoside, rhamnopyranoside, gluvopyranoside or arabinopyranoside unit linked to MeSA glucoside. Yeast has been shown to possess hydrolytic capabilities towards such glycosides, which can vary significantly depending on the sugar moiety of the substrate (Ugliano *et al.* 2006). To gain insights on the ability of yeast to hydrolyse the various precursors, a Turbiana grape must was semipurified by column liquid chromatography and the methanolic fraction was further purified in 16 different fractions using semipreparative-HPLC. Enzymatic hydrolysis of these fractions by commercial glycosidases revealed the presence of MeSA precursors in three different fractions (fractions number 5, 7 and 8). The presence of MeSA precursors in different fractions could be explained by the existence of six different forms of glycosidic precursors (Carlin *et al.*, 2019b). Fractions number 5, 7 and 8 were incubated with yeast resting cells. Data reported in Table 3 showed that yeast was able to release MeSA from all the three fractions, indicating that yeast can hydrolyze different precursors substrates. It was interesting to note that yeast produced more MeSA in fraction 7, while enzymatic hydrolysis with commercial glycosidase gave highest MeSA release in fraction 8. In light of these results, we conclude that the increase of MeSA observed

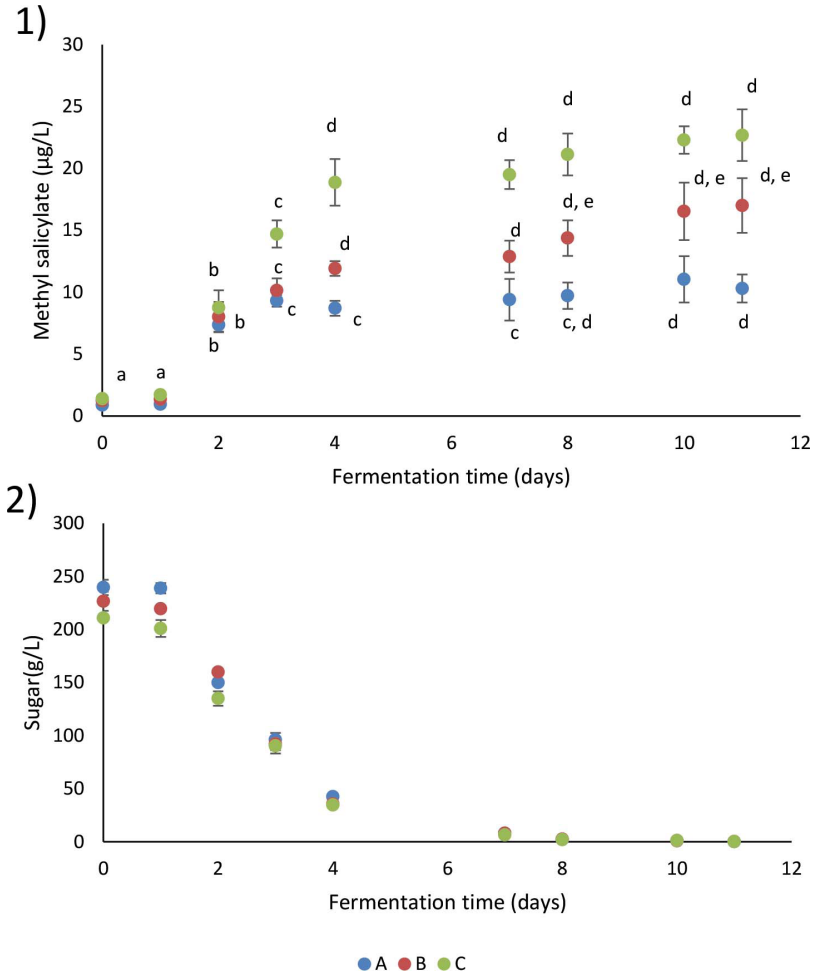

FIGURE 3. Evolution of MeSA concentration during fermentation of three Lugana musts A, B, C. Within each must series, different letters denote statistidcally sidngificant diffrerences at $p < 0.05$; 2) Evolution of the concentration of sugar in the tree musts A,B,C.

during fermentation was due to the hydrolysis of glycosidic precursors catalysed by yeast.

2.2. Fate of MeSA during wine aging

In order to evaluate the behaviour of MeSA in wine during time, a sample subset formed by 7 Lugana commercial wines were submitted to model aging. MeSA concentration was analysed after 1 month of bottle aging at 40 °C.

Results (Table 4) showed a general increase in free MeSA, which is consistent with the presence of glycosidic precursors undergoing acid hydrolysis (Carlin *et al.* 2019b). However, two out of the seven wines tested shown a different trend and interestingly these were the two wines with minimum and maximum MeSa content before aging. Specifically, for the wine with the lowest MeSA content, aging did not result in a change in MeSA content, which could be explained assuming that the low free MeSA content was also associated with low content of glycosidic precursors. More surprising was the observation of a major MeSA loss in the wine having the highest initial MeSA content also showed the greatest decline. This observation indicates that MeSA can also undergo degradation during bottle storage, resulting in a net loss of MeSA if precursor content is low. Hydrolytic degradation of MeSA in the presence of amino acids has been reported (Cheng *et al.*, 2021), although the likelihood of this reaction at wine pH will need to be investigated. When comparing the data of Table 4 with those in Table 2 it is also worth observing that, although the highest MeSA concentration detected (208 µg/L) was observed in a 13 years old Verdicchio, instead MeSA content in a 16 years old Verdicchio was relatively low (20.9 µg/L), supporting the hypothesis that MeSA content of aged wine results from the combined contribution of precursor content as well as free MeSA hydrolytic degradation.

3. Sensory Impact of MeSA

In a study concerning Verdicchio wines, Carlin *et al.* (2019a) mentioned that the odour threshold of MeSA was comprised between 50 and 100 µg/L. More recently, Poitou *et al.* (2021) reported a threshold of 75 ± 25 µg/L for MeSA in model wine, relating above-threshold levels of MeSA to Esca disease. Mansfiled *et al.* (2011) proposed that MeSA could contribute to green and cedar attributes of Frontenac wines and possibly other wines from Vitis riparia. MeSA quantitative data presented herein, however clearly indicate that high levels

TABLE 3. MeSA released from HPLC fraction when incubated with commercial glycosidase enzyme (AR2000) or with yeast resting cell.

Fraction n°	AR 2000 (µg/L)	Yeast resting cells (µg/L)
5	1.15 ± 0.07	0.09 ± 0.01
7	60.3 ± 0.9	5.4 ± 0.4
8	81 ± 2	1.65 ± 0.09

TABLE 4. MeSA concentrations in the model aging experiment.

Sample n°	Control (µg/L)	Model aging (µg/L)	Variation (µg/L)
1	19.0 ± 1.5	22.4 ± 3.1	3.4
2	37.7. ± 0.7	47.7 ± 1.4	10.0
3	20.9 ± 0.8	26.8 ± 0.4	6.0
4	192 ± 10	127 ± 8	-64.0
5	26.7 ± 1.9	30.0 ± 1.4	3.3
6	49.8 ± 1.5	61.2 ± 9.1	11.5
7	8.66 ± 0.69	7.81 ± 0.71	-0.9

of MeSA can be considered of genuinely varietal origin in the case of Lugana, and to a lesser extent Verdicchio, wines. To assess the odour impact of MeSA, its olfactory detection threshold was determined in white wine using the Best Estimated Threshold (BET) method. Individual threshold ranging from 14.14 µg/L to 113 µg/L (Figure 4), showing an unimodal distribution. The detection threshold of MeSA in white wine was then calculated at 38 µg/L. This value was lower than what reported by Carlin *et al.* (2019) and by Poitou *et al.* (2021). Accordingly, in 17 % of the Verdicchio and 50 % Lugana wine samples analyzed in the present study MeSA exceeded this concentration potentially contributing to perceived aroma.

Nevertheless, the individual odour threshold for 26 % of the judges was at 28 µg/L, whereas for 21 % of the panelist was lower down to about 14 µg/L. From this point of view, MeSA could impact Lugana aroma to a significant extent, as 69 % of the wines showed a concentration of MeSA higher than 28 µg/L, and 92 % of the Lugana wines showed concentration of MeSA higher than 14 µg/L.

CONCLUSION

In this paper the presence of MeSA in Lugana wines was assessed in comparison to other Italian monovarietal white and red wines. MeSA appeared to be a varietal marker of Lugana wines, also characterizing, to a lower extent, Verdicchio wines which are also obtained from Turbiana grape but produced in Marche region. The olfactory detection threshold of MeSA was established at 38 µg/L in white wines. Accordingly, MeSA concentrations found in Lugana wines are often higher than or at least close to its odour threshold suggesting that this compound play a role in Lugana wines aroma.

Free MeSA is already present in Lugana grape must although in very low concentrations. During alcoholic fermentation yeast catalysed the hydrolysis of glycosidic precursors releasing the aglycone, whereas other possible pathways such as *de-novo* biosynthesis or esterification of salicylic acid were discarded. Yeast hydrolytic activity seemed to differ according to the glycosidic structure of the precursors

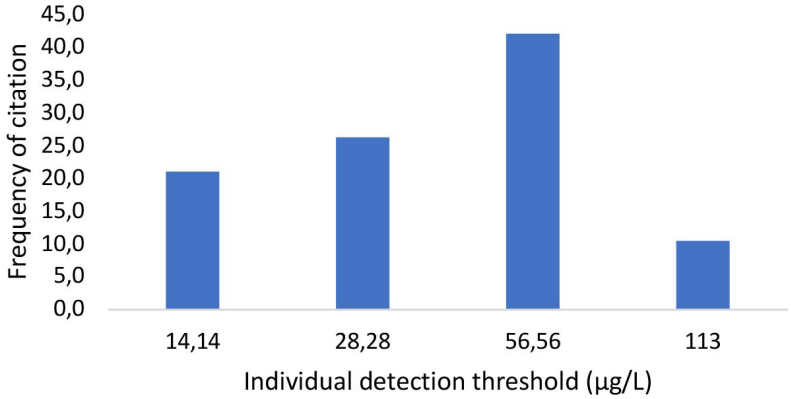

FIGURE 4. Individual detection threshold distribution for MeSA for a panel of 19 judges.

Additional MeSA can be released during aging presumably through acid hydrolysis of glycosidic precursors, although decline in MeSA content can also occur during aging. This work allowed to clearly establish the importance of MeSA for Lugana and Verdicchio wines. Further investigation should address the factors influencing grape content of glycosidically-bound MeSA as well the influence of different yeasts, fermentation and post-fermentation practices on MeSA release during fermentation and aging.

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