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ORIGINAL RESEARCH ARTICLE

Free methyl salicylate and its glycosides mapping in monovarietal Italian white wines

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ABSTRACT

Aroma is an identifying characteristic of wines and is of crucial importance for the consumer appreciation. Among all odour-active compounds, some key molecules appear only after ageing and give a strong contribution to both identity and added value of the wine bottle. Methyl salicylate (MeSA) is an odorant which usually appears after ageing and could provide an increase in fruity aromas and freshness. This odour-active ester can be found mainly bound to one or more carbohydrates, forming a glycoside. In wines, the hydrolysis of glycosides contributes to the aroma evolution of wine. In our previous works, MeSA was found in relevant content, both in bound and free form, in some genetically related Italian varieties such as Trebbiano di Lugana, Trebbiano di Soave (both employed in the production of Lugana wines), and Verdicchio. In this research, a straightforward filter-and-shot LC-MS/MS method was used for the determination of 7 different MeSA glycosides in 246 samples representative of 18 different monovarietal Italian white wines. MeSA glycosides were found in relevant concentrations in Verdicchio and Lugana wines, whereas they were determined for the first time in all others. Results were in accordance with the literature since Lugana and Verdicchio emerged for the total amount of glycosides, together with Erbaluce, whose richness was not known to date. This trend was observed for all analytes, with stronger intensity, especially in MeSA–glucoside, MeSA–gentiobioside and MeSA–violutoside. The triglycosylated form was not detected in any case. The same trend was also observed for the free form, whose value was not above the olfactory threshold for any sample. MeSA in all its forms also showed a qualitative trend, with MeSA - glucoside and MeSA–primeveroside emphasised in richer cultivars with also a characteristic pattern. The knowledge of the concentration of MeSA glycosides could be considered a potential predictor of the potential evolution of white wines towards balsamic-like nuances.

KEYWORDS: Methyl salicylate, aroma precursors, glycosides, white wine, Lugana, Verdicchio, Erbaluce

INTRODUCTION

Among the distinctive characteristics that distinguish a wine, aroma is one of the most important. Some grape cultivars are known to give wines with a highly recognisable aroma profile, whereas some others display their character as a consequence of winemaking style and evolution during cellaring and bottle ageing (Flamini *et al*., 2010; Piergiovanni *et al*., 2022). From a commercial point of view, distinctive sensory characteristics, including varietal aroma characters, are among the key aspects increasing the added value in wines with consequent positive effects in terms of price placement and quality perception. In wine, aroma compounds are present both as bound and free. The free form, which represents a minor fraction, contributes to the aromatic bouquet, whereas the conjugated forms act as a reservoir of odours which are released over time (Ugliano, 2013). These hidden smells constitute a potential whose controlled expression gives a significant contribution to wine complexity, characteristic nuances as well as added value. The several mechanisms through which bound aroma compounds in wines can be liberated have been extensively described (Parker *et al*., 2018).

Within these molecules, methyl salicylate (MeSA) is a key odorant whose presence is highly impacting for some wine cultivars known to be particularly rich in its precursors. This is an odour-active compound known to provide pleasant aromatic nuances of wintergreen, which could be related to floral, spicy, and slightly balsamic notes (Bauer *et al*., 2008). Its structure is based on salicylic acid (SA) with a methyl group linked to the carboxylic function through an ester bond. Because of its pleasant odour, MeSA is synthetically produced by the fragrance industry and widely used in cosmetics and personal care products or as a food additive (Deng *et al*., 2017a; Greene *et al*., 2017; Lapczynski *et al*., 2007).

MeSA (DrugBank Accession Number DB09543) is also a natural bioactive compound, used as a major active ingredient of topical analgesics, having both stimulatory and inhibitory actions on transient receptor potential vanilloid 1 (TRPV1) channel. The latter action has been suggested to partly explain the analgesic effects (Ohta *et al*., 2009)

In natural products, MeSA is known to occur in many cultivars of tea (Deng *et al*., 2017a), elderberry (Ricci *et al*., 2018), green pepper (Buttery *et al*., 1969), tomato (Tieman *et al*., 2010), sweet birch bark (Murphy *et al*., 2021), wintergreen leaves (Greene *et al*., 2017) and grapes (Granum *et al*., 2023) where it is involved in many plant defence mechanisms (Hippauf *et al*., 2010; Simpson *et al*., 2011). Recent studies on grapes and wines demonstrated a strong correlation between MeSA and the presence of several cryptogamic diseases, which makes its content eligible to be considered a chemical marker for this issue (Jansen *et al*., 2011; Poitou *et al*., 2021). MeSA was also present in higher quantities in wines produced without SO_2 (Pelonnier-Magimel *et al*., 2022).

In many plants, MeSA originates from salicylic acid esterification, catalysed by salicylic acid carboxyl methyltransferase (SAMT) (Sheng *et al*., 2018). This synthetic pathway has been assessed to be significant in many plant leaves, whereas there is no evidence of its occurrence in berry fruits such as grapes (Zou *et al*., 2021). In these fruits, MeSA originates as a secondary metabolite during the ripening stage and is mainly present bonded to one or more sugars (Slaghenaufi *et al*., 2020); the resulting configuration is defined as a glycoside. Glycosylation, which is not restricted only to MeSA, is a common plant phenomenon that concerns many small and low-polar metabolites (aglycones) and is based on the enzymatic activity of glycosyltransferases (GTs) (Flamini *et al*., 2014); therefore, glycosylation provides to bonded molecules a better water-solubility, stability and simplifies accumulation, and transport (Lukić *et al*., 2016). Glycosides survive through all winemaking steps, so MeSA is present in wine both as free and conjugated, in different amounts depending on many aspects like grape cultivar, agronomic and oenological parameters (Boido *et al*., 2013; Mao *et al*., 2014).

The olfactory power of MeSA has been estimated by measuring the BET (Best Estimate Threshold). Surprisingly, it resulted in higher values in model wine (76.2 ± 25.5 µg/L; Poitou *et al*., 2021) than in white wine (38 µg/L; Slaghenaufi *et al*., 2022), suggesting perceptual interactions between MeSA and other wine VOCs, as already proven for guaiacol (Yoder *et al*., 2012). A study on cherry wine found that its olfactory threshold in ethanol aqueous solution (9 % v/v) was 20 µg/L, and after omission/addition experiments, it was found that ethyl decanoate and MeSA at sub-threshold concentration had great effects on cherry wine aroma due to their perceptual interaction (Niu *et al*., 2019). Furthermore, perceptual interactions have also been reported for ethyl salicylate, a compound characterised by chemical and olfactory features similar to MeSA (Cruz *et al*., 2021; Ferreira *et al*., 2022). Moreover, once bound to sugars, MeSA is not olfactory detectable, so it must be converted into free form to contribute to wine aroma (Esti and Tamborra, 2006; Ghaste *et al*., 2015; Hjelmeland and Ebeler, 2015).

Recently, many published papers focusing on the contribution of MeSA in wine aroma highlighted a strong correlation between grape cultivars and MeSA content, suggesting that some cultivars are rich in this compound. In the Italian white wine scenario, a relevant content of MeSA was detected mainly in Lugana and Verdicchio (Carlin *et al*., 2019a and 2019b; Crespan *et al*., 2021). Verdicchio and Lugana are genetically related Italian white wines produced, respectively, in the Marche region and on the southern side of the Garda Lake (Vantini *et al*., 2003). The MeSA content in these wines was evaluated to be sensorially detectable for most aged samples assessed by sensory analysis (Slaghenaufi *et al*., 2021). This result was supported by a recent article which evaluated the MeSA content in many commercial white and red monovarietal wines produced using eight different Italian varieties cultivated in north and central Italy (Slaghenaufi *et al*., 2022);

results confirmed that only Lugana and Verdicchio wines contained MeSA above its odour-detection threshold (ODT).

However, these findings are related to the free form and did not account for the conjugated ones whose hydrolysis can be crucial during ageing, especially for the non-aromatic cultivars whose odour profile could be enriched by glycosides hydrolysis (Fracassetti *et al*., 2020; Slaghenaufi and Ugliano, 2018). In previous works, our research group investigated many aspects concerning the occurrence of some MeSA glycosides in wines (Carlin *et al*., 2019a and 2019b). The presence of monoglycosylated, diglycosylated, and triglycosylated forms have been evaluated, both from a qualitative and quantitative perspective, within a sample set composed of Italian wines (Verdicchio, Trebbiano di Soave, Trebbiano di Lugana, Turbiana) which are produced from genetically-related cultivars, and also considering many representatives of international and national cultivars (Carlin *et al*., 2019b). Results showed a relevant content of the monoglycoside and some diglycosides in Lugana and Verdicchio-related grapes, whereas triglycosides were under the limit of quantitation for all samples.

This study aimed at conducting a comprehensive evaluation of the MeSA content in some of the most representative Italian white wine cultivars: in particular, both free and glycosylated MeSA, including monoglycosylated, diglycosylated, and triglycosylated forms, were investigated within 246 monovarietal wine samples produced using 18 cultivars sourced from 9 regions of Italy. Quantitative MeSA data will be used to map the occurrence of MeSA glycosides in Italian white wines, to add a further piece to the exploration of their ageing potential, and to increase the overall knowledge of key Italian autochthonous white cultivars (Carlin *et al*., 2022).

MATERIALS AND METHODS

1. Solvents and standards

Free methyl salicylate (MeSA) and methyl salicylate 2-O-β-d-glucoside (MeSAG) were purchased from Sigma Aldrich and Methyl salicylate 2-O-β-d-xylopyranosyl (1–6)-d-glucopyranoside (MeSA-primeveroside or Gaultherin) was supplied from iChemical Technology (Shanghai, China). Plastic syringes and the 0.22 µm cartridge filter were supplied by Millex-GV (Millipore, Tullagreen, Ireland). All solvents for LC-MS analysis were purchased in compliance with the MS grade requirement from Sigma-Aldrich (Milan, Italy).

2. Wine samples

The sample set 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 produced without malolactic fermentation and wood ageing were collected from the main regions of production: 14 Albana (Emilia-Romagna, ALB); 16 Cortese (Piemonte, CRT); 13 Arneis (Piemonte, ARN); 15 Erbaluce (Piemonte, ERB); 13 Falanghina (Campania, FAL); 12 Fiano (Campania, FIA); 14 Garganega (Veneto, GAR); 17 Gewürztraminer (Trentino Alto Adige, GWR); 13 Greco di Tufo (Campania, GRE); 21 Lugana (Veneto, LUG); 13 Müller-Thurgau (Trentino Alto Adige, MLR); 12 Nosiola (Trentino Alto Adige, NSL); 13 Pallagrello bianco (Campania, PAL); 12 Pinot grigio (Friuli Venezia Giulia /Veneto/Trentino Alto Adige, PG); 14 Ribolla gialla (Friuli Venezia Giulia, RIB); 11 Verdicchio (Marche, VERD); 14 Vermentino (Sardegna, VEM); 8 Vernaccia (Toscana, VER). The bottles were stored in a cellar at 4 °C until analysis (performed ten months after sampling). Sample origin and geographical distribution are shown in Figure 1.

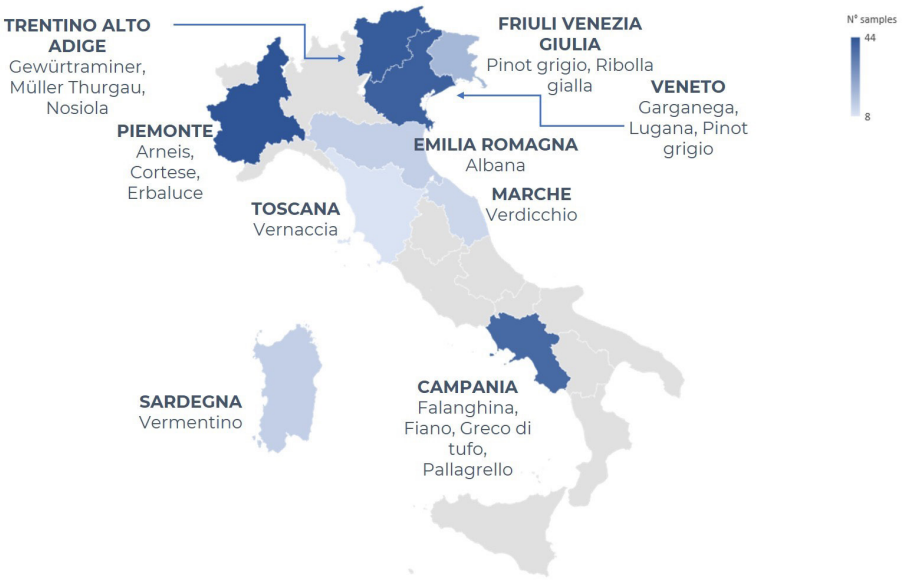


FIGURE 1. Map of Italy with regional distribution of varieties and samples.

3. Sample preparation and UHPLC-MS/MS analysis of MeSA glycosides

The sample preparation procedure was the same used by Carlin *et al*. for UHPLC-MS/MS Ion Trap for the quantification of MeSA glycosides (Carlin *et al*., 2019a). Wine samples were filtered at 0.22 µm after bottle opening and injected; no other treatment was required.

The separation was performed at 40 ° C in reverse phase with an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) using an Acquity UPLC® BEH HSS-T3 (1.8 µm, 2.1 × 50 mm) precolumn coupled to an Acquity UPLC® C18 HSS-T3 (1.8 µm, 2.1 mm × 150 mm) column both from Waters Corporation (Milford, MA, USA). The mobile phase consisted of water (A) and methanol (B), both acidified at 0.1 % v/v with formic acid. Elution was performed at 0.28 mL/min with the following multistep linear gradient: 0–1 min, 100 % A isocratic; 1–3 min, 100–90 % A; 3–18 min, 90–60 % A; 18–21 min, 60–0 % A; 21–25.5 min, 0 % A isocratic; 25.5–25.6 min, 0–100 % A; 25.6–28 min 100 % isocratic.

The ionisation was performed in electrospray (ESI) using an AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA) operating with the same optimal setting described in detail by Carlin *et al*. (2019a). The best-performing transitions were selected to be used as the quantifier (Q) and qualifier (q). The following [M+Na^+] masses were used for quantification purposes: 337.09 for MeSAG, *m/z* 469.13 for the three isomers gaultherin, canthoside A and MeSA-vicianoside (violutoside), *m/z* 499.14 for MeSA-gentiobioside and m/z 483.15 for MeSA-rutinoside; Q/q ratio and retention times were used as confirmation criteria for each compound. Mass spectrometer instrumental parameters are reported in the Supplementary information (Table S1).

As many as 15 freshly prepared calibration samples with concentrations within 0.02 µg/L and 2000 µg/L of MeSAG and gaultherin were used for the acquisition of calibration curves.

MultiQuant and Analyst from AB Sciex LLC (Framingham, MA, USA) were used for data acquisition and elaboration, respectively.

4. Analysis of free MeSA

For quantification of free MeSA, SPME extraction followed by GC-MS analysis was used, following the procedure described by Slaghenaufi *et al.* (2022). Five µL of internal standard 2-octanol (4.2 mg/L in ethanol) are added to 5 mL of wine diluted with 5 mL of deionised water in a 20 mL glass vial. Three grams of NaCl are added prior to GC-MS analysis. Samples were equilibrated for 1 min at 40 °C. Subsequently, SPME extraction was performed using a 50/30 µm divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, PA, USA) exposed to sample headspace for 60 minutes. GC-MS analysis was carried out on an HP 8060 (Agilent Technologies) gas chromatograph coupled to a 5977B quadrupole mass spectrometer equipped with a Gerstel MPS3 autosampler (Mülheim/Ruhr, Germany). Separation was performed using a DB-WAX UI capillary column (30 m × 0.25 mm, 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 follows: start at 40 °C for 3 min, raise to 230 °C at 4 °C/min and maintain for 20 min. With this configuration, retention time (t_r) was 32.68 min, and linear retention index (LRI) was 1771. Mass spectrometer operated in electron ionisation (EI) at 70 eV with ion source temperature at 250 °C and quadrupole temperature at 150 °C. Mass spectra were acquired in SIM mode (quantitation ion (*m/z*) 121, qualifier ions (*m/z*) 92, 152). A calibration curve was prepared using seven concentration points and three replicate solutions per point in still-white wine. Five µL of internal standard 2-octanol (4.2 mg/L in ethanol) were added to each calibration point, which was then submitted to SPME extraction and GC-MS analysis as described for the samples. Calibration curves were obtained using Chemstation software (Agilent Technologies, Inc.) by linear regression, plotting the response ratio (analyte peak area divided by internal standard peak area) against concentration ratio (added analyte concentration divided by internal standard concentration).

5. Model ageing protocols

In order to evaluate the evolution of MeSA during wine ageing, a subset of six wine samples for each variety was submitted to an accelerated ageing protocol based on the procedures described by Slaghenaufi *et al*. (2019) and Slaghenaufi and Ugliano (2018) with minor differences. Wine bottles were opened in a polyethylene glove box under nitrogen, and free SO_2 levels were determined and then, where needed, adjusted to 30 ± 3 mg/L. Sixty mL of each wine were then placed in glass vials with polypropylene screw caps with butyl/Teflon septa. Vials were kept under vacuum in thermally sealed plastic oxygen barrier bags at either 10 °C (Control), 40 °C (T40) and 60 °C (T60) (± 0.2 °C) for 30 days. All samples were produced in duplicate.

RESULTS

1. MeSA glycosides in Italian white wines

Experimental data of MeSA glycosides in the 246 wines analysed are reported in Table S2 and plotted in Figure 2. The concentration of the triglycosylated form of MeSA (MSTG-A) was under the quantitation limit in all samples, so it was not reported.

Lugana and Verdicchio were the cultivars which showed the highest total amount of MeSA glycosides, with values over 1000 µg/L (1286 and 1101 µg/L, respectively). Moreover, Erbaluce emerged from other cultivars with a mean total value of 674.9 µg/L, whereas the other 15 displayed lower concentrations. The average total value for the entire sample set, excluding the three previously mentioned cultivars, was 109.3 µg/L, spanning from 328.3 µg/L of Garganega wines and 29.5 µg/L of Gewürztraminer ones.

From a quantitative point of view, the monoglycosylated form (MeSAG) and two diglycosides (MeSA–gentiobioside

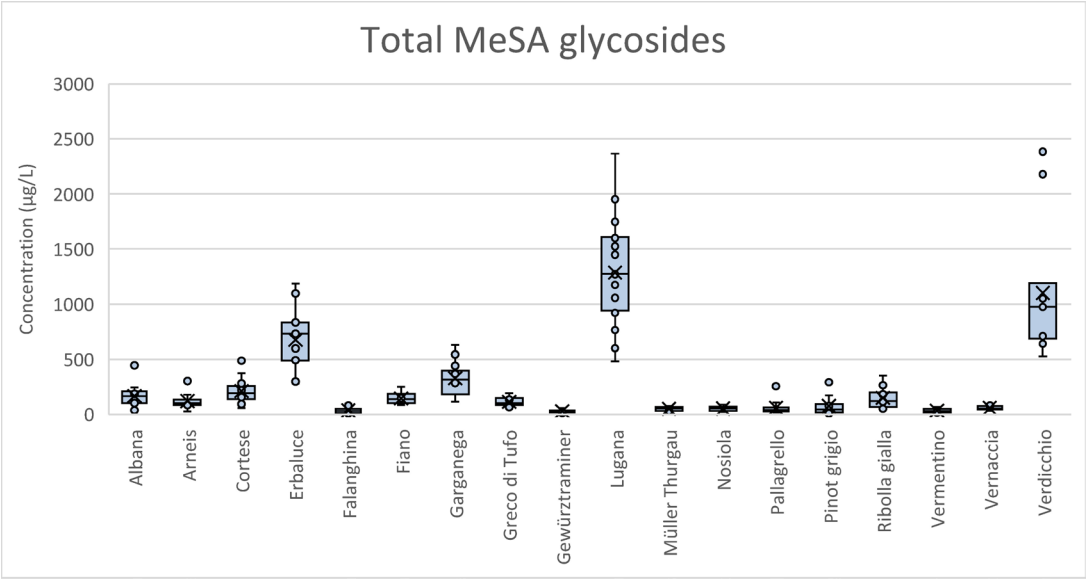


FIGURE 2. Boxplot of the total MeSA glycoside concentrations in the cultivars considered.

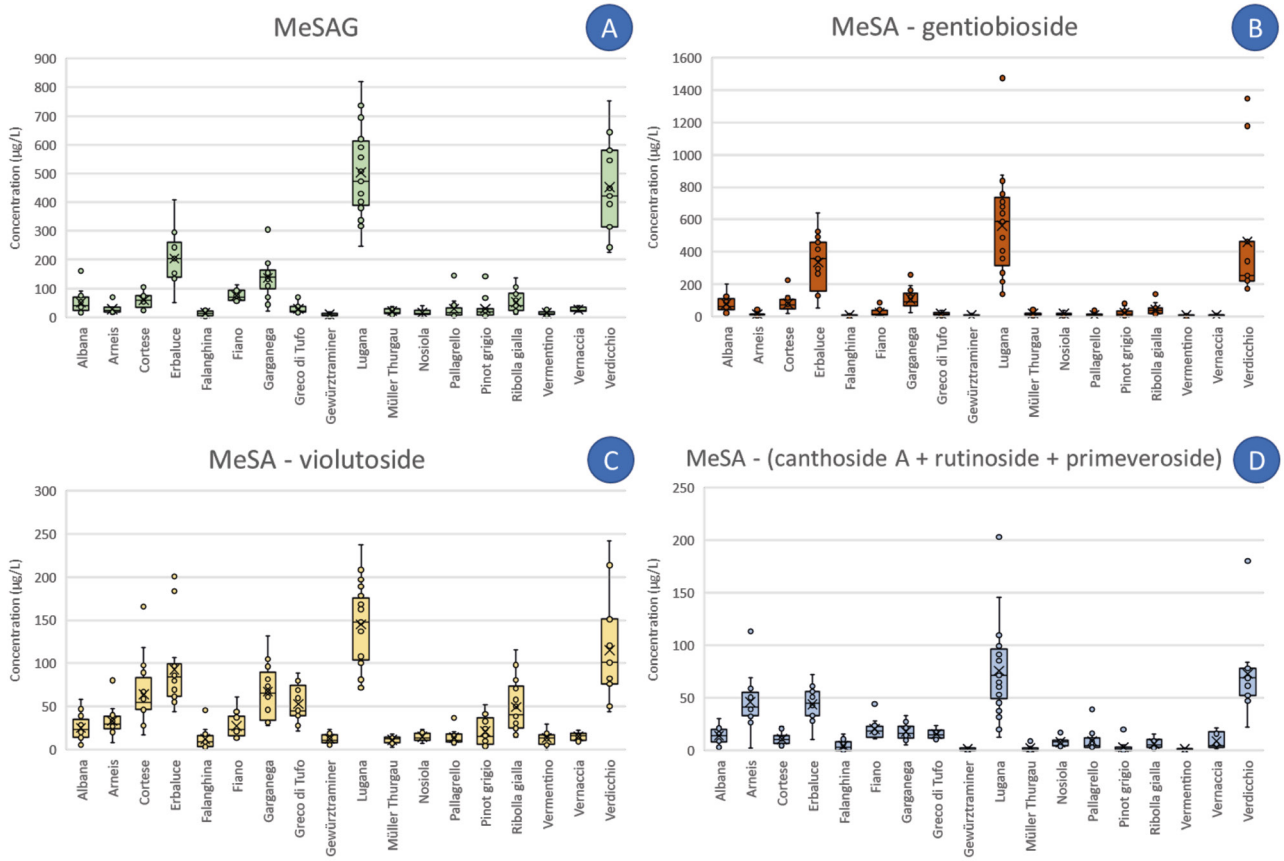
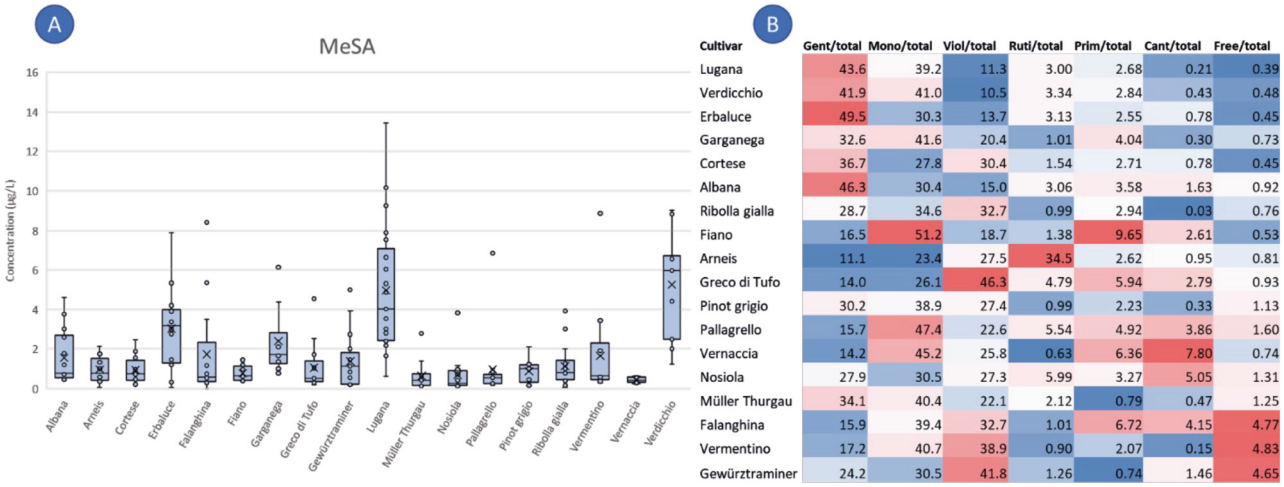


FIGURE 3. Boxplots of the MeSA concentration over its glycosylated forms MeSAG (A), MeSA–gentiobioside (B), MeSA–violutoside (C) sum of less concentrated ones (D) in the cultivars considered.

and MeSA violutoside) were the most abundant forms for almost all cultivars (Figure 3). This distribution was in total accordance with the trend observed for other white varieties reported in our previous article (Carlin *et al*., 2019a).

The sum of these three glycosides accounted for more than 80 % of bound MeSA (from 85.1 % of Vernaccia di San Gimignano to 96.7 % of Ribolla gialla) in every sample except Arneis, which displayed an average value of 62.0 % due to its relevant amount of MeSA–rutinoside. For MeSAG (Figure 3A), MeSA–gentiobioside (Figure 3B), MeSA–violutoside (Figure 3C) and the sum of MeSA–canthoside A + rutinoside + primeveroside (Figure 3D) the same trend appeared.



Cultivar	Gent/total	Mono/total	Viol/total	Ruti/total	Prim/total	Cant/total	Free/total
Lugana	43.6	39.2	11.3	3.00	2.68	0.21	0.39
Verdicchio	41.9	41.0	10.5	3.34	2.84	0.43	0.48
Erbaluce	49.5	30.3	13.7	3.13	2.55	0.78	0.45
Garganega	32.6	41.6	20.4	1.01	4.04	0.30	0.73
Cortese	36.7	27.8	30.4	1.54	2.71	0.78	0.45
Albana	46.3	30.4	15.0	3.06	3.58	1.63	0.92
Ribolla gialla	28.7	34.6	32.7	0.99	2.94	0.03	0.76
Fiano	16.5	51.2	18.7	1.38	9.65	2.61	0.53
Arneis	11.1	23.4	27.5	34.5	2.62	0.95	0.81
Greco di Tufo	14.0	26.1	46.3	4.79	5.94	2.79	0.93
Pinot grigio	30.2	38.9	27.4	0.99	2.23	0.33	1.13
Pallagrello	15.7	47.4	22.6	5.54	4.92	3.86	1.60
Vernaccia	14.2	45.2	25.8	0.63	6.36	7.80	0.74
Nosiola	27.9	30.5	27.3	5.99	3.27	5.05	1.31
Müller Thurgau	34.1	40.4	22.1	2.12	0.79	0.47	1.25
Falanghina	15.9	39.4	32.7	1.01	6.72	4.15	4.77
Vermentino	17.2	40.7	38.9	0.90	2.07	0.15	4.83
Gewürztraminer	24.2	30.5	41.8	1.26	0.74	1.46	4.65

FIGURE 4. Boxplots of the free MeSA concentration (A) and a heatmap of the relative percentual distribution of MeSA over its measured forms (B) in the cultivars considered. Colour intensities from blue (low) to red (high) highlighted what were the richest and the poorest cultivars for every MeSA form.

Pie charts reported in Figure S1 displayed the relative distribution of MeSA over Lugana (Figure S1A), Erbaluce (Figure S1B), Verdicchio (Figure S1C) and the mean of other 15 cultivars (Figure S1D). Lugana and Verdicchio had a specular distribution of MeSA between MeSAG and MeSA–gentiobioside with almost 40 % of the total each. Erbaluce gave an increased amount of MeSA–gentiobioside and a simultaneously decreased content of MeSAG but with percentages of other forms very similar to the previously mentioned cultivars.

2. Free MeSA and its relation with its glycosides

In addition to bound forms, also free MeSA was measured in all 246 samples (numeric data not shown), and the boxplot representing their distribution was reported in Figure 4A. Free MeSA gave a mean of 1.69 µg/L, spanning from Verdicchio to Vernaccia di San Gimignano (5.26 and 0.44 µg/L, respectively). The sample with the highest concentration was a Lugana with 13.45 µg/L whereas several samples among Falanghina, Ribolla gialla, Nosiola and Müller-Thurgau showed negligible contents (under 0.20 µg/L).

Merging all data from free and bound MeSA, it was possible to have a comprehensive overview of this odorant and its precursors. In the heatmap reported in Figure 4B, relative percentages of each form over the cultivars were displayed. Colour intensities from blue (low) to red (high) highlighted what were the richest and the poorest cultivars for every MeSA form. A trend was observed over the diagonal.

DISCUSSION

The data reported in Figure 2 and Table S2 showed the existence of a clear varietal distinction associated with the MeSA glycoside content of different wines. In accordance with our previous works (Carlin *et al*., 2019a), Lugana and Verdicchio clearly stood out for their high content of glycosides, with an average total amount of 1286 and 1101 µg/L, respectively. This is in agreement with previously published data concerning the high content of free MeSA in Lugana and Verdicchio (Carlin *et al*., 2019b; Fracassetti *et al*., 2020; Slaghenaufi *et al*., 2022). As the grape cultivars used to produce these wines are genetically related (Crespan *et al*., 2021), the observation of similar and significantly higher content of different MeSA forms in these wines is not surprising. Conversely, the relatively high MeSA precursors content of Erbaluce wines, showing a total average content of 674.9 µg/L (more than half of what was found in Lugana and Verdicchio), was reported here for the first time. To the best of our knowledge, methyl salicylate was not previously determined in Erbaluce wines made from fresh grapes. "Erbaluce" or "Erbaluce di Caluso" is a white genotype of *Vitis vinifera* L. cultivated in the provinces of Torino, Biella, and Vercelli, surrounding the town of Caluso in Piemonte, North West Italy (Vincenzi *et al*., 2012). To date, the Erbaluce variety has been used to produce conventional wines as well as for Passito wines (*Passito* di Caluso) and for sparkling wines with the bottle-fermentation method (Produzioni and Agroalimentari, 2016). Low concentrations of MeSA have been found in Erbaluce dehydrated grapes (4.13−9.33 µg/L as free form and 15.9−4.71 µg/L as glycosylated form; Rolle *et al*., 2012). However, MeSA has not been detected in those grapes affected by *Botrytis cinerea* in noble form and showing higher weight loss. In a study carried out on 23 wine samples of Caluso *Passito* DOC aged from 4 to 28 years, made from dehydrated Erbaluce grapes, very low concentrations were found (1 ± 1 µg/L as averaged concentration; Giordano *et al*., 2009). Therefore, MeSA concentration could decrease during the grape withering process and become non detectable in most of the samples analysed of *Passito* wines (Giordano *et al*., 2009).

Garganega wines, which were for the most belonging to the Soave appellation, also showed relatively high MeSA precursor content (328.3 µg/L vs 109.3 µg/L), although in this case, the possible presence of small portions of

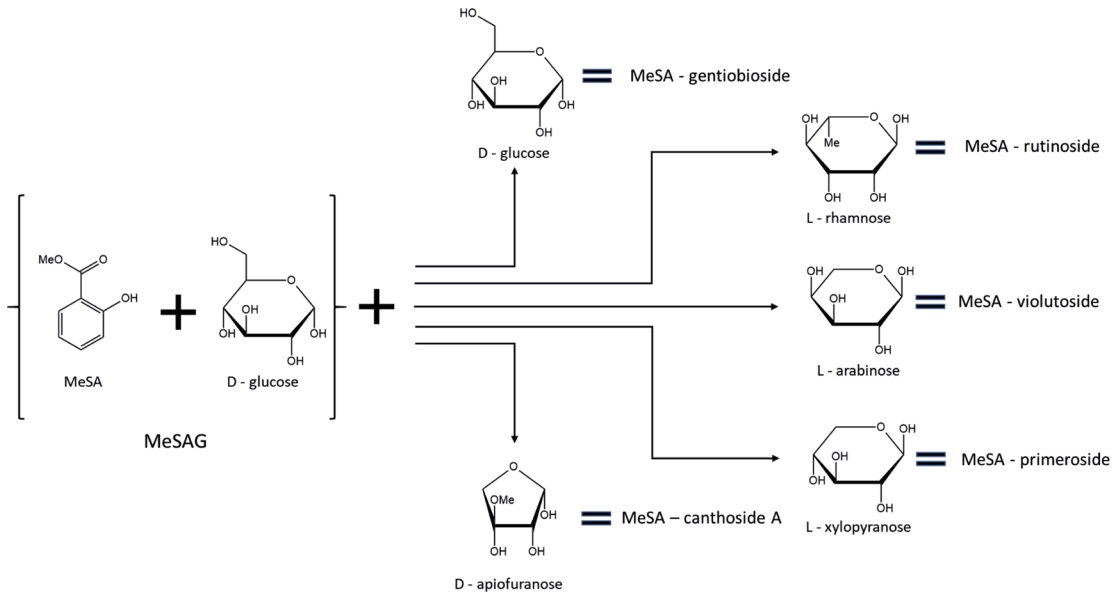


FIGURE 5. Schematic representation of MeSA monoglycosides and diglycosides.

Lugana in the final blend must be considered. The Soave appellation regulation allows indeed a 5 % w/w maximum addition of different grapes from the province of Verona, in which both Soave and Lugana wines are produced (Produzioni and Agroalimentari, 2020).

Additionally, from data reported in Table S2, a relevant content of MeSA–rutinoside was detected in Arneis samples. MeSA–rutinoside, whose structure was based on MeSAG with an L–rhamnose bound through a 1→6 glycosidic bond, was identified among the most important odour precursors in several passion fruit varieties (*Passiflora edulis, Passiflora flavicarpa, Passiflora ligularis and Passiflora molissima*) (Chassagne *et al*., 1998). Arneis was the cultivar with the greatest average content of this glycoside, even higher than Lugana and Verdicchio (41.4 vs 38.6 and 36.7 µg/L, respectively), whereas MeSA–rutinoside was the most abundant glycoside for this cultivar (it accounted for up to 34.5 % of its total MeSA precursors), which seemed to be an identity characteristic.

In terms of structural distribution, the first trend detected was a very similar distribution of every glycoside over the different varieties. The only monoglycoside (MeSAG) was, in all varieties, either the first or the second MeSA form for concentration. It accounted for a minimum of 23.4 % in Arneis (whose distribution profile was highly characterised by MeSA–rutinoside) to a maximum of 51.2 % in Fiano, for an average value of 36.6 % which is approximately a 1:2 ratio of mono-to-diglycosides. This fact was not surprising since MeSAG was the only naturally occurring monoglycoside of methyl salicylate and was also the basic structure of all MeSA glycosides (Mao *et al*., 2014). In particular, all diglycosides considered in this article were composed of a MeSAG unit linked to another sugar through a glycosidic bond in position 6 (Figure 5).

In MeSAG (MeSa 2-*O*-β-D-glucoside), methyl salicylate was connected to the hydroxy function in the anomeric carbon of a D–glucose in glucopyranose form. In diglycosides, a 1→6 glycosidic bond links a further sugar to glucose, as reported in Table 1. Within all six naturally occurring MeSA diglycosides, there is MeSA–lactoside where the sugar connection is 1→4 but this was never detected in wines (Mao *et al*., 2014).

MeSa 2-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside (MeSA–violutoside), MeSa 2-*O*-β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (MeSA–primeveroside), MeSa 2-*O*-β-D-apiofuranosyl(1→ 6)-β-D-glucopyranoside (MeSA–canthoside A) have the same molecular weight with some structural differentiation. Of all varieties, MeSA–violutoside

TABLE 1. Detected MeSA glycosides and related structural information.

Glycoside name	MW (g/mol)	Unit 1	Unit 2	Glycosidic bond
MeSAG	314.0	D–glucose	-	-
MeSA–canthoside A	446.1	D–glucose	D–apiose	1→6
MeSA–primeveroside	446.1	D–glucose	L–xylose	1→6
MeSA–violutoside	446.1	D–glucose	L–arabinose	1→6
MeSA–rutinoside	460.1	D–glucose	L–rhamnose	1→6
MeSA–gentiobioside	476.1	D–glucose	D–glucose	1→6

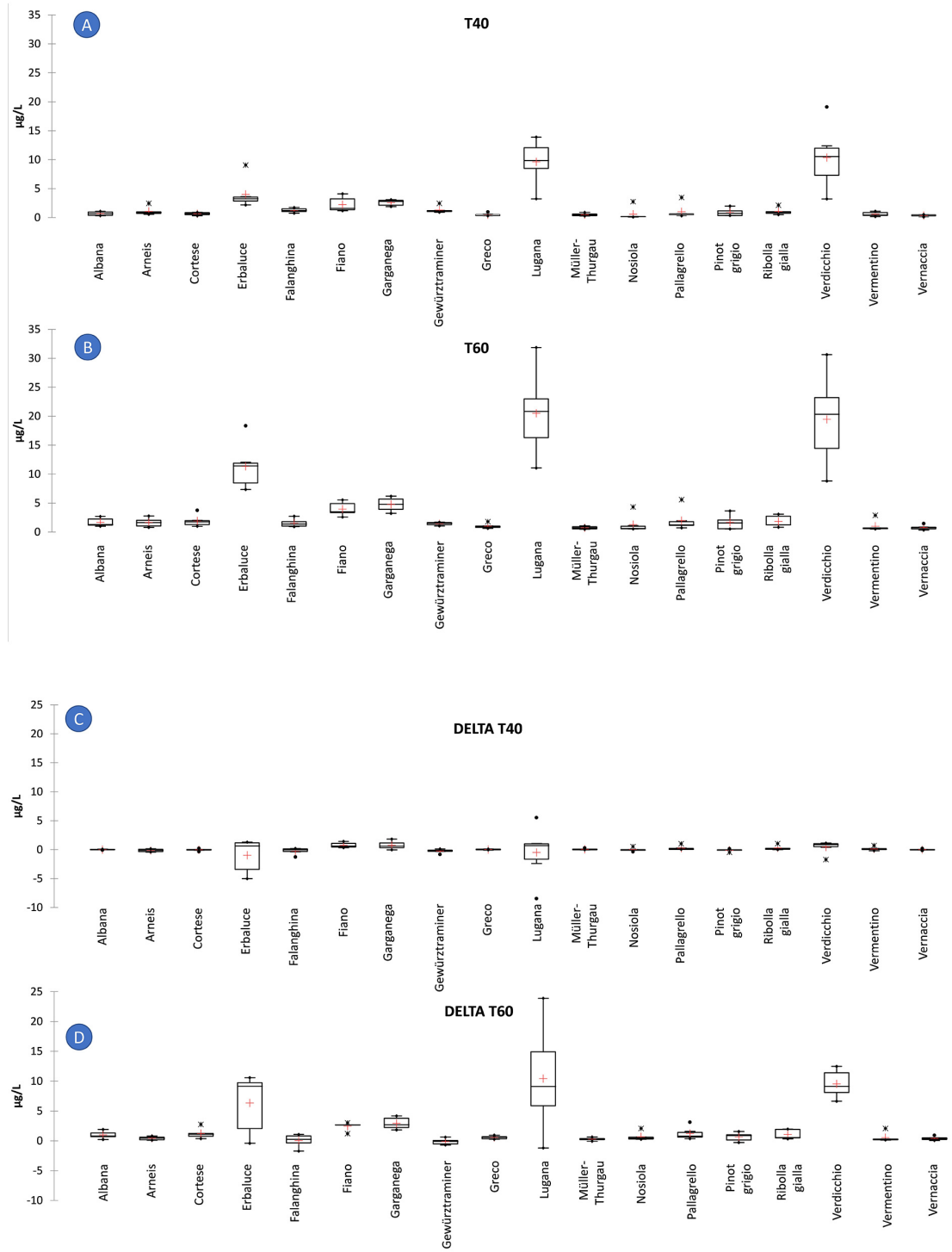


FIGURE 6. Content of free MeSA in T40 (A) and T60 (B) wines and the difference between T40 and Control (C) and T60 and Control (D) wines.

was absolutely the most abundant of them, whereas MeSA–Canthoside A was the less concentrated (Table S2). MeSA–violutoside (Figure 3C) followed the same trend across the cultivars even if, in this case, the concentration absolute differences were smaller. Due to their lower content, MeSA–primeveroside and MeSA–Canthoside A were reported in Figure 3D as a sum with MeSA–rutinoside. The same distribution was observed with lower dispersion. MeSA–Canthoside A, which was the less abundant, was also the only glycoside composed of a branched chain pentose (apiose) which was present in a furanose five-member ring rather than

linear ones that cyclise in pyranosic six-member rings (like xylose and arabinose) (Loscos *et al*., 2007).

The same trend could be observed for MeSA 2-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (MeSA–rutinoside) even though its content was minor if compared to the total amount (data shown as a sum with other two minor analytes in Figure 3D). In this analyte, the second unit besides glucose was an L–rhamnose, which is a sugar with an increased molecular weight due to a methyl group more than the previously mentioned glycosides.

Finally, MeSA 2-O-β-D-glucopyranosyl(1→6)-β-D-gluco pyranoside (MeSA–gentiobioside) was in this research, the most abundant diglycosylated form of methyl salicylate (Figure 3B). Gentiobiose is a disaccharide composed of glucose units and was also the analyte with the highest molecular weight. MeSAG and MeSA–gentiobioside were present both in relevant concentrations, and this trend was in strong accordance with the broad content of glucose, the most abundant natural monosaccharide.

Despite the absolute values, there are also relevant differences in the pattern of MeSA derivatives for each cultivar. Analysing the pie charts reported in Figure 4, a different distribution of MeSA over its glycosides emerged, with an increased MeSA–gentiobioside percentage in Lugana, Verdicchio and Erbaluce if compared to the means of the poorest cultivars. In particular, in Erbaluce wines MeSA–gentiobioside accounted for up to half of the whole MeSA amount but with a reduction of the MeSAG percentage. On the opposite, a lower percentage of MeSA–violutoside was detected in those three varieties if compared to the mean of others. Other apparent trends were not shown, but interestingly, the relative compositions of Lugana and Verdicchio were in accordance with their common genetic origins. This finding supports the hypothesis that both the absolute quantities accumulated in the berry, as well as the pattern of the bound forms are likely under genetic control. For other cultivars except for Arneis, there was a more homogeneous distribution even though MeSAG, MeSA–gentiobioside and MeSA–violutoside were clearly the most abundant.

About free MeSA, analysing data reported in Figure 4A, the same trend of glycosylated forms was observed. From literature data and previous knowledge, only Lugana and Verdicchio were expected to give concentrations over the average, whereas no data were available for wines from other cultivars (Carlin *et al*., 2019; Fracassetti *et al*., 2020; Slaghenaufi *et al*., 2022). Data shown in Figure 4A clearly showed the same trend detected for all glycosides,

TABLE 2. Significant differences between varieties of the same ageing treatments, according to Kruskal–Wallis, (α = 0.05). Accelerated ageing treatments: T40 = 40 °C and T60 = 60 °C for 30 days.

T40				T60			
Variety	Groups			Variety	Groups		
Verdicchio	A			Lugana	A		
Lugana	A			Verdicchio	A		
Erbaluce		B		Erbaluce		B	
Garganega		B	C	Garganega			C
Gewürztraminer		B	C	Cortese			C
Falanghina			C	Ribolla gialla			C
Ribolla gialla			C	Pinot grigio			C
Pallagrello			C	Pallagrello			C
Pinot grigio			C	Falanghina			C
Albana			C	Albana			C
Arneis			C	Arneis			C
Cortese			C	Gewürztraminer			C
Nosiola			C	Nosiola			C
Vernaccia			C	Vernaccia			C
Vermentino			C	Greco di Tufo			C
Greco di Tufo			C	Vermentino			C
Müller-Thurgau			C	Müller-Thurgau			C

TABLE 3. The significant difference between wines of the same variety and different ageing treatments, according to Kruskal–Wallis (α = 0.05).

	Albana	Arneis	Cortese	Erbaluce	Falanghina	Fiano	Garganega	Greco di Tufo	Gewürztraminer	Lugana	Müller-Thurgau	Nosiola	Pallagrello	Pinot grigio	Ribolla gialla	Vernaccia
Control	B	A	B	B	A	B	B	B	A	B	A	A	A	A	B	B
T40	B	A	B	B	A	B	B	B	A	B	A	A	A	A	B	B
T60	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

with Lugana, Verdicchio and Erbaluce higher in content if compared to others. In this case, the situation was less defined since a broader variability was detected, reasonably due to different winemaking styles and types of closures (Parker *et al*., 2020). Although for wines of identical vintage and conservation, analysed two years after harvest, none of them has reached a concentration of free MeSA higher than the sensory threshold (ODT in wine, 38 µg/L) (Slaghenaufi *et al*., 2022) it cannot be ruled out that even at concentrations below the threshold it may contribute to the overall aroma, as found in cherry wine (Niu *et al*., 2019).

Looking at the situation as a whole, from the relative perspective (Figure 4B), the odour-active form accounted for less than 5 % (less than 1 % in most cases), confirming that free MeSA was just the tip of the iceberg whereas most of it was stored in silent form. Interestingly, when displaying cultivars organised based on their total glycosides content in decreasing concentration (up→down) and free MeSA relative percentages in the same decreasing way (left→right), higher values were oriented along the diagonal revealing an unexpected trend. Varieties with a higher total content of free MeSA, like Lugana, Verdicchio and Erbaluce, were more inclined to accumulate MeSA–gentiobioside and MeSAG, whereas cultivars with lower concentrations, like Gewürztraminer, Vermentino and Falanghina tended to have a higher proportion of free MeSA. Something similar was also observed from an opposite point of view since low percentages were distributed mostly in the high, right and left, down corners.

Further insights into the relationship between free MeSA and precursor content were obtained by submitting a subset of 108 wines (six for each variety) to an accelerated ageing experiment. Free MeSA content of the wines after ageing is shown in Figure 6, and statistically significant differences in Tables 2 and 3.

Samples at T40 showed a similar content to control wines, with the only exceptions being Lugana and Verdicchio, which showed an average accumulation of about 5 µg/L. Conversely, more than half of the varieties, ten out of eighteen, at T60, showed a significant increase compared to control wines. Although in most of the varieties, the average content showed increases over two times the initial content, in absolute terms, the difference turns out in almost all cases to be small since the average differences reached a maximum of 2.9 µg/L. We found a larger increase of MeSA in Verdicchio, Lugana and Erbaluce varieties, close to 10 µg/L. As already observed for non-aged wines, Lugana and Verdicchio were the varieties with the highest content, followed by Erbaluce. This observation was consistent with the presence of glycosidic precursors undergoing acid hydrolysis (Carlin *et al*., 2019a).

Although the increase in MeSA content was widespread under the conditions of accelerated ageing, for several samples, a constant (or even slightly negative) MeSA balance during ageing was observed, in agreement with the observations of Slaghenaufi *et al*. (2021). As can be seen in Figure 6, this was not due to a variety-specific effect, as in almost all varieties, there were samples which did not accumulate MeSA during ageing. Besides MeSA release from the precursor, hydrolytic degradation of MeSA in the presence of amino acids (Cheng *et al*., 2021) and transesterification (Gutierrez *et al*., 2022) have been reported, although the likelihood of this reaction in wine should be investigated. The absence of MeSA accumulation could therefore be due to a balance of the reactions leading to its release from precursors as well as its degradation. Nevertheless, a positive correlation between the variation of free MeSA during ageing (T60) and the total glycoside precursor content in control wines was observed (r Pearson = 0.812, r^2 = 0.6597) (Figure 7A), clearly indicating the importance of total glycosidic precursors content in odour-active MeSA accumulation during ageing.

In a further attempt to investigate the factors associated with the presence of outliers in which the final MeSA content after ageing was either too low or too high compared to precursor content, the possible effect of pH was investigated. Wines were divided into two groups according to ANOVA (α = 0.05), the first group including wines which showed a net accumulation of MeSA with ageing (T60) and the second group which did not. We found a statistically significant difference (Kruskal–Wallis, α = 0.05) between the two groups, with the samples not accumulating MeSA showing higher pH, indicating that low pH could promote the release of MeSA from precursors (Figure 7B), representing an additional modulation factor of the chemical reactions leading to MeSA during ageing.

CONCLUSIONS

Methyl salicylate is an impactful odorant in wines whose scientific interest is continuously rising. To date, except for Lugana and Verdicchio, only little was known about MeSA occurrence in Italian white single-cultivar wines. In this

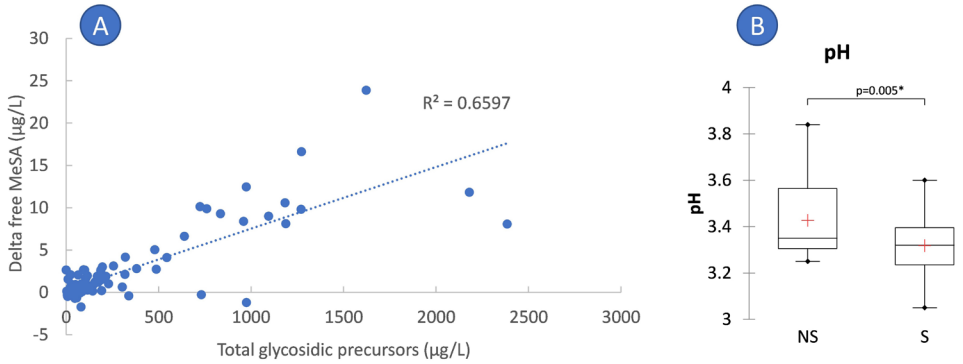


FIGURE 7. Correlation between total MeSA precursors and delta of free MeSA in T60 (Panel A), pH of wines (Panel B) with significant (S) and non-significant (NS) increase of free MeSA after ageing.

paper, a comprehensive overview representative of MeSA and its glycosides was provided over wines from the most relevant white autochthonous varieties cultivated in Italy. Lugana, Verdicchio and Erbaluce stood out for their high content of both MeSA precursors as well as odour-active form. While recent reports have shown that Lugana and Verdicchio both contain high amounts of MeSa, the fact that also Erbaluce behaves similarly is totally new information. The same trend over the cultivars could be detected for all forms, free, monoglycosylated and diglycosylated, whereas the triglycoside MSTG-A was not detected in any case. MeSAG, MeSA–gentiobioside and MeSA–violutoside were the most abundant forms, accounting for almost 80 % of all MeSA in almost all cultivars. A trend was detected in terms of precursors distribution since these three precursors were the forms which accumulated most in the richest cultivars, whereas the remaining glycosides and the free form showed lower relative variations. Precursor content was positively associated with increased content of MeSA during ageing. According to the ageing experiment, Lugana, Verdicchio and Erbaluce were the cultivars expected to release the highest amount of odour-active MeSA over the years, which also appeared to be promoted by low pH. The release of this hidden balsamic wintergreen-like potential further completes the aroma bouquet unveiling an evolution potential for these wines, which could be impactful for both consumer appreciation and bottle-added value.

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